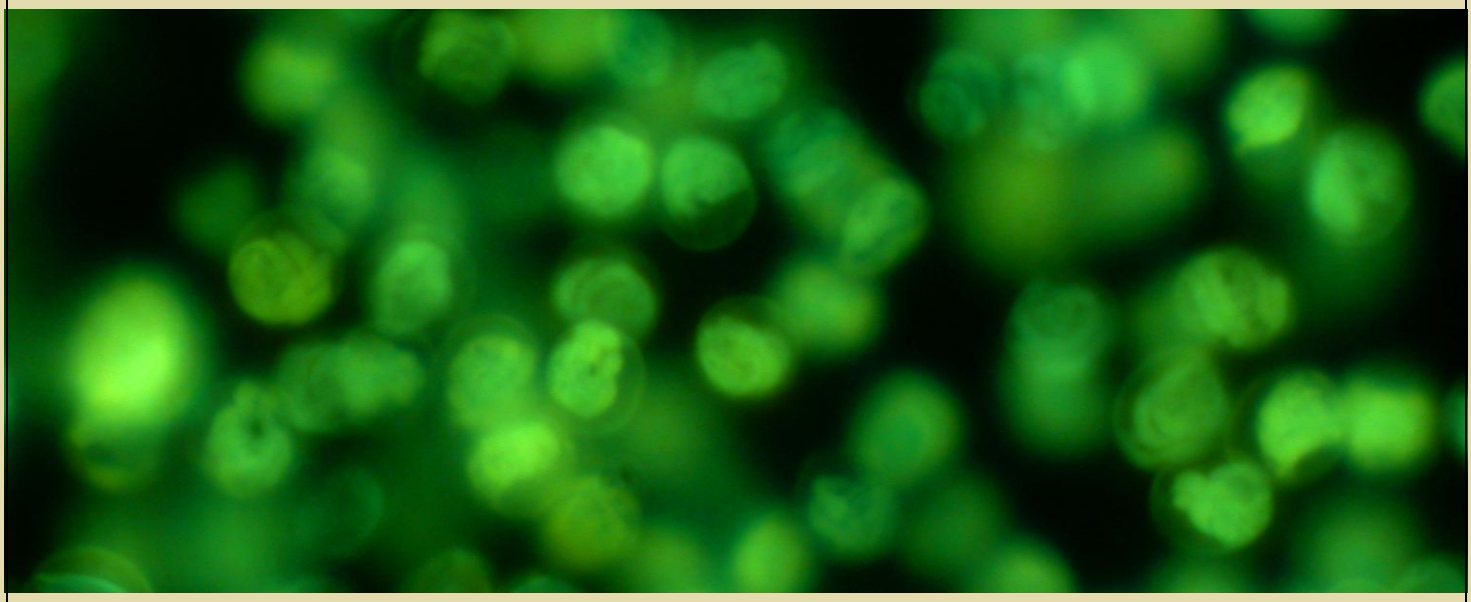
TESE DE DOUTORAMENTO

Análise dos padróns de diversidade xenética e epidemioloxía das especies e variedades de *Cryptosporidium* que parasitan humanos mediante o desenrolo dun protocolo multilocus de tipado xenético de alto rendemento



José Luis Abal Fabeiro

Departamento de Anatomía Patolóxica e Ciencias Forenses

Facultade de Medicina

Santiago de Compostela, 2015

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
FACULTADE DE MEDICINA
DEPARTAMENTO DE ANATOMÍA PATOLÓXICA E CIENCIAS FORENSES

Tese de doutoramento

Análise dos padróns de diversidade xenética e epidemioloxía das especies e variedades de *Cryptosporidum* que parasitan humanos mediante o desenrolo dun protocolo multilocus de tipado xenético de alto rendemento

Memoria para a obtención do grao de Doutor presentada por

José Luis Abal Fabeiro

Asdo.: José Luis Abal Fabeiro

Santiago de Compostela, Novembro de 2015

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AUTORIZACIÓN DOS DIRECTORES

Dr. Xulio M. Maside Rodríguez, Profesor do Departamento de Anatomía Patolóxica e Ciencias Forenses da Universidade de Santiago de Compostela, e

Dra. Carolina Bartolomé Husson, investigadora contratada da Universidade de Santiago de Compostela,

Como Directores da presente Tese de Doutoramento titulada "**Análise dos padróns de diversidade xenética e epidemioloxía das especies e variedades de *Cryptosporidium* que parasitan humanos mediante o desenrolo dun protocolo multilocus de tipado xenético de alto rendemento"** que presenta Don José Luis Abal Fabeiro, alumno do Programa de Doutoramento en Medicina Molecular, para optar ó Grao de Doutor:

Autorizamos a presentación da presente tese, considerando que reúne os requisitos esixidos no artigo 34 do regulamento de Estudos de Doutoramento e que non incorre nas causas de abstención establecidas na lei 30/1992.

E para que conste, firmamos a presente en Santiago de Compostela, a 13 de Novembro de 2015

Asdo: Dr. Xulio Maside Rodríguez

Asdo: Dra. Carolina Bartolomé Husson

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

O traballo presentado na presente tese doutoral foi realizado na Universidade de Santiago de Compostela baixo a dirección dos doutores Xulio M. Maside Rodríguez e Carolina Bartolomé Husson como parte do proxecto PGIDIT06PXIB228073PR da Xunta de Galicia.

Fotografía de cuberta: Dr. José Llovo Taboada

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

A toda a miña familia

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AGRADECEMENTOS

Ós meus directores de tese Carolina e Xulio, pola súa axuda e guía durante todos estes anos. Grazas polos vosos consellos e o voso esforzo durante a realización da presente tese. Tiña pasado por varios laboratorios de investigación con anterioridade pero podo dicir, sen lugar a dúbidas, que foi o primeiro que sentín como *propio*.

Ós meus compañeiros de despacho e laboratorio Xavi, Luis e Rosa, grazas tamén pola vosa axuda e polos momentos compartidos. Moitísima sorte cos vosos respectivos proxectos.

Ó persoal do Servizo de Microbioloxía e Parasitoloxía do CHUS, especialmente a Pepe Llovo e a Tiago, por deixarme traballar con total liberdade e por ensinarme tantas curiosidades no laboratorio. Aprendín moito ó longo de tantas horas e de tantas conversas.

Ós meus amigos, por todos estes anos vividos (e os que virán!). Facedes que TODO sexa moito máis doado. Non hai palabras suficientes para agradecervos a vosa amizade e o voso apoio: sempre estivéchedes, estades e estaredes aí.

E, finalmente, á miña familia, porque a pesares de que en ocasións pode non ser sinxelo comprender por qué alguén segue adiante con determinados proxectos –os cales se alongan no tempo–, sempre estivéchedes aí para amosarme o voso cariño e comprensión. O voso apoio foi fundamental para chegar ata o final.

Esta tese foi posible grazas a vós, tamén é para vós. Moitas grazas a todos.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ÍNDICE

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCIÓN 1

1. Antecedentes históricos 3
2. Ciclo vital do protozoo 4
3. Clasificación taxonómica e posicisión filoxenética do xénero *Cryptosporidium* 9
   3.1 Especies descritas e criterios para a asignación de novas especies 13
   3.2 Xenotipos e subtipos identificados no xénero *Cryptosporidium* 14
4. Características xenómicas do xénero *Cryptosporidium* 16
   4.1 Características moleculares do xénero *Cryptosporidium*: baixos niveis de polimorfismo e de recombinación 18
5. Ferramentas e métodos empregados para detecta-la presenza de *Cryptosporidium* 20
   5.1 Detección mediante tinturas e microscopía 20
   5.2 Técnicas de inmunoanálise 22
   5.3 Métodos de diagnóstico molecular 23
      5.3.1 Técnicas baseadas no estudo de isoenzimas e alozimas 23
      5.3.2 Diagnóstico baseado en PCR 23
         5.3.2.1 O uso de RAPDs 24
         5.3.2.2 Estudos dos padróns de bandas obtidos con enzimas de restrición 25
         5.3.2.3 PCR cuantitativa 26
         5.3.2.4 Polimorfismos de conformación de cadea sinxela (SSCP) 26
         5.3.2.5 Análise da variación en microsatélites e minisatélites 27
         5.3.2.6 Segundo espazador interno do ARN: ITS-2 28
         5.3.2.7 Variación no *locus gp60* 29
6. Manifestacións clínicas e sintomatoloxía da criptosporidiose 30
7. Capacidade infectiva, reservorios e vías de transmisión 32
   7.1 Taxas de incidencia e outros aspectos epidemiolóxicos xerais da criptosporidiose en humanos 35
8. A criptosporidiose en Europa e en España 37
9. A criptosporidiose en Galicia 38

XUSTIFICACIÓN e OBXECTIVOS 41

1. Xustificación 43
2. Obxectivos 45

PUBLICACIÓNS 47

1. Multilocus patterns of genetic variation across *Cryptosporidium* Species suggest balancing selection at the *gp60* locus 49
   1.1 Material suplementario 61
2. High-throughput genotyping assay for the large-scale genetic characterization of *Cryptosporidium* parasites from human and bovine samples 71
   2.1 Material suplementario 83
3. Aetiology and epidemiology of human criptosporidiosis cases in Galicia (NW Spain), 2000-2008 93
   3.1 Material suplementario 109

DISCUSIÓN 113

1. Análise da variabilidade xenética en dez *loci* codificadores de copia única 115
2. Desenrolo dun novo método diagnóstico da criptosporidiose de alto rendemento 118
3. Epidemioloxía da criptosporidiose na área sanitaria de Santiago de Compostela 120

CONCLUSIÓNS 125

BIBLIOGRAFÍA 129

INTRODUCIÓN

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Antecedentes históricos

A primeira descrición dun representante do xénero *Cryptosporidium* levouna a cabo Ernest Edward Tyzzer no ano 1907 mentres estudaba glándulas gástricas de ratos de laboratorio. Nelas identificou as fases de reprodución sexual e asexual, e detectou a capacidade de formar esporas dun protozoo ó que denominou *Cryptosporidium muris* (TYZZER 1907), que posteriormente foi incluído dentro dun novo xénero –*Cryptosporidium*, (TYZZER 1910)– e unha nova familia –Cryptosporidiidae, (LÉGER 1911). Anos máis tarde Tyzzer describiu unha segunda especie deste xénero, *C. parvum*, de menor tamaño que a anterior e que parasitaba o intestino delgado de ratos e coellos (TYZZER 1912). Mais os traballos de Tyzzer non foron considerados como veterinaria ou clinicamente relevantes polos seus contemporáneos, polo que nas cinco décadas posteriores á identificación de *C. muris* o estudo destes parasitos quedou reducido ó entorno taxonómico. Nese período atopáronse e describíronse novas especies de *Cryptosporidium* en diversos grupos de vertebrados –peixes, réptiles, aves e mamíferos– ás que se lles foron asignando nomes baseados nunha suposta especificidade de hóspede (FAYER e XIAO 2008). Foi a partires destes descubrimentos cando rexurdiu o interese veterinario polo estudo deste xénero de protozoos, especialmente cando se demostrou que a súa infección provocaba perdas económicas importantes nas explotacións de gando aviar (SLAVIN 1955) e bovino (PANCIERA 1971; BARKER e CARBONELL 1974; MEUTIN *et al.* 1974; POHLENZ *et al.* 1978; TZIPORI 1983; TZIPORI *et al.* 1983; HEINE *et al.* 1984; DE GRAAF *et al.* 1999).

Xa ben entrada a década dos setenta describíronse os primeiros casos de criptosporidiose en humanos (MEISEL *et al.* 1976; NIME *et al.* 1976), aínda que o interese clínico por este xénero de parasitos non cobrou grande importancia ata os anos oitenta, cando se diagnosticaron diarreas severas causadas por *Cryptosporidium* spp. en persoas afectadas pola síndrome de inmunodeficiencia adquirida (SIDA) (ANON. 1982; MA e SOAVE 1983; PITLIK *et al.* 1983a; PITLIK *et al.* 1983b), nas que a infección podía prolongarse durante meses. Dende entón, os estudos epidemiolóxicos constataron que *Cryptosporidium* é un dos parasitos oportunistas que se atopan con maior frecuencia neste tipo de doentes (CURRENT e GARCIA 1991; HUNTER e NICHOLS 2002; CAMA *et al.* 2003; SINGH *et al.* 2003). Ademais, traballos realizados na década dos oitenta puxeron de manifesto a susceptibilidade das persoas sans inmunocompetentes a sufriren de fortes diarreas despois de ter contacto con gando infectado con *Cryptosporidium* (CURRENT 1983), o que veu a confirmar non só a

capacidade infectiva destes parasitos, senón tamén a natureza zoonótica desta enfermidade (CURRENT e LONG 1983; CASEMORE *et al.* 1985b; MIRON *et al.* 1991).

A mediados da década dos oitenta rexistrouse o primeiro andazo epidémico asociado á inxestión de auga contaminada con ooquistes de *Cryptosporidium* spp. (D'ANTONIO *et al.* 1985). A multiplicación dos casos de andazos reportados con posterioridade (LEVINE *et al.* 1990; MOORE *et al.* 1993; SMITH *et al.* 2006; WALDRON *et al.* 2011b; CHALMERS 2012) – algúns dos cales chegaron a implicar a ducias de milleiros de persoas, como o descrito en Milwaukee (Wisconsin, EEUU) a principios da década dos noventa (MACKENZIE *et al.* 1994; MACKENZIE *et al.* 1995)– puxo de manifesto a importancia do control sanitario das augas de consumo público para previ-lo contaxio destes patóxenos (NETO *et al.* 2006; CASTRO-HERMIDA *et al.* 2008b; CASTRO-HERMIDA *et al.* 2010; AUSTIN *et al.* 2012; CASTRO-HERMIDA *et al.* 2015).

Estes antecedentes, xunto co notable impacto económico e sanitario que ten a criptosporidiose e a súa consideración de parasito emerxente a nivel mundial (CLARK e SEARS 1996; MATUKAITIS 1997; CORSO *et al.* 2003; XIAO *et al.* 2004a; SAVIOLI *et al.* 2006; YODER *et al.* 2012), explican a importancia que cobrou o estudo desta enfermidade, que se evidencia na grande cantidade de artigos publicados sobre este xénero de protozoos (máis de 5000, cando antes da década dos oitenta se contaba apenas cunha trintena) (CURRENT e GARCIA 1991; SHORE GARCÍA 2007) e na multiplicación de métodos e técnicas desenrolados para a súa detección.

2. Ciclo vital do protozoo

O xénero *Cryptosporidium*, a diferenza doutras especies do *phylum* Apicomplexa como *Toxoplasma* ou *Plasmodium*, presenta un ciclo monoxeno monocompartimental, xa que tódalas súas etapas ocorren dentro dun único hóspede (BOUZID *et al.* 2013). Trátase dun ciclo complexo, pois nel obsérvanse tanto fases sexuais como asexuais (CURRENT e GARCIA 1991), con distintos estadios de desenrolo claramente diferenciados (KEUSCH *et al.* 1995; THOMPSON *et al.* 2005): ooquiste, esporozoito, trofozoito, merozoito e dous gamontes (**Figura 1**).

O ciclo vital de *Cryptosporidium* iníciase pola inxesta de ooquistes viables (**Figura 1, a**), os cales presentan un diámetro medio de entre 4 e 7 μm e son a forma de resistencia e de propagación destes organismos. Un ooquiste maduro está constituído por paredes internas e externas, unhas estruturas granulares ou corpos residuais, un glóbulo membranoso e catro

esporozoitos nus (PETRY 2004; FAYER e XIAO 2008). Sen embargo, a diferenza doutros coccidios, mostra unha sutura nun extremo da cuberta pola cal parecen saír os esporozoitos durante o proceso de desenquistación (DUBEY *et al.* 2002; FAYER e XIAO 2008) (**Figura 2**).

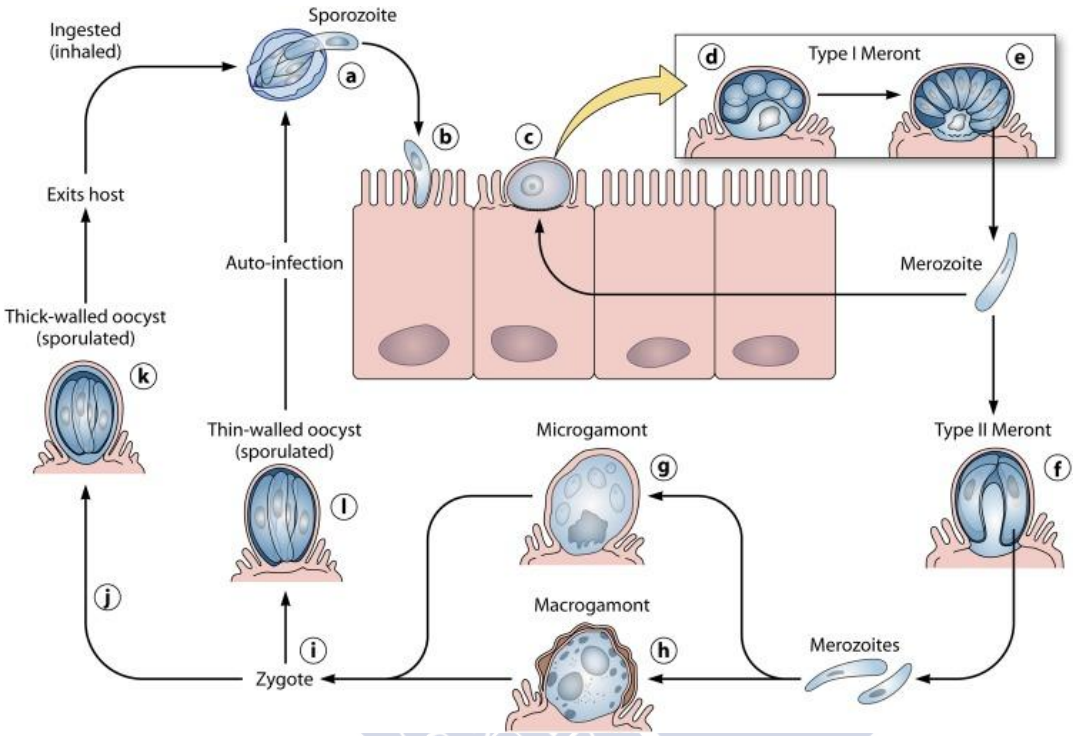


Figura 1. Ciclo vital de *Cryptosporidum* spp. (adaptado de BOUZID *et al.* 2013)

No intestino os ooquistes liberan os esporozoitos que conteñen no seu interior (**Figura 1, a**) (FAYER e XIAO 2008; BOUZID *et al.* 2013; LENDNER e DAUGSCHIES 2014). Estes esporozoitos están rodeados por unha única membrana, son haploides e conteñen a maioría das estruturas e orgánulos típicos dos Apicomplexa (**Figura 3**). Así, na súa parte anterior atópase a película ou complexo apical, formado polos aneis polares, o conoide (conxunto de microtúbulos dispostos en espiral) e unha serie de orgánulos que van xogar un papel fundamental nos procesos de desprazamento, locomoción, adhesión e invasión da célula hóspede por parte do parasito (O'HARA *et al.* 2005; FAYER e XIAO 2008; LENDNER e DAUGSCHIES 2014). Entre eles están os corpos electrodensos (estruturas granulares opacas ós electróns) (HUANG *et al.* 2004), os micronemas (complexos secretores de substancias

proteolíticas de forma helicoidal) (HARRIS *et al.* 2003) e as roptrias (orgánulos membranosos orixinados polo retículo endoplasmático e o complexo de Golgi e que están encargados do recoñecemento da célula hóspede) (SAM-YELLOWE 1996). Na súa posición central os esporozoitos presentan un núcleo cun nucléolo ben diferenciado, ribosomas, microtúbulos subpeliculares –que participan tamén nos procesos de locomoción– aparello de Golgi, gránulos de amilopectina (TETLEY *et al.* 1998; FAYER e XIAO 2008), e un mitosoma (TETLEY *et al.* 1998; FAYER e XIAO 2008). Este último é un orgánulo non descrito noutros coccidios, remanente dunha antiga mitocondria, que se atopa limitado por unha dobre membrana e rodeado de múltiples segmentos de retículo endoplasmático rugoso (PUTIGNANI *et al.* 2004; KEITHLY *et al.* 2005; PUTIGNANI 2005). Os esporozoitos presentan ademais dúas estruturas globulares, unha de grande tamaño en posición distal e outra máis pequena na parte central da célula, ámbalas dúas de orixe e función descoñecidas (FAYER e XIAO 2008) (**Figura 3**).

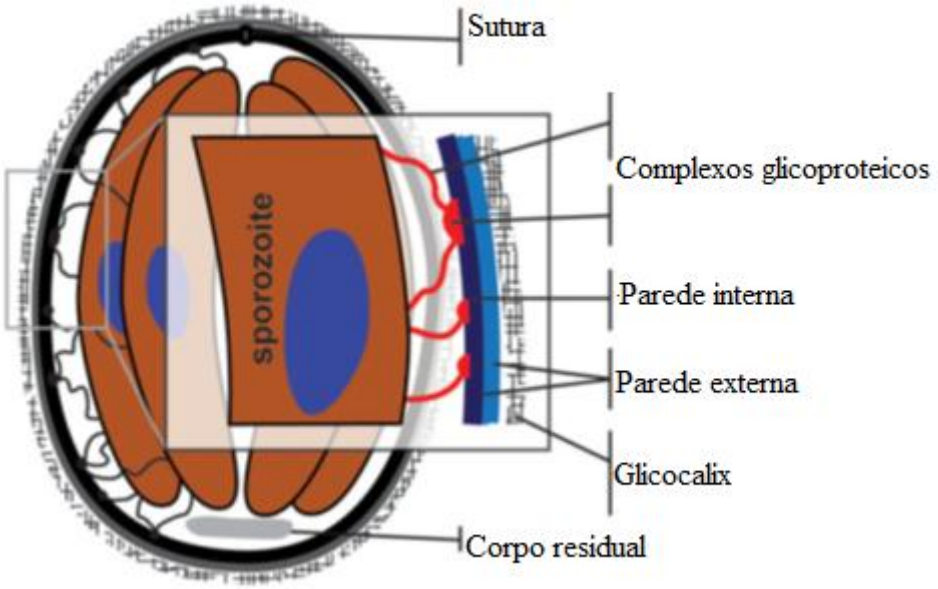


Figura 2. Esquema dun ooquiste (adaptado de LENDNER e DAUGSCHIES 2014).

Os esporozoitos liberados teñen capacidade motora –mediante movementos de contracción, extensión e deslizamento (*gliding*), típicos de Apicomplexa (ARROWOOD *et al.* 1991; GUT e NELSON 1994; FORNEY *et al.* 1998; LENDNER e DAUGSCHIES 2014)– e aproxímanse cara a parte anterior das células do epitelio intestinal. Atravesan a súa capa

mucosa (ATUMA *et al.* 2001; LENDNER e DAUGSCHIES 2014) e invaden o enterocito grazas á acción do complexo apical e outros orgánulos que median no proceso de invasión e internalización (O'HARA *et al.* 2005; WETZEL *et al.* 2005) (**Figura 1, b**). As roptrias esténdense cara o lugar de acoplamento, mentres que os glóbulos densos e os micronemas se dirixen cara a rexión apical (HUANG *et al.* 2004) onde van liberar un conxunto de moléculas que facilitan a ancoraxe e introdución do parasito na célula hóspede (SMITH *et al.* 2005; THOMPSON *et al.* 2005; BOROWSKI *et al.* 2008; BLACKMAN e CARRUTHERS 2013). Neste proceso de contacto, invasión e internalización participan un bo número de ligandos e glicoproteínas que se expresan na superficie do esporozoito –como as lectinas ou o os complexos GP60 e GP900, entre outros (CEVALLOS *et al.* 2000a; CEVALLOS *et al.* 2000b; STRONG *et al.* 2000; SESTAK *et al.* 2002; HASHIM *et al.* 2006; JAKOBI e PETRY 2006; BHAT *et al.* 2007; O'CONNOR *et al.* 2007; LENDNER e DAUGSCHIES 2014)–, os cales se poden unir a distintos receptores, estruturas ou complexos que se atopan na superficie da célula a infectar. Outros factores adicionais, como a temperatura corporal do hóspede ou a secreción de sustancias producidas durante a dixestión –como as sales biliares ou a tripsina– parecen influír tamén no desenrolo destes procesos (CURRENT e HAYNES 1984; CHEN *et al.* 2004a).

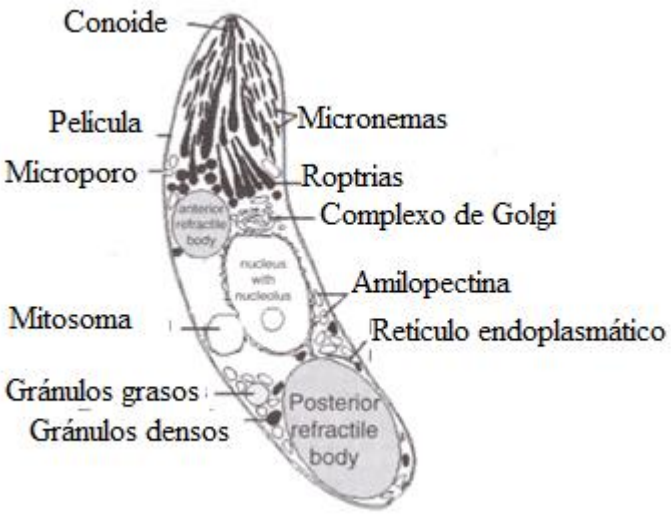


Figura 3. Estrutura interna dun esporozoito de *Cryptosporidium* spp. (ORTEGA-PIERRES 2009).

No momento en que se produce o recoñecemento da célula hóspede e unha vez liberado o contido dos orgánulos secretores do complexo apical, o enterocito rodea ó parasito

por medio dunhas protuberancias que se forman na súa membrana apical, adheríndoo á superficie celular. Durante dito proceso prodúcese unha fusión incompleta entre a membrana do parasito e a da célula hóspede, creando unha interface na que se formará o vacúolo parasitóforo (CURRENT e REESE 1986; MARCIAL e MADARA 1986; LUMB *et al.* 1988; UMEMIYA *et al.* 2005). Este vacúolo é utilizado polo parasito para captar nutrientes procedentes da célula hóspede (HUANG *et al.* 2004; FAYER e XIAO 2008; VALIGUROVA *et al.* 2008; LENDNER e DAUGSCHIES 2014) e activar certas rutas de sinalización celular dirixidas a muda-la estrutura e o normal funcionamento dos enterocitos infectados (FORNEY *et al.* 1999; CHEN e LARUSSO 2000; ELLIOTT *et al.* 2001; CHEN *et al.* 2004b; LENDNER e DAUGSCHIES 2014).

Rematado o proceso de ancoraxe, o esporozoito muda a súa forma aplatanada e pasa a se converter nun trofozoito esférico cun único núcleo prominente (**Figura 1, c**), que se multiplica asexualmente. Como resultado dese proceso orixínanse merontes que conteñen ata oito núcleos no seu interior (merontes tipo I) (**Figura 1, d**) e que se van diferenciar noutros tantos merozoitos mononucleados (**Figura 1, e**). Estes poden abandona-lo vacúolo parasitóforo e infecta-los enterocitos máis próximos, onde poden iniciar un novo ciclo multiplicativo asexual como o descrito anteriormente (**Figura 1, c e d**), ou ben poden entrar nun ciclo multiplicativo distinto que conduce a unha fase de reprodución sexual. Neste caso os merozoitos transfórmanse en merontes tipo II, que se dividen dúas veces para produciren catro merozoitos tipo II (**Figura 1, f**). Estes son liberados ó lumen intestinal e se adhiren a novas células epiteiliais onde se transforman en microgamontes (**Figura 1, g**) –masculinos– e macrogamontes (**Figura 1, h**) –femininos– (TZIPORI e WIDMER 2000; BOUZID *et al.* 2013). Os microgamontes sofren múltiples divisións nucleares dando lugar a microgametos, equivalentes a células espermáticas, que son liberados da célula hóspede e van fertilizar ós macrogametos, células uninucleadas inmóbiles semellantes a óvulos.

Despois da fertilización fórmase un cigoto diploide (**Figura 1, i**), o cal durante a esporogonia sufre unha meiose reducional e da lugar a catro esporozoitos haploides con plena capacidade infectiva. No proceso os cigotos transfórmanse en ooquistes, que poden ser excretados ó medio ambiente por vía fecal para dar comezo a un novo ciclo (**Figura 1, k**) ou quedarse no hóspede e comezar un novo proceso de autoinfección (**Figura 1, l**) (FAYER e XIAO 2008; BOUZID *et al.* 2013; LENDNER e DAUGSCHIES 2014). Este último tipo de ooquistes –xunto cos merontes ou merozoitos tipo I– son os responsables dos procesos de autoinfección endóxena dos hóspedes, que ocasionalmente derivan en infeccións crónicas.

3. Clasificación taxonómica e posición filoxenética do xénero *Cryptosporidium*

Os criptosporidios clasifícanse no *phylum* Apicomplexa (LEVINE *et al.* 1980; UPTON e CURRENT 1985; FAYER e UNGAR 1986; LEVINE 1988; TZIPORI e GRIFFITHS 1998), formado por máis de 300 xéneros e unhas 4800 especies. Todas elas son de vida parasitaria e moitas son axentes causais de enfermidades humanas, entre as que podemos destaca-la malaria (*Plasmodium* spp.), a toxoplasmose (*Toxoplasma* spp.) ou a babebiose (*Babesia* spp.) (FAYER e XIAO 2008; BOSCH *et al.* 2015). A localización taxonómica de *Cryptosporidium* dentro do *phylum* fundamentouse tradicionalmente en certas características biolóxicas (principalmente a morfoloxía e tipo de ciclo vital), que os achegaban ó grupo dos coccidios (**Táboa 1**) (LEVINE 1980; LEVINE *et al.* 1980; FAYER e UNGAR 1986; O'DONOGHUE 1995; UPTON 2000).

Táboa 1. Clasificación taxonómica de *Cryptosporidium* spp

Clasificación	Nome	Características biolóxicas
Filo	Apicomplexa (Levine 1970)	Endosimbiontes unicelulares e parasitarios con complexo apical.
Clase	Conoidasida (Levine 1988)	Complexo apical completo (con conoide)
Subclase	Coccidiasina (Leuckart, 1879)	Gamontes con desenrolo intracelular
Orde	Eucoccidiorida (Leger e Duboscq, 1910)	Ciclo biolóxico con merogonia. A infección prodúcese principalmente en hóspedes vertebrados.
Suborde	Eimeriorina (Leger 1911)	Micro e macrogametos con desenrolo independente.
Familia	Cryptosporidiidae (Leger, 1911)	Ciclo monoxeno monocompartimental. Liberan ooquistes maduros que conteñen catro esporozoitos nus (sen cuberta esporoquística).

Os criptosporidios sempre tiveron a consideración de coccidios inusuais xa que presentan peculiaridades propias que os diferencian do resto dos compoñentes da subclase Coccidiasina (CURRENT 1985; TETLEY *et al.* 1998; SPANO e CRISANTI 2000; CHAPPELL e OKHUYSEN 2002; ABRAHAMSEN *et al.* 2004; XU *et al.* 2004; THOMPSON *et al.* 2005; BARTA e THOMPSON 2006; SHORE GARCÍA 2007; FAYER e XIAO 2008; VALIGUROVA *et al.* 2008; MOGI e KITA 2010; GUBBELS e DURAISINGH 2012; BOUZID *et al.* 2013; LENDNER e DAUGSCHIES 2014), entre as que destacan:

- Carecen de esporoquistes, micropilo, gránulos polares, apicoplasto e mitocondrias funcionais, o cal se interpreta como evidencia de que as súas rutas metabólicas son máis sinxelas que as do resto de coccidios descritos.
- Os ooquistes son pequenos e parecen amosar dúas morfoloxías ou tipos segundo o grosor que presenten as súas paredes celulares: os de parede grosa ("*thick-walled*") e os de parede fina ("*thin-walled*"). Así, os primeiros saen do hóspede coas feces (**Figura 1, k**), dado que poden sobrevivir mellor no exterior; mentres que os segundos presentan unhas paredes moito máis finas, que permiten unha saída máis doada e rápida dos esporozoitos dentro do mesmo hóspede (**Figura 1, l**).
- Os criptosporidios desenrolan a maior parte do seu ciclo vital no tracto gastrointestinal de vertebrados sen requiriren dunha fase extraintestinal para completa-la súa maduración e activa-la súa capacidade infectiva.
- Son resistentes ós antibióticos e a outros axentes usados habitualmente para controla-las infeccións causadas por outros coccidios, así como a un grande número de desinfectantes.
- Tódalas fases intracelulares do ciclo vital de *Cryptosporidium* desenvólvense na superficie apical das células ás cales parasita –xusto por debaixo das microvellosidades dos enterocitos– e, dentro destas, nunha posición intracelular pero extracitoplasmática, sen invadi-lo citoplasma da célula hóspede (**Figura 1, c**). Ademais, a unión entre o parasito e a célula vén mediada por unha estrutura multimembranosa –o orgánulo alimentario– que se forma na base do vacúolo parasitario e que facilita a absorción de nutrientes procedentes da célula infectada.

É interesante detallar un pouco máis polo miúdo esta última característica dos criptosporidios, dado que esta forma de adhesión á membrana no extremo apical das células hóspede é moi semellante á empregada por algunhas especies de gregarinas –un grupo ancestral de parasitos que afecta principalmente a vermes e outros invertebrados mariños (ADL *et al.* 2005; LANE e ARCHIBALD 2008)–, pois ambos organismos forman unha interface especializada entre eles e a célula parasitada na superficie da mesma para obter nutrientes do hóspede (**Figura 4**), amosando polo tanto estratexias de adaptación e de supervivencia como parasitos semellantes (BARTA e THOMPSON 2006; BUTAEVA *et al.* 2006; VALIGUROVA *et al.* 2007; ALDEYARBI e KARANIS 2015; BARTOSOVA-SOJKOVA *et al.* 2015; CLODE *et al.* 2015; RYAN e HIJJAWI 2015). A estas similitudes na súa localización dentro do hóspede e nos modos de adhesión e de nutrición atopados entre as gregarinas e os criptosporidios súmaselle

a coincidencia de certas características morfolóxicas, estruturais e biolóxicas comúns para ambos grupos de parasitos (HIJJAWI *et al.* 2002; HIJJAWI *et al.* 2004; ROSALES *et al.* 2005; VILOGUROVA e KOUDELA 2005; BARTA e THOMPSON 2006; TOSO e OMOTO 2007; ORTEGA-PIERRES 2009; CLODE *et al.* 2015), como son :

- A ausencia de apicoplasto.
- A presenza dun ciclo vital monoxeno.
- A formación durante os seus respectivos ciclos vitais duns ooquistes que se atopan rodeados por unha parede celular formada por dúas capas e que amosan uns tamaños semellantes.
- Ciclo vital con tres estadios celulares diferenciados: merogonia, gametogonia e esporogonia.
- A existencia de posibles fases de desenvolvemento extracelular.

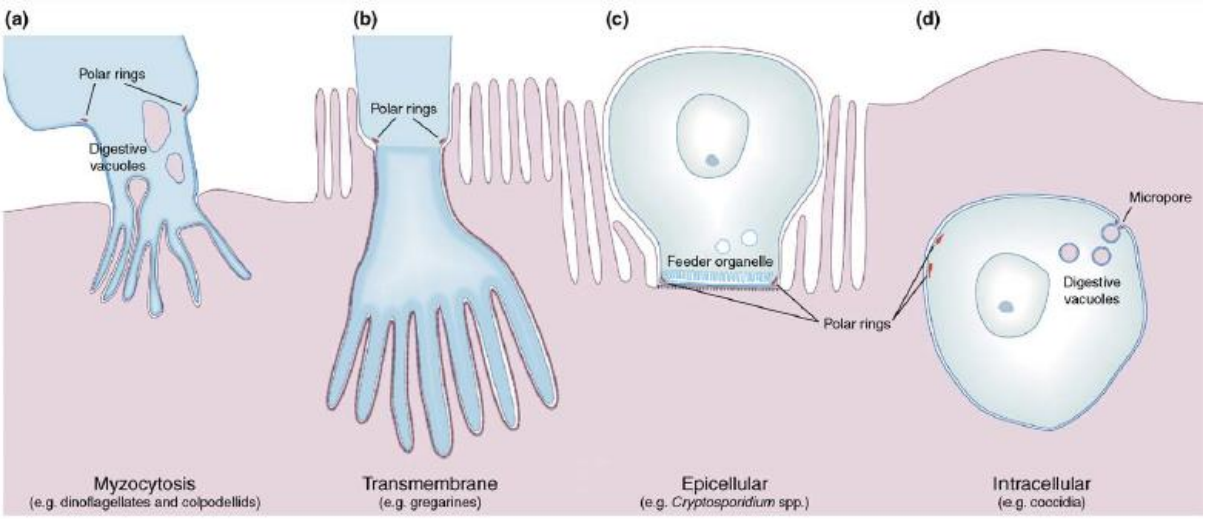


Figura 4. Diferentes tipos de interacción observadas entre as células hóspede e os parasitos dinoflaxelados (a), gregarinas (b), *Cryptosporidium spp*. (c) e coccidios (d). A interacción que amosan os dinoflaxelados, coñecida como mizocitosis, tamén se denomina *vampirismo celular*. As gregarinas amosan unha unión más estreita coa célula hóspede, onde a localización intracelular pode ser parcial ou completa, pero sempre nunha posición epicelular. No caso de *Cryptosporidium*, esta adhesión epicelular ó hóspede observada nos dous grupos anteriores evolucionou cara un grao maior de especialización, mediada por un orgánulo membranoso encargado da captación de nutrientes dende o hóspede. Pola súa parte, os coccidios soen localizarse no citoplasma da célula parasitada e emprega tanto o transporte transmembrana como microporos para obter nutrientes da célula hóspede (BARTA e THOMPSON 2006).

Se xa estas características biolóxicas facían dubidar á comunidade científica sobre a posición taxonómica destes organismos, a incorporación de ferramentas de análise molecular ó estudo dos criptosporidios provocou que a súa localización filoxenética fose mudando ó longo do tempo. Así, mentres a principios dos anos 90 situábanse preto do xénero *Plasmodium* (BARTA *et al.* 1991) –pese a que xa existían estudos previos que poñían en cuestión a filoxenia establecida para o *phylum* Apicomplexa (JOHNSON *et al.* 1990)–, en análises posteriores foise desbotando a hipótese inicial de que *Cryptosporidium* pertencera ó grupo dos coccidios. Hoxe en día considérase un clado diferenciado que está próximo ás gregarinas (inclúese dentro da subclase Cryptogregaria), coas cales comparte un antepasado común na base do *phylum* (MORRISON e ELLIS 1997; CARRENO *et al.* 1999; ZHU *et al.* 2000a; LEANDER *et al.* 2003a; LEANDER *et al.* 2003b; THOMPSON *et al.* 2005; BARTA e THOMPSON 2006; TEMPLETON *et al.* 2010; CAVALIER-SMITH 2014; CLODE *et al.* 2015) (**Figura 5**).

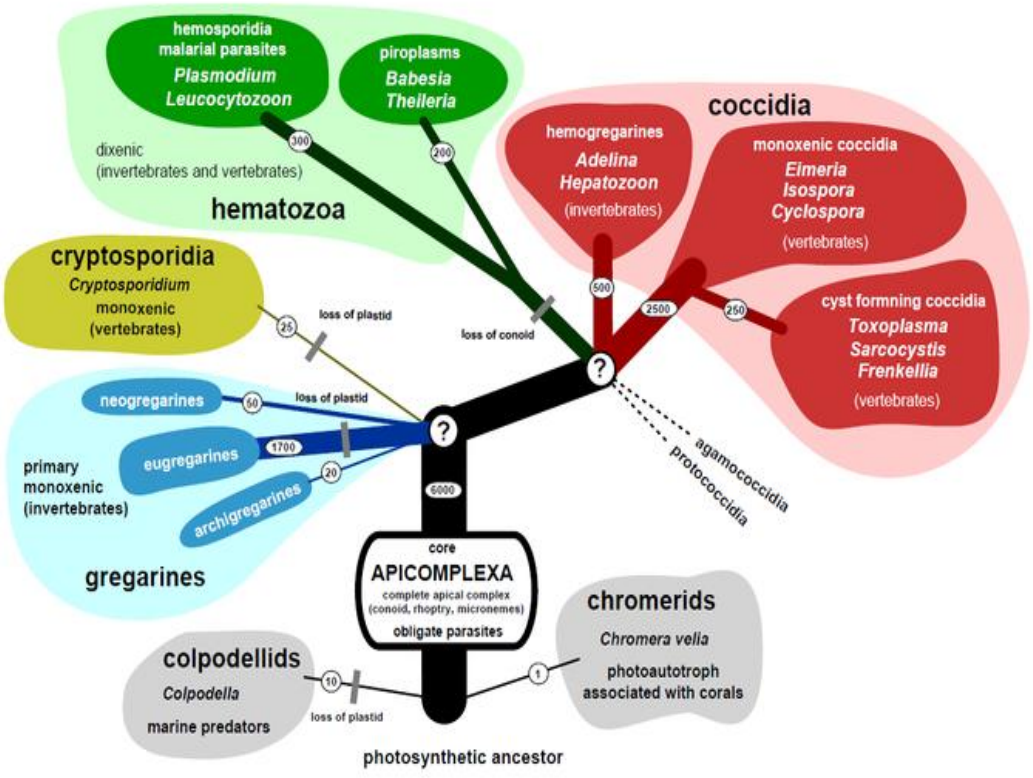


Figura 5. Relacións filoxenéticas hipotéticas do *phylum* Apicomplexa (adaptado de PORTMAN e SLAPETA 2014).

3.1 Especies descritas e criterios para a asignación de novas especies

Os criterios empregados durante moitos anos para a identificación das diferentes especies que compoñen o xénero *Cryptosporidium* foron as características morfométricas dos ooquistes e a súa aparente especificidade de hóspede (FAYER e XIAO 2008). Nembargante, as limitacións desta metodoloxía fixeron que a discriminación entre especies fose complexa, xa que o tamaño dos ooquistes varía pouco entre especies e a especificidade de hóspede non sempre existe (FALL *et al.* 2003; XIAO *et al.* 2004a). De feito, xeráronse confusións que motivaron o descarte dalgunha especie por ser redundante ou por pertencer a outro xénero de coccidios (como foi o caso de *C. vulpis* ou *C. crotali*, especies adscritas na actualidade ó xénero *Sarcocystis*) (FAYER e XIAO 2008; FAYER 2010; RYAN *et al.* 2014). Estas rectificacións foron posibles grazas á aplicación de técnicas de bioloxía molecular que ofrecen maior resolución para a correcta asignación de especies e xenotipos de *Cryptosporidium* (MORGAN *et al.* 1999c; MORGAN *et al.* 1999d).

Desta maneira, conforme ás indicacións do Código Internacional de Nomenclatura Zoolóxica (ICZN, siglas en inglés) do ano 2006, para describir unha especie de *Cryptosporidium* débense avalia-las súas características morfolóxicas, biolóxicas e xenéticas (EGYED *et al.* 2003; FALL *et al.* 2003; XIAO *et al.* 2004a; XIAO *et al.* 2007a; FAYER e XIAO 2008; JIRKU *et al.* 2008; FAYER 2010; RYAN *et al.* 2014). Para elo requírese:

- A realización de estudos morfométricos dos ooquistes, incidindo nas diferenzas de tamaño (tamaño medio e gama de tamaños observados) e forma (cálculo da relación da medida lonxitude:anchura).
- A análise das súas propiedades biolóxicas, como a especificidade de hóspede, a capacidade dos esporozoitos para saíren do ooquiste, a localización da infección no hóspede, a súa patoxenicidade ou a periodicidade da mesma.
- A caracterización molecular, baseada na obtención de secuencias nucleotídicas dos *loci* mellor estudados, como por exemplo os xenes da *SSU rRNA*, da proteína HSP70 ou da proteína 1 da parede do ooquiste (COWP1).

Na actualidade coñécense ó redor dunhas 30 especies de *Cryptosporidium* (**Táboa 2**) (FAYER e XIAO 2008; ORTEGA-PIERRES 2009; PLUTZER e KARANIS 2009; RYAN *et al.* 2014), aínda que na literatura segue sen haber consenso sobre a validez dalgunhas delas (SLAPETA

2012; XIAO *et al.* 2012). Por exemplo, algúns criptosporidios detectados en peixes (*C. nasorum* ou *C. scophthalmi*) (FAYER e XIAO 2008; RYAN *et al.* 2014) considéranse *nomina nuda* por non cumprir a totalidade das normas establecidas polo ICZN (XIAO *et al.* 2007a), o cal se debe xeralmente á ausencia de secuencias nucleotídicas que os identifiquen como especies diferentes ás xa descritas.

3.2 Xenotipos e subtipos identificados no xénero *Cryptosporidium*

Ademais das especies anteriormente citadas, existen ata 60 xenotipos de *Cryptosporidium* (FAYER e XIAO 2008; PLUTZER e KARANIS 2009; RYAN *et al.* 2014) baseados nas secuencias de *loci* coma o da *SSU rRNA*, da actina ou da proteína HSP70 (MORGAN *et al.* 1999a; THOMPSON *et al.* 2005; FAYER e XIAO 2008; FAYER 2010), que agardan unha análise máis pormenorizada para determinar se corresponden a linaxes de especies xa descritas ou se consideran novas especies. Entre eles hai illados de pato (ZHOU *et al.* 2004), porco (SUÁREZ-LUENGAS *et al.* 2007), cabalo (RYAN *et al.* 2003a) ou mangosta (ABE *et al.* 2004). Nesta situación estiveron *C. bovis* (FAYER *et al.* 2005), *C. canis* (FAYER *et al.* 2001), *C. ryanae* (FAYER *et al.* 2008), *C. ubiquitum* (FAYER *et al.* 2010) ou *C. xiaoi* (FAYER e SANTIN 2009), anteriormente consideradas como linaxes doutras especies.

Por outra parte, a análise de marcadores altamente polimórficos coma microsatélites, minisatélites e algúns xenes funcionais permitiu sub-estruturar liñaxes dentro dalgunhas das especies xa descritas. Entre estes marcadores destaca o *locus gp60*, (STRONG e NELSON 2000; GLABERMAN *et al.* 2001; GLABERMAN *et al.* 2002; ALVES *et al.* 2003b; FAYER e XIAO 2008; LI *et al.* 2014; RYAN *et al.* 2014) que codifica para dúas glicoproteínas que participan nos procesos de recoñecemento, unión, ancoraxe e invasión da célula hóspede (THOMPSON *et al.* 2005; LENDNER e DAUGSCHIES 2014) e amosa unha variabilidade nucleotídica superior á reportada para outros xenes (STRONG *et al.* 2000; COHEN *et al.* 2006). A secuencia deste xene é moi variable e os seus distintos alelos son empregados para identificar liñaxes do parasito dentro dunha especie, denominadas *familias de subtipos*. Ademais, na rexión 5' deste *locus* hai un microsatélite que inclúe un número variable de codóns que codifican para o aminoácido serina (TCA, TCG e TCT), cuxas secuencias son utilizadas para definir subdivisións (ou *subtipos*) dentro de cada unha das familias de subtipos descritas (ver seccións posteriores) (SULAIMAN *et al.* 2005; FAYER e XIAO 2008; XIAO 2010; RYAN *et al.* 2014).

Táboa 2. Especies descritas dentro do xénero *Cryptosporidium*

Especie	Descrición	Localización da infección	Tamaño do ooquiste (*μ*m)	Hóspedes
C. andersoni	LINDSAY *et al.* 2000	Gástrica (abomasum)	7.4 (6.0–8.1) × 5.5 (5.0–6.5)	Mamíferos
C. baileyi	CURRENT *et al.* 1986	Cloaca, bolsa Fabricio	4.8 (4.8–5.7) × 6.4 (5.6–7.5)	Aves
C. bovis	BARKER e CARBONELL 1974; FAYER *et al.* 2005	Intestino delgado	4.9 (4.8–5.4) × 4.6 (4.2–4.8)	Mamíferos
C. canis	FAYER *et al.* 2001	Intestino delgado	4.95 × 4.75	Mamíferos
C. cuniculus	INMAN e TAKEUCHI 1979; ROBINSON *et al.* 2010	Intestino delgado	5.9 (5.6-6.4) × 5.4 (5-5.9)	Mamíferos
C. erinacei	KVAC *et al.* 2014	Intestino	4.9 (4.5-5.8) x 4.4 (4.0-4.8)	Mamíferos
C. fayeri	RYAN *et al.* 2008	Intestino delgado	4.9 (4.5-5.1) × 4.3 (3.8–5.0)	Mamíferos
C. felis	ISEKI 1979	Intestino delgado	4.5 (5.0–4.5) × 5.0 (6.0–5.0)	Mamíferos
C. fragile	JIRKU *et al.* 2008	Estómago	6.2 (5.5–7.0) × 5.5 (5.0–6.5)	Anfibios
C. galli	PAVLASEK 1999; RYAN *et al.* 2003b	Gástrica	8.3 (8.0–8.5) × 6.3 (6.2–6.4)	Aves
C. hominis	MORGAN-RYAN *et al.* 2002	Intestino delgado	4.8 (6.4–5.4) × 5.2 (4.4–5.9)	Mamíferos
C. macropodum	POWER e RYAN 2008	Descoñecida	4.9 (4.5-6.0) x 5.4 (5.0-6.0)	Mamíferos
C. meleagridis	SLAVIN 1955	Intestino delgado	5.0 (4.5-6.0) × 4.4 (4.2-5.3)	Mamíferos e aves
C. molnari	ÁLVAREZ-PELLITERO e SITJA-BOBADILLA 2002	Estómago, intestino delgado	4.7 (3.2-5.5) x 4.5 (3.0-5.0)	Peixes
C. muris	TYZZER 1907; TYZZER 1910	Estómago	6.3 (5.5–7.0) × 8.4 (7.5–9.8)	Mamíferos

Táboa 2. Continuación.

Especie	Descrición	Localización da infección	Tamaño do ooquiste (*µ*m)	Hóspedes
C. parvum	TYZZER 1912	Intestino delgado	4.9 (4.5-5.4) × 4.4 (4.2-5.2)	Mamíferos
C. ryanae	FAYER *et al.* 2008	Descoñecida	3.2 (2.9-4.4) x 3.7 (2.9-3.7)	Mamíferos
C. scrofarum	KVAC *et al.* 2013	Intestino	5.2 (4.8-5.9) x 4.8 (4.2-5.3)	Mamíferos
C. serpentis	LEVINE 1980	Estómago	6.2 (5.6–6.6) × 5.3 (4.8–5.6)	Réptiles
C. suis	RYAN *et al.* 2004	Intestino delgado e groso	4.6 (4.9-4.4) x 4.2 (4.0-4.3)	Mamíferos
C. tyzzeri	REN *et al.* 2012	Intestino	4.64 x 4.19	Mamíferos
C. ubiquitum	FAYER *et al.* 2010	Intestino	5.0 (4.7-5.3) x 4.7 (4.3-4.9)	Mamíferos
C. varanii	PAVLASEK e RYAN 2008	Estómago e intestino delgado	4.7 (4.2–5.2) × 5 (4.4–5.6)	Réptiles
C. viatorum	ELWIN *et al.* 2012b	Intestino	5.4 (4.8-5.7) x 4.7 (4.1-5.2)	Mamíferos
C. wrairi	VETTERLING *et al.* 1971	Intestino delgado	4.6 (4.0–5.0) × 5.4 (4.8–5.6)	Mamíferos
C. xiaoi	FAYER e SANTIN 2009	Descoñecida	3.9 (2.9 – 4.4) x 3.4 (2.9 – 4)	Mamíferos

Nota: destácanse en negriña e subliñadas as especies atopadas en humanos

4. Características xenómicas do xénero *Cryptosporidium*

Existen sete iniciativas en curso dirixidas a estuda-los xenomas do xénero *Crytposporidium* (*C. baileyi*, *C. hominis*, *C. meleagridis*, *C. muris*, *C. parvum*, *C. ubiquitum* e o do xenotipo *Cryptosporidium* spp. chipmunk) (http://www.ncbi.nlm.nih.gov/bioproject/), que se atopan en distintos grados de elaboración. Na actualidade só están dispoñibles as secuencias dos cromosomas de *C. parvum* (illado Iowa II) (ABRAHAMSEN *et al.* 2004), namentres que o xenoma de *C. muris* (illado RN66) está ensamblado en secuencias consenso de fragmentos solapantes (ou *contigs*, en inglés) e o de *C. hominis* (illado TU502) (XU *et al.* 2004) en

scaffolds –que son series sinténicas de *contigs*– (ORTEGA-PIERRES 2009). As secuencias de todos os xenomas son accesibles en dous repositorios públicos: GenBank (http://www.ncbi.nlm.nih.gov/genbank/) e CryptoDB (http://www.cryptodb.org) (PUIU *et al.* 2004; HEIGES *et al.* 2006).

Se atendemos unicamente ás especies que presentan unha ensamblaxe máis avanzada (*C. parvum* e *C. hominis*) observamos que posúen xenomas moi compactos (KEELING 2004), de pouco máis de 9 Mb, repartidos en oito grupos de ligamento de entre 0,9 e 1,4 Mb de lonxitude, e que conteñen cerca de 4.000 xenes (ABRAHAMSEN *et al.* 2004; XU *et al.* 2004) (**Táboa 3**). A homoloxía nucleotídica promedio entre as dúas especies é do 96,7% (XU *et al.* 2004; BOUZID *et al.* 2010; MAZURIE *et al.* 2013), conteñen un número parecido de secuencias de rRNAs e tRNAs e exhiben un elevada proporción de pares Adenina-Timina, tanto nas rexións codificadoras como nas non codificadoras (70% e 75%, respectivamente) (XU *et al.* 2004) (**Táboa 3**).

Comparados con outros xenomas de Apicomplexa, os do xénero *Cryptosporidium* son moi reducidos. Conteñen uns 1.200 xenes menos que *Plasmodium falciparum* e 3.800 menos que *Toxoplasma gondii*. Inclúen menos intróns, presentes unicamente nun 5–20% dos seus xenes, fronte ó 54% dos de *P. falciparum* ou ó 74% dos de *Theileria parva*, e as rexións interxénicas comprenden unha fracción do xenoma unha orde de magnitude menor á observada en *P. falciparum* (GARDNER *et al.* 2002; BANKIER *et al.* 2003; ABRAHAMSEN *et al.* 2004; XU *et al.* 2004; GARDNER *et al.* 2005; AJIOKA 2007; GAJRIA *et al.* 2008) (**Táboa 3**).

Os criptosporidios carecen de xenes implicados na síntese de aminoácidos (FAYER e XIAO 2008) e nalgunhas rutas bioquímicas básicas, como o ciclo de Krebs ou a fosforilación oxidativa (DENTON *et al.* 1996; KEITHLY *et al.* 1997; ABRAHAMSEN *et al.* 2004; XU *et al.* 2004; FAYER e XIAO 2008); no canto, obsérvase un incremento no número de proteínas transportadoras, que dobra ás descritas en *P. falciparum* (ABRAHAMSEN *et al.* 2004; FAYER e XIAO 2008).

Outra característica molecular das especies que compoñen o xénero *Cryptosporidium* é a ausencia de xenoma mitocondrial e o do apicoplasto (ZHU *et al.* 2000b; BARTA e THOMPSON 2006; MOGI e KITA 2010). Así, a pesares de que no ADN nuclear existen varios xenes de orixe mitocondrial (RIORDAN *et al.* 2003) e de que presentan un mitosoma –remanente dunha antiga mitocondria (RIORDAN *et al.* 2003; PUTIGNANI *et al.* 2004; ALCOCK *et al.* 2012; HEINZ e LITHGOW 2013)–, este orgánulo non é funcional.

Táboa 3. Características xenómicas de *Cryptosporidium* spp. comparado con outros representantes do *phylum* Apicomplexa .

	C. hominis	***C. parvum***	***P. falciparum***	***T. gondii***	***T. parva***
Cromosomas	8	8	14	14	4
Tamaño (Mb)	9,16	9,11	22,84	63,5	8,31
GC (%)	31,7	30,3	19,4	52,2	34,1
Número de xenes	3994	3952	5268	7793	4035
Lonxitude media dos xenes (pb)	1576	1720	2283	2265	1407
Rexións codificadoras (%)	69	74	53	30	68,4
Xenes con intróns (%)	5-20	5	54	74	74
GC das rexións codificantes (%)	32,3	31,9	23,7	57,2	-
Número de tRNAs	45	45	43	174	47
Número de 5S rRNAs	6	6	3	4	3
Número de 5.8S, 18S e 28S rRNAs	5	5	7	420	2

4.1 Características moleculares do xénero *Cryptosporidium*: baixos niveis de polimorfismo e de recombinación

A maioría dos traballos moleculares adicados ó estudo da estrutura xenética das poboacións naturais das diferentes especies e subtipos de *Cryptosporidium* céntranse en avalia-los niveis de polimorfismo destes parasitos analizando os cambios atopados no número de repeticións de determinadas secuencias micro e minisatélites, como por exemplo os *loci* ML1, MS9 ou as repeticións de serina atopadas no *locus gp60* (STRONG *et al.* 2000; LEAV *et al.* 2002; ALVES *et al.* 2003a; SULAIMAN *et al.* 2005; ALVES *et al.* 2006; WIDMER 2009; WIDMER e SULLIVAN 2012). No entanto, estes estudos teñen principalmente unha finalidade epidemiolóxica, encamiñados a describi-las características dun andazo dado (determina-las especies e subtipos infectantes e a súa relación entre eles) e baséanse nunha serie de marcadores que non son os máis idóneos para describi-los padróns de diversidade xenética das poboacións, dadas as características evolutivas peculiares dos micro e minisatélites (GOLDSTEIN e POLLOCK 1997; ELLEGREN 2004). Por contra, apenas hai datos sobre os niveis de diversidade en rexións codificadoras (caso dos *loci* da *SSU RNA*, actina, *hsp70* ou *COWP1*) aínda que se sabe que, coa excepción dalgún *locus* como *gp60* (STRONG et al. 2000; WIDMER 2009), estas son pouco variables (PAIN *et al.* 2005; BOUZID *et al.* 2010).

Pola súa banda, os estudos sobre recombinación son moi importantes para o coñecemento da bioloxía dun parasito dado que ditos eventos provocan o incremento da súa variabilidade xenética e poden favorece-la súa adaptación ós organismos ós cales parasita, que é un dos parámetros empregados na delimitación de especies (MORGAN-RYAN *et al.* 2002; FAYER *et al.* 2010; WIDMER e SULLIVAN 2012). Os criptosporidios mostran fases sexuais durante o seu ciclo vital (ver seccións anteriores) e xenes homólogos ós implicados nos procesos de recombinación meiótica atopados noutros organismos eucariotas, aínda que presentan certas modificacións na secuencia dalgunhas das proteínas que participan nos procesos de replicación e recombinación do ADN (ZHU *et al.* 1999; MILLERSHIP e ZHU 2002; ABRAHAMSEN *et al.* 2004; MILLERSHIP *et al.* 2004; RIDER *et al.* 2005; ROY e PENNY 2007; RIDER e ZHU 2008; RIDER e ZHU 2010). Sen embargo, a pesares de contar cunha serie de marcadores xenéticos que permiten detectar procesos de recombinación dentro do xénero *Cryptosporidium* –que adoitan coincidir coas secuencias empregadas na realización dos estudos poboacionais (ver enriba) (FENG *et al.* 2002; MALLON *et al.* 2003a; XIAO e FENG 2008; DRUMO *et al.* 2012; HERGES *et al.* 2012; WIDMER e SULLIVAN 2012; DE WAELE *et al.* 2013; FENG *et al.* 2013; LI *et al.* 2013; QUÍLEZ *et al.* 2013; FENG *et al.* 2014; ZHAO *et al.* 2014)– ata o día de hoxe non existe evidencias claras de que se produzan entrecruzamentos entre especies diferentes de *Cryptosporidium* (ZHOU *et al.* 2003b). Do que si existen evidencias é de procesos de recombinación sexual –xa fora en observacións naturais ou en estudos experimentais– entre subtipos da mesma especie durante unha infección mixta nun hóspede (WIDMER *et al.* 1998; MALLON *et al.* 2003a; MALLON *et al.* 2003b; WIDMER 2004; TANRIVERDI *et al.* 2007; TANRIVERDI *et al.* 2008; WIDMER e LEE 2010; HERGES *et al.* 2012; DE WAELE *et al.* 2013; LI *et al.* 2013; CACCIÒ *et al.* 2015), aínda que ditas evidencias non son concluíntes respecto á existencia de recombinación meiótica en *Cryptosporidium*. Isto débese a que cabe a posibilidade de que ditos xenotipos quiméricos poidan tratarse de recombinantes de PCR –artefactos obtidos durante o proceso de amplificación no momento de estudar unha infección mixta– (PAABO *et al.* 1990; ZHOU *et al.* 2003b; RUECKER *et al.* 2011). Así as cousas, aínda que os procesos de recombinación se consideran posibles nas especies do xénero *Cryptosporidium*, pénsase que estes só se manifestan de maneira puntual (DRUMO *et al.* 2012) e implican unicamente a algúns xenes ou rexións cromosómicas (LI *et al.* 2013; FENG *et al.* 2014; GUO *et al.* 2015; WIDMER *et al.* 2015).

5. Ferramentas e métodos empregados para detecta-la presenza de *Cryptosporidium*

Existen numerosas ferramentas que permiten o diagnóstico da criptosporidiose, dentro das cales podemos destaca-lo exame histolóxico de biopsias –tanto en microscopio óptico como empregando microscopía electrónica–, a identificación de ooquistes con métodos de tintura ou coloración fluorescente/inmunofluorescente, a detección de antíxenos nas feces mediante enzimoinmunoensayo (ELISA) ou a amplificación e opcional secuenciación de fragmentos de ADN (FAYER e XIAO 2008). Na actualidade, as técnicas que se empregan de maneira máis rutineira na maior parte dos laboratorios son o diagnóstico mediante microscopía e a inmunoanálise (KATANIK *et al.* 2001; GARCÍA *et al.* 2003; SHORE GARCÍA 2007).

5.1 Detección mediante tinturas e microscopía

A detección de ooquistes presentes en mostras mediante microscopía é, sen dúbida, a técnica máis empregada para o estudo e confirmación da criptosporidiose (FAYER e XIAO 2008). Xeralmente, antes da observación é preciso concentra-las mostras. Un dos métodos máis habituais para elo é o de Ritchie, baseado no uso de disolucións de sacarosa de diferente concentración (MCNABB *et al.* 1985; ARROWOOD e STERLING 1987; FAYER e XIAO 2008). Os ooquistes teñen un tamaño similar –entre 4 e 7 μm– ós lévedos e outros organismos unicelulares, polo que é preciso empregar tinturas específicas para a súa correcta identificación. Dentro das máis empregadas podemos atopar:

1. **Tintura Giemsa**: Foi un método moi empregado na década dos setenta (POHLENZ *et al.* 1978; TZIPORI *et al.* 1980). Presenta o problema de que tanto os ooquistes como os fungos tínguense de cor púrpura, o que pode xerar confusións no diagnóstico (AUSINA RUÍZ e MORENO GUILLÉN 2006). As bacterias tamén se tinguen de púrpura, pero estas son distinguibles facilmente polo seu pequeno tamaño.

2. **Modificación do método Ziehl-Neelsen**: Permite sortea-lo problema observado co método anterior, xa que os ooquistes de *Cryptosporidium* tenden a se tinguir de cor rosa ou vermello, no canto que os fungos o fan de cor azul verdoso (HENRIKSEN e POHLENZ 1981; CASEMORE 1991; AUSINA RUÍZ e MORENO GUILLÉN 2006). Un dos

problemas desta tintura é que é moi complexo diferenciar entre os ooquistes de *Cryptosporidium* e os de *Cyclospora* spp. ou *Isospora* spp. (FAYER e XIAO 2008).

3. **Safranina – azul de metileno**: Os ooquistes de *Cryptosporidium* tínguense dunha cor rosa alaranxado brillante, no canto que os fungos, bacterias e os quistes de *Entamoeba* spp. e *Giardia lamblia* o fan de cor azul púrpura (BAXBY *et al.* 1984). As esporas bacterianas e os restos fecais tínguense da mesma cor que os ooquistes (AUSINA RUÍZ e MORENO GUILLÉN 2006).

4. **Fluorescencia con auramina–fenol**: Os ooquistes tínguense dunha cor verde fluorescente e pódense observar directamente baixo filtros ultravioletas. Esta técnica é moito máis sensible que as anteriores, xa que tingue tanto as paredes exteriores do ooquiste como as estruturas interiores debido a que o fenol facilita a penetración da tintura a través das paredes dos quistes (NICHOLS e THOM 1984; CASEMORE *et al.* 1985a; AUSINA RUÍZ e MORENO GUILLÉN 2006; FAYER e XIAO 2008) (**Figura 6**). Esta tintura é a que se emprega de rutina para o diagnóstico da criptosporidiose nos laboratorios do Servizo de Microbioloxía e Parasitoloxía do Hospital Clínico Universitario de Santiago de Compostela.

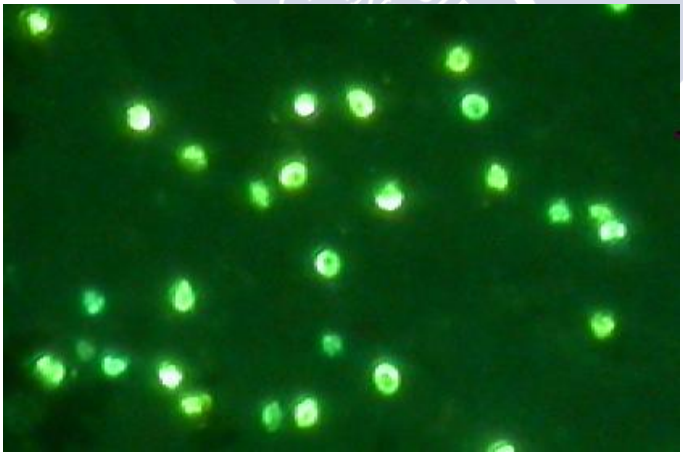


Figura 6. Ooquistes de *Cryptosporidium* spp. tinguidos mediante o método da auramina-fenol (imaxe cedida polo Dr. José Llovo Taboada)

5.2 Técnicas de inmunoanálise

Permiten o estudo, recoñecemento e cuantificación de moléculas utilizando reaccións baseadas no recoñecemento antíxeno-anticorpo. Entre estas destacan:

1. **Inmunofluorescencia directa ou indirecta con anticorpos monoclonais:** Combina microscopía e inmunoanálise (fundaméntase nunha reacción inmunolóxica cun marcador fluorescente), polo que é máis sensible e específica que os métodos descritos anteriormente aínda que require máis tempo de desenrolo e resulta máis cara que as anteriores (XIAO e HERD 1993; AUSINA RUÍZ e MORENO GUILLÉN 2006; FAYER e XIAO 2008).

2. **ELISA (Enzyme-linked ImmunoSorbent Assay)**: Baséase no emprego de enzimas como marcadores inmunoquímicos que van permitir detecta-la presenza de ooquistes (CHAPMAN *et al.* 1990; UNGAR 1990; ROSENBLATT e SLOAN 1993; SHORE GARCÍA 2007). Como no caso anterior, presenta unha alta especificidade, sensibilidade e precisión; e normalmente úsase en forma de kit comercial (KATANIK *et al.* 2001; GARCÍA *et al.* 2003; FAYER e XIAO 2008).

3. **Método cuantitativo por citometría de fluxo**: A citometría de fluxo é unha técnica biofísica que se apoia no emprego dunha luz láser para contar e clasificar células segundo as súas características morfolóxicas e a presenza de diferentes biomarcadores (xeralmente fluoróforos unidos a anticorpos). Este método é unhas 10 veces máis sensible que os métodos de inmunofluorescencia clásicos, pero para levalo a cabo é necesario contar cun equipamento do que carecen a maioría dos laboratorios clínicos (VALDEZ *et al.* 1997; AUSINA RUÍZ e MORENO GUILLÉN 2006; BARBOSA *et al.* 2008).

5.3 Métodos de diagnóstico molecular

Aínda que os métodos de diagnóstico molecular son máis caros e laboriosos que os anteriormente descritos, son moitísimo máis sensibles (WEBSTER 1993; MCLAUCHLIN *et al.* 2003), até o punto de detectar a presenza dun único ooquiste na mostra (FAYER e XIAO 2008). Outra vantaxe é que son os únicos que permiten identificar tanto a especie como o subtipo de *Cryptosporidium* presente na mostra analizada, o cal é esencial á hora de aborda-lo estudo da

epidemioloxía e das vías de transmisión deste parasito (CAMA *et al.* 2003; ZHOU *et al.* 2003a; MCCUIN e CLANCY 2005; SHORE GARCÍA 2007). Ademais, a súa aplicación foi, e segue sendo, decisiva para caracteriza-las distintas especies deste xénero. Nembargante, aínda que na actualidade están publicados os xenomas dalgunhas especies de *Cryptosporidium*, na maioría dos estudos moleculares limítanse á análise dun número reducido de *loci*, entre os que se atopan secuencias de ADN ribosómico, xenes codificantes –como *COWP1*, *hsp70* ou *gp60*– e mini e microsatélites (JIANG e XIAO 2003; CACCIÒ *et al.* 2005; FAYER e XIAO 2008).

As principais ferramentas moleculares empregadas na determinación de especies son:

5.3.1 Técnicas baseadas no estudo de isoenzimas e alozimas

As primeiras ferramentas moleculares empregadas na distinción de especies de *Cryptosporidium* presentes en mostras humanas foron os estudos de isoenzimas e alozimas, que puxeron de manifesto unha grande heteroxeneidade na composición proteica e enzimática destes parasitos (MEAD *et al.* 1990; OGUNKOLADE *et al.* 1993; AWAD-EL-KARIEM *et al.* 1998). Non obstante, e debido principalmente a que era preciso contar cun amplo número de ooquistes purificados, foron rapidamente substituídas pola amplificación de secuencias nucleotídicas mediante a técnica de reacción en cadea da polimerasa (PCR, acrónimo en inglés), a cal presenta a vantaxe de ser máis sensible, específica, e de presentar resultados máis facilmente interpretables (WIDMER e SULLIVAN 2012).

5.3.2 Diagnóstico baseado en PCR

A PCR é un método que permite obter un grande número de copias dunha secuencia determinada de ADN a partires dunhas poucas secuencias moldes empregando cebadores moleculares específicos. O primeiro intento de diagnóstico da criptosporidiose mediante PCR e secuenciación en mostras fecais de procedencia humana e bovina levouse a cabo a principios da década dos noventa, empregando clons (LAXER *et al.* 1991). Anos máis tarde conseguiuse a amplificación de fragmentos da subunidade pequena do ADN ribosómico (tamén denominada *SSU rRNA* ou *18S rRNA*) (JOHNSON *et al.* 1993; JOHNSON *et al.* 1995), secuencia que presenta varias copias no xenoma de *Cryptosporidium* polo que resulta relativamente doada a súa amplificación mediante PCR. Na súa secuencia altérnanse rexións

semiconservadas con rexións hipervariables (LE BLANCQ *et al.* 1997; WIDMER *et al.* 1999; XIAO *et al.* 1999a), o cal permite o deseño de cebadores tanto para detectar unicamente a presenza de ooquistes nunha mostra, como para determinar a especie (RYAN *et al.* 2014). Por esta razón foi considerado o marcador universal para describir molecularmente as diferentes especies de *Cryptosporidium* (XIAO *et al*. 1999b; XIAO *et al*. 2001b; NICHOLS *et al*. 2003; LIM *et al*. 2011; NGUYEN *et al*. 2012), aínda que a presenza de varias copias da *SSU rRNA* no xenoma destes parasitos que son distintas entre si (LE BLANCQ *et al.* 1997; XIAO *et al.* 1999a; STRONG e NELSON 2000; NAVARRO-I-MARTÍNEZ *et al.* 2003; ABRAHAMSEN *et al.* 2004; XU *et al.* 2004) pode derivar en que a detección dun parálogo descoñecido se atribúa erroneamente a unha nova especie. Isto, unido á escasa resolución do método como consecuencia da limitada variación entre especies ou xenotipos de *Cryptosporidium* en dito *locus* (GIBBONS-MATTHEWS e PRESCOTT 2003; ABEYWARDENA *et al.* 2014), fixo necesario estende-lo estudo a outros xenes (SANTÍN e ZARLENGA 2009). Deste xeito ampliouse o catálogo de marcadores de especie cos seguintes *loci*: *actina* (SULAIMAN *et al.* 2002), *β-tubulina* (WIDMER *et al.* 1998; ROCHELLE *et al.* 1999; SULAIMAN *et al.* 1999), proteína 1 da parede celular do ooquiste (*COWP1*) (PATEL *et al*. 1998; HOMAN *et al.* 1999; MCLAUCHLIN *et al.* 1999; TROTZ-WILLIAMS *et al.* 2006) e *hsp70* (SULAIMAN *et al.* 2000; GOBET e TOZE 2001) (**Figura 7**).

As técnicas baseadas na PCR resultaron ter unha sensibilidade maior á das técnicas de inmunofluorescencia empregadas durante a década dos 80 para a identificación de ooquistes (WEBSTER 1993; WEBSTER *et al*. 1993; BALATBAT *et al*. 1996; LENG *et al*. 1996b; FISCHER *et al*. 1998; WIDMER 1998), polo que o seu uso estendeuse rapidamente entre a comunidade científica. A continuación amósanse algunhas das análises moleculares máis comunmente empregadas para interpretar as secuencias obtidas tra-lo proceso de amplificación mediante PCR e que non requiren da secuenciación directa do fragmento amplificado:

5.3.2.1 O uso de RAPDs

Durante os anos 90 aplicouse a amplificación aleatoria de ADN polimórfico (RAPDs no seu acrónimo inglés), mediante cebadores de secuencia arbitraria, para tipar diferentes especies de *Cryptosporidium* (MORGAN *et al*. 1996; TIAN *et al*. 2001; TIAN *et al*. 2002). Esta técnica foi a primeira que permitiu diferenciar xeneticamente a *C. parvum* de *C. hominis* (MORGAN *et al.* 1995). Presenta vantaxes como a rapidez, a escasa cantidade de ADN que precisa, e sobre

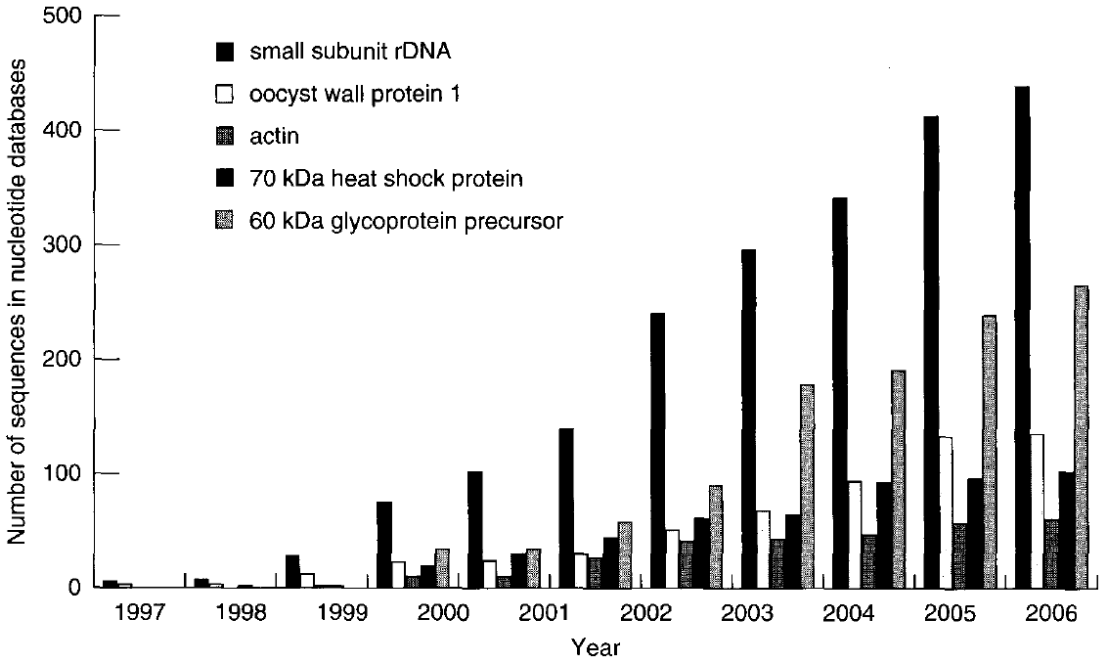


Figura 7. Aumento do número de secuencias dispoñibles dalgúns dos xenes máis estudados dos criptosporidios – *SSU rRNA*, *COWP1, actina, hsp70* e *gp60* – entre os anos 1997 e 2006 nas bases de datos públicas de secuencias (ORTEGA-PIERRES 2009).

todo, que non require coñecemento previo da secuencia do organismo a estudar. Entre as súas limitacións están a complexa interpretación dos resultados e a súa escasa reproducibilidade.

5.3.2.2 Estudos dos padróns de bandas obtidos con enzimas de restrición

Un método particularmente estendido para a identificación da especie de *Cryptosporidium* presente en mostras fecais e ambientais é a análise do polimorfismo na lonxitude dos fragmentos de restrición (PCR-RFLPs, nas súas siglas en inglés) (AWAD-EL-KARIEM *et al.* 1994; LENG *et al.* 1996a; SPANO *et al.* 1997; KIMBELL *et al.* 1999; XIAO *et al.* 1999b; LOWERY *et al.* 2000; PEDRAZA-DÍAZ *et al.* 2001; STURBAUM *et al.* 2001; XIAO *et al.* 2001a; NICHOLS *et al.* 2003; COUPE *et al.* 2005; FELTUS *et al.* 2006; TROTZ-WILLIAMS *et al.* 2006; FENG *et al.* 2007; FAYER e XIAO 2008; GHERASIM *et al.* 2012). Baséase no recoñecemento de secuencias específicas de nucleótidos que serven como punto de corte para determinadas enzimas de restrición, o que orixina patróns de bandas que difiren entre especies. É unha

tecnoloxía rápida, sinxela e económica. A maioría dos estudos que empregan esta técnica baséanse na lonxitude dos fragmentos da *SSU RNA*, particularmente nun fragmento de dito xene duns 830 pares de bases que se dixire coas enzimas *Ase*I (ou *Vsp*I) e *Ssp*I e que permite a determinación de numerosas especies de *Cryptosporidium* que afectan a humanos (XIAO *et al.* 1999b; XIAO *et al.* 2004b; FAYER e XIAO 2008) (**Figura 8**), aínda que tamén existen estudos baseados en RFLPs para outros xenes como *COWP*1 (SPANO *et al.* 1997; PATEL *et al.* 1998; HOMAN *et al.* 1999), *β-tubulina* (CACCIÒ *et al.* 1999) ou *hsp*70 (GOBET e TOZE 2001).

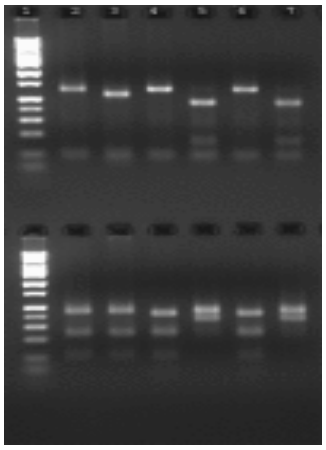


Figura 8. Diferenciación de *Cryptosporidium* spp. mediante a análise por RFLPs do xene *SSU RNA*. Os produtos de PCR foron dixeridos coa enzima *Vsp*I (panel superior) e coa enzima *Ssp*I (panel inferior). No carril 1 aparece o *ladder*, no 2 *C. parvum*, no 3 *C. hominis* e nos seguintes diferentes variantes do xenotipo cervino (FELTUS *et al.* 2006).

5.3.2.3 PCR cuantitativa

Na última década introducíronse novas técnicas na determinación de especies de *Cryptosporidium*, entre as que destaca a PCR cuantitativa – ou PCR a tempo real (qPCR). Na qPCR empréganse diferentes pigmentos fluorescentes que se van ir incorporando ó amplicón nos sucesivos ciclos da PCR, o cal permite cuantifica-la cantidade de produto amplificado. No caso da criptosporidiose, esta característica pode resultar útil para estima-los niveis de concentración de ooquistes que presentan as mostras, xa sexan de orixe medioambiental (JOTHIKUMAR *et al.* 2008; HADFIELD *et al.* 2011; LALONDE *et al.* 2013; MARY *et al.* 2013; STAGGS *et al.* 2013; YANG *et al.* 2013; YANG *et al.* 2014) ou clínica (HADFIELD *et al.* 2011). Nembargante, a súa capacidade de diferenciación entre especies é relativamente baixa (RYAN *et al.* 2014).

5.3.2.4 Polimorfismos de conformación de cadea sinxela (SSCP)

A metodoloxía denominada polimorfismo de conformación de cadea sinxela (SSCP, nas súas siglas en inglés) baséase no distinto comportamento electroforético que amosan segundo a

súa secuencia nucleotídica as cadeas monocatenarias de ADN nun xel de poliacrilamida non desnaturalizante (febras con secuencias distintas presentan migracións diferentes). Esta técnica ven sendo utilizada para a detección e xenotipado de *Cryptosporidium* aínda que o seu uso non está demasiado estendido (GASSER *et al.* 2001; JEX *et al.* 2007; JEX *et al.* 2008; POWER *et al.* 2011).

A maioría das técnicas descritas ata agora resultan moi útiles tanto para demostra-la presenza de ooquistes de *Cryptosporidium* nas mostras analizadas como para caracteriza-las especies/xenotipos presentes nas mesmas. Pola contra, carecen de resolución suficiente como para realizar clasificacións en categorías inferiores ás de especie, como en familia de subtipos ou en subtipos. Esta maior precisión ten utilidade práctica pois permite, por exemplo, identifica-la liñaxe causante dun andazo ou determina-la natureza zoonótica ou antroponótica dunha infección, dado que algunha familia de subtipos só se describiu en humanos (IIc de *C. parvum*) (XIAO 2010). O mesmo ocorre con algúns dos métodos descritos anteriormente, como é o caso dos marcadores RFLPs, que non presentan a resolución suficiente para levar a cabo análises a nivel intraespecífico (WIDMER e SULLIVAN 2012), aínda que se fixeron algúns intentos co *locus gp60* (WU *et al.* 2003; COHEN *et al.* 2006; MUTHUSAMY *et al.* 2006). Debido a estas limitacións, buscáronse marcadores alternativos que, unha vez amplificados e secuenciados, permitiran desenmascarar niveis de variabilidade por debaixo do de especie e establecer subdivisións intraespecíficas, como sería o caso de microsatélites e minisatélites (AIELLO *et al.* 1999; CACCIÒ *et al.* 2000; CACCIÒ *et al.* 2001; ALVES *et al.* 2003a; MALLON *et al.* 2003b; WIDMER *et al.* 2004; TANRIVERDI *et al.* 2006; TANRIVERDI e WIDMER 2006; GATEI *et al.* 2007), o do segundo espazador interno do xene da *SSU rRNA* (internal transcribed spacer-2, *ITS-2*) (MORGAN *et al.* 1999b; GASSER *et al.* 2003; GASSER *et al.* 2004; CHALMERS *et al.* 2005; SCHINDLER *et al.* 2005), e, sobre todo, o do xene da glicoproteína GP60 (GLABERMAN *et al.* 2001; PENG *et al.* 2001; LEAV *et al.* 2002; ALVES *et al.* 2003b; SULAIMAN *et al.* 2005; RYAN *et al.* 2014):

5.3.2.5 Análise da variación en microsatélites e minisatélites

Son secuencias repetidas en tándem constituídas por motivos de un a seis pares de bases (microsatélites) ou máis (minisatélites). Presentan taxas de mutación moi superiores ás das secuencias de copia única, como consecuencia dunha maior taxa de erros da ADN polimerasa

durante a replicación do ADN (GOLDSTEIN e POLLOCK 1997; ELLEGREN 2004; TANRIVERDI e WIDMER 2006). Esto redunda nunha maior variabilidade, que favoreceu o seu uso para detectar diferenzas entre distintas poboacións do parasito. Así, na actualidade atópanse dispoñibles un bo número de marcadores, como o ML1 (CACCIÒ *et al.* 2000), o MS1 (KHRAMTSOV *et al.* 1995), as variacións no número de repeticións de serina que presenta o *locus gp60* (SULAIMAN *et al.* 2005), o MS9 ou o TP14 (MALLON *et al.* 2003a; MALLON *et al.* 2003b), entre outros (FAYER e XIAO 2008; WIDMER e SULLIVAN 2012). Na maioría dos casos estas secuencias só están dispoñibles para o estudo da variabilidade de *C. hominis* e *C. parvum*.

Habitualmente fanse análises simultáneas de varios *loci* hipervariables ou MLTs (*multilocus typing*) co fin de incrementa-lo poder de discriminación das mesmas (WIDMER e SULLIVAN 2012). O seu estudo pode afrontarse de dúas maneiras diferentes, xa sexa mediante a detección de diferenzas de lonxitude dos fragmentos amplificados (MLFTs, *multilocus fragment size-based typing*) ou ben avaliando a heteroxeneidade das secuencias obtidas (MLSTs, *multilocus sequence typing*). No primeiro caso (MLFTs), empréganse electroforeses de xeles de poliacrilamida (FENG *et al.* 2000; TANRIVERDI *et al.* 2006; TANRIVERDI e WIDMER 2006) ou tecnoloxías como GeneScan® (Applied Biosystems) (ALVES *et al.* 2003a; MALLON *et al.* 2003a; MALLON *et al.* 2003b; NGOUANESAVANH *et al.* 2006; FAYER e XIAO 2008), que permite asignar xenotipos ós diversos padróns de lonxitude observados en cada un dos *locus* analizados. Pola súa banda, a secuenciación multilocus (MLSTs) baséase na detección de variabilidade xenética nas secuencias de produtos previamente amplificados por PCR (SULAIMAN *et al.* 2001; GATEI *et al.* 2007; FAYER e XIAO 2008; FENG *et al.* 2011b). En comparación coa aproximación anterior, ten a vantaxe de que permite detectar substitucións nucleotídicas dun único nucleótido (SNPs) (GLABERMAN *et al.* 2001; SULAIMAN *et al.* 2001; PENG *et al.* 2003a; GATEI *et al.* 2006; GATEI *et al.* 2007), o cal permite describir con maior precisión a estrutura poboacional e a epidemioloxía destes parasitos (AIELLO *et al.* 1999; CACCIÒ *et al.* 2000; CACCIÒ *et al.* 2001; ENEMARK *et al.* 2002; GATEI *et al.* 2006; GONCALVES *et al.* 2006; FAYER e XIAO 2008; XIAO 2010).

5.3.2.6 Segundo espazador interno do ARN: ITS-2

A análise das secuencias do segundo espazador interno do ARN (ITS-2) mediante SSCP tamén pode empregarse na subtipaxe de *C. parvum* e *C. hominis* (GASSER *et al.* 2003;

GASSER *et al.* 2004; CHALMERS *et al.* 2005; SCHINDLER *et al.* 2005), pero é unha técnica que apenas se emprega na actualidade xa que –como ocorre tamén para o caso do ITS-1– existen grandes diferenzas entre as secuencias de ambos marcadores mesmo dentro dun mesmo illado (LE BLANCQ *et al.* 1997; GIBBONS-MATTHEWS e PRESCOTT 2003).

5.3.2.7 Variación no *locus gp60*

Sen lugar a dúbidas, a ferramenta máis empregada para subclasificar as especies de *Cryptosporidium* fundaméntase na secuenciación do *locus gp*60 (GLABERMAN *et al*. 2001; GLABERMAN *et al*. 2002; ALVES *et al*. 2003b; PENG *et al*. 2003b; WU *et al*. 2003; ZHOU *et al*. 2003a; SULAIMAN *et al*. 2005; ABE *et al*. 2006; ALVES *et al*. 2006; FELTUS *et al*. 2006; MUTHUSAMY *et al*. 2006; THOMPSON *et al*. 2007; XIAO *et al*. 2007b; FENG *et al*. 2011a). A diferenza das outras dianas empregadas para o subtipado molecular –como é o caso dos microsatélites, dos minisatélites e das rexións ITS-1 e ITS-2, que son rexións non funcionais, o *locus gp60* codifica dúas glicoproteínas (GP40 e GP15) (STRONG *et al.* 2000) que se localizan na superficie da rexión apical do parasito, as cales median o recoñecemento e invasión da célula hóspede (CEVALLOS *et al.* 2000b; PRIEST *et al.* 2000; WINTER *et al.* 2000; LENDNER e DAUGSCHIES 2014).

A grande diversidade haplotípica que amosa este *locus* e a importantísima repercusión que tivo a súa utilización como ferramenta de subtipaxe reflíctese no feito de que a nomenclatura empregada na maioría dos traballos para referirse ás distintas variantes intraespecíficas de *Cryptosporidium* baséase exclusivamente nas variantes atopadas para dito marcador. Dita nomenclatura consiste en codificar cada especie –ou xenotipo– mediante un número romano (*e.g*. I para *C. hominis*, II para *C. parvum*, III para *C. meleagridis*, IV para *C. fayeri*, V para *C. cuniculus*, VII para *C. wrairi* ou XII para *C. ubiquitum*) (GLABERMAN *et al.* 2001; AKIYOSHI *et al.* 2006; FAYER e XIAO 2008; LV *et al.* 2009; POWER *et al.* 2009; WALDRON *et al.* 2009; XIAO *et al.* 2009; FENG *et al.* 2011a; LI *et al.* 2014; RYAN *et al.* 2014). Este número vén seguido dunha letra minúscula que denota a familia alélica de GP60 á que pertence a secuencia estudada (abranguendo da Ia–Ij para *C. hominis*, da IIa–IIo para *C. parvum*, ou da IIIa–IIIg para *C. meleagridis*) (SULAIMAN *et al.* 2005; FAYER e XIAO 2008; XIAO 2010; RYAN *et al.* 2014). Cada familia alélica agrupa secuencias que teñen entre un 98-100% de homoloxía entre elas (sen ter en conta a secuencia do microsatélite que presenta este *locus* no extremo 5').

Na literatura é frecuente que os autores diferencien variantes (subtipos) dentro de ditas familias, determinados pola secuencia do microsatélite do *locus gp60*. O xenotipo codifícase segundo o tipo e número de tripletes sinónimos deste microsatélite de serina: TCA (representado pola letra A), TGC (representado pola letra G), e TCT (representado pola letra T) (FAYER e XIAO 2008; XIAO 2010; FENG *et al.* 2011a). Así, o xenotipo IeA11G3T3 indica que o parasito atopado pertence á especie *C. hominis*, familia de subtipos Ie e que o microsatélite contén 11 repeticións do codón TCA, tres de TCG e tres de TCT (RYAN *et al.* 2014).

Ademais, nalgunhas familias do *locus gp60* describíronse variacións no número de repeticións doutras secuencias (CHALMERS *et al.* 2008; XIAO 2010; RYAN *et al.* 2014), as cales se representan cunha letra R acompañada do número de copias de dita secuencia. Así por exemplo, na familia IIa de *C. parvum*, algúns subtipos teñen dúas copias da secuencia "ACATCA" corrente abaixo das repeticións de serina, o cal se representaría mediante a abreviatura "R2".

Cando unha familia alélica non mostra variacións no número de repeticións dos trinucleótidos de serina, pero si presenta polimorfismo na secuencia do xene que se atopa despois do microsatélite, os seus subtipos diferéncianse mediante a adición de letras ó final (SULAIMAN *et al.* 2005; FAYER e XIAO 2008; XIAO 2010; RYAN *et al.* 2014). Isto ocorre, por exemplo, coa familia alélica IIc, cuxos subtipos son todos A5G3 pero amosan variación na rexión 3' do *locus* (IIcA5G3a, IIcA5G3b, IIcA5G3c, etc.).

Este grado de precisión na subclasificación dos illados que permite a secuencia do *locus gp60* ten moito interese epidemiolóxico, xa que permite identificar linaxes con distintas propiedades patoxénicas (CAMA *et al.* 2007) ou de especificidade de hóspede –caso do subtipo IIc de *C. parvum* que só parasita a humanos (XIAO 2010)–, permitindo levar a cabo estudos epidemiolóxicos máis rigorosos e profundos sobre a criptosporidiose que aqueles que empregan unicamente datos a nivel de especie.

6. Manifestacións clínicas e sintomatoloxía da criptosporidiose

A enfermidade soe cursar cunha gastroenterite aguda autolimitada, que se manifesta en forma de diarrea acuosa e abundante, e que pode conter mucosidades (PALMER e BIFFIN 1990; HUNTER *et al.* 2004a; CHALMERS e DAVIES 2010). Aínda que en ocasións pode cursar case asintomática, a infección vai acompañada con frecuencia de vómitos, febre, perda de peso e

do apetito, dores abdominais, cólicos, malestar xeral, flatulencias, náuseas, deshidratación e/ou fatiga. Estes síntomas duran entre dúas e tres semanas (MACKENZIE *et al.* 1994; INSULANDER *et al.* 2005; THOMPSON *et al.* 2005; BOUZID *et al.* 2013), aínda que nalgúns doentes pode chegar a durar máis dun mes (ISAACS *et al.* 1985; WOLFSON *et al.* 1985; FAYER e XIAO 2008; CHALMERS e DAVIES 2010), chegando incluso a establecerse unha posible relación entre as infeccións causadas por este grupo de parasitos con enfermidades crónicas do intestino (WOLFSON *et al.* 1985; CURRENT e GARCIA 1991; MACKENZIE *et al.* 1995; HUNTER *et al.* 2004a; HUNTER *et al.* 2004b; BUSHEN *et al.* 2007; CHALMERS e DAVIES 2010; KOEHLER *et al.* 2013).

A gravidade sintomatolóxica e a duración da parasitación son función do estado immunolóxico e nutricional do hóspede (HUNTER e NICHOLS 2002; THOMPSON *et al.* 2005; CHALMERS e DAVIES 2010; KOTLOFF *et al.* 2013), de xeito que nos países en vías de desenvolvemento a criptosporidiose pode chegar a comprometer o normal desenrolo dos nenos por estar intimamente asociada coa malnutrición, a perda de peso e a deshidratación, chegando a ser letal nos casos máis graves (TUMWINE *et al.* 2003; RAMÍREZ *et al.* 2004; CHALMERS e DAVIES 2010; BOUZID *et al.* 2013; KOTLOFF *et al.* 2013; LEVINE *et al.* 2013; STRIEPEN 2013). Os doentes inmunocomprometidos –persoas afectados polo VIH, transplantados, que reciben tratamentos de quimioterapia, etc.– adoitan presentar un cadro clínico máis grave: diarreas crónicas máis severas e prolongadas (THOMPSON *et al.* 2005), acompañadas de fortes perdas de peso (GOLDSTEIN *et al.* 1996) e doutras enfermidades atípicas, como a colanxite, a pancreatite, a sinusite ou a pneumatose intestinal (MANABE *et al.* 1998; HUNTER e NICHOLS 2002; SHORE GARCÍA 2007; CHALMERS e DAVIES 2010; BOUZID *et al.* 2013). Isto supón maior risco de secuelas (TANYUKSEL *et al.* 1995; SREEDHARAN *et al.* 1996; HUNTER e NICHOLS 2002; RAJA *et al.* 2014), e un maior perigo para a vida dos doentes (JURANEK 1995; MANABE *et al.* 1998; SHORE GARCÍA 2007; FAYER e XIAO 2008).

A infección amosa dous períodos diferenciados: prepatente e patente. O período prepatente abrangue dende a inxestión do parasito ata a excreción dos primeiros ooquistes formados dentro do hóspede (FAYER e XIAO 2008). A súa duración varía en función da especie de *Cryptosporidium*, do estado de saúde e inmunitario do hóspede e da dose infectiva inicial (DUPONT *et al.* 1995; CHAPPELL *et al.* 1996). Así, esta fase dura entre 2 e 12 días no gando bovino e ovino (TZIPORI *et al.* 1981; TZIPORI *et al.* 1983; FAYER *et al.* 2005; THOMPSON *et al.* 2005), de 4 a 22 días en humanos (DUPONT *et al.* 1995; RAMÍREZ *et al.* 2004; CHAPPELL *et al.* 2006) –cunha media de 7,2 días (JOKIPII e JOKIPII 1986)–, de 2 a 9 días

en porcos (ENEMARK *et al.* 2003), de 6 a 21 días en ratos (MATSUI *et al.* 1999), de 5 e 6 días en gatos (ISEKI 1979; AUGUSTIN-BICHL *et al.* 1984) e de 4 a 24 días en polos (CURRENT *et al.* 1986).

O período patente ven determinado polo número de días durante os cales o hóspede excreta ooquistes viables. Ten una duración de 1 a 18 días en gando bovino (ANDERSON e BULGIN 1981; TZIPORI *et al.* 1981; TZIPORI *et al.* 1983), de 9 a 15 días en porcos (MOON e BEMRICK 1981), de 7 a 10 días en gatos (ISEKI 1979), e de ata 18 días en polos (FAYER e XIAO 2008). Nos humanos inmunocompetentes pode durar entre 2 e 24 días (SHORE GARCÍA 2007; FAYER e XIAO 2008), aínda que pode prolongarse máis de 30 días no caso dos doentes inmunocomprometidos días (JOKIPII e JOKIPII 1986; CHAPPELL *et al.* 2006; SHORE GARCÍA 2007).

7. Capacidade infectiva, reservorios e vías de transmisión

O xénero *Cryptosporidium* presenta unhas características biolóxicas (presenza de formas de resistencia) e ambientais (son especies ubicuas, atópanse en todo o entorno natural agás o aire) (UNITED STATES ENVIRONMENTAL PROTECTION AGENCY 2001a; UNITED STATES ENVIRONMENTAL PROTECTION AGENCY 2001b; FAYER e XIAO 2008) que o converten nun risco potencial para un grande número de seres vivos. Proba de elo é súa detección en preto de 200 especies –todas elas de vertebrados– entre as que se atopan mamíferos (>100 especies), aves (>30), réptiles (>50) e case unha decena de especies de anfibios e peixes (O'DONOGHUE 1995; FAYER *et al.* 2000; FAYER 2004; XIAO *et al.* 2004a; NAVARRO-I-MARTÍNEZ *et al.* 2011; RYAN *et al.* 2014; REBOREDO-FERNÁNDEZ *et al.* 2015a; REBOREDO-FERNÁNDEZ *et al.* 2015b).

A maioría das infeccións detectadas en humanos son causadas por *C. hominis* e *C. parvum* (CACCIÒ *et al.* 2005; CHALMERS *et al.* 2009; XIAO 2010; CHALMERS *et al.* 2011). A familia de subtipos atopada con máis frecuencia é a Ib de *C. hominis* –e, en menor medida, as Ia, Id, Ie e If–, e as familias de subtipos IIa, IIc e IId de *C. parvum* (JEX e GASSER 2010; XIAO 2010; WALDRON e POWER 2011; RYAN *et al.* 2014). Nembargante, a pesares da grande incidencia destas dúas especies, non se pode ignorar a presenza documentada dunha ducia de especies deste xénero como *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. fayeri* ou *C. cuniculus* que tamén parasitan a humanos (XIAO 2010; WALDRON *et al.* 2011a; ELWIN *et al.* 2012a; RYAN *et al.* 2014) (**Táboa 2**).

É importante sinalar que a dose infectante da criptosporidiose é moi baixa –no caso de *C. parvum*, a ID50 ou dose necesaria para infectar ó 50% do grupo experimental sitúase entre 9 e 1042 ooquistes (DUPONT *et al*. 1995; OKHUYSEN *et al*. 1999; FAYER *et al*. 2000; CHAPPELL *et al*. 2006)–, o que fai que a súa capacidade de transmisión sexa elevada. Esta realízase por vía fecal–oral de catro maneiras diferentes (SHORE GARCÍA 2007; FAYER e XIAO 2008):

- Transmisión de persoa a persoa (ou *vía antroponótica*), por contacto directo con persoas infectadas (KOCH *et al.* 1985; EL-SIBAEI *et al.* 2003; HELLARD *et al.* 2003; HUNTER *et al.* 2004b; ARTIEDA *et al.* 2012). É unha vía de transmisión bastante común e dáse principalmente nos empregados de garderías infantís, entre o persoal sanitario ou aquel que está ó coidado de persoas infectadas. A transmisión dentro dunha mesma familia tamén é frecuente, particularmente cando a infección primaria se produce en nenos. O risco de infección vese incrementado naquelas prácticas sexuais que implican contacto oral–anal.
- Transmisión directa de animais a persoas (ou *vía zoonótica*) debido ó contacto con animais infectados (GRACZYK *et al.* 1997; ASHBOLT *et al.* 2003; STANTIC-PAVLINIC *et al.* 2003; HUNTER *et al.* 2004b; WEBB *et al.* 2014). É pouco frecuente, e aínda que nalgúns casos afecta a individuos inmunocomprometidos (MORGAN *et al.* 2000), na maioría das ocasións maniféstase como enfermidade profesional en veterinarios, granxeiros e gandeiros (CURRENT 1983; ENRÍQUEZ *et al.* 2001).
- Transmisión debida á contaminación de alimentos con ooquistes viables de *Cryptosporidium* (DAWSON 2005; KNIEL e JENKINS 2005; BLACKBURN *et al.* 2006; SMITH *et al.* 2006; ROSENTHAL *et al.* 2015). Esta pode producirse tanto de xeito directo (polo acceso de gando infectado ós cultivos, pola manipulación incorrecta dos alimentos ou polo uso de esterco contaminado) como indirecto (por irrigación, lavado ou procesado dos cultivos con auga contaminada) (QUIROZ *et al.* 2000; FAYER e XIAO 2008). Normalmente este tipo de transmisión vincúlase ó consumo de produtos frescos como froitas, verduras ou leite (MILLARD *et al.* 1994; GELLETLIE *et al.* 1997; ORTEGA *et al.* 1997; ROBERTSON e GJERDE 2001; CALVO *et al.* 2004; ORTEGA-PIERRES 2009; ROSENTHAL *et al.* 2015), aínda que tamén se atopan ooquistes viables nas carnes procesadas nalgúns matadoiros e tendas de alimentación (por exemplo, nas salchichas) (KANETA e NAKAI 1998; KOYAMA *et al.* 2005; MORIARTY *et al.* 2005) e en moluscos bivalvos –como ostras, ameixas, berberechos

ou mexillóns (TAMBURRINI e POZIO 1999; GRACZYK *et al.* 2003; FAYER 2004; GÓMEZ-COUSO *et al.* 2004a; GIANGASPERO *et al.* 2009; ORTEGA-PIERRES 2009).

- Transmisión indirecta debida á contaminación dos recursos hídricos con ooquistes infectivos, xa sexan destinados ó consumo humano ou ó lecer (caso das piscinas ou parques acuáticos) (FAYER 2004; INSULANDER *et al.* 2005; CAUSER *et al.* 2006; SMITH *et al.* 2006; SEMENZA e NICHOLS 2007; CASTRO-HERMIDA *et al.* 2008b; CASTRO-HERMIDA *et al.* 2008a; MASON *et al.* 2010; BALDURSSON e KARANIS 2011; DREELIN *et al.* 2014; SPANAKOS *et al.* 2015). A maioría das infeccións prodúcense a partir de ooquistes procedentes das feces de gando –principalmente bovino, aínda que tamén ovino e caprino, e de animais salvaxes (e.g. FARIZAWATI *et al.* 2005; CASTRO-HERMIDA *et al.* 2006b; CASTRO-HERMIDA *et al.* 2011b; BUDU-AMOAKO *et al.* 2012; REBOREDO-FERNÁNDEZ *et al.* 2015a).

A capacidade de sobrevivir no exterior durante meses (FAYER *et al.* 2000; MEDEMA e SCHIJVEN 2001), grazas á protección que ofrece a parede celular (ROBERTSON *et al.* 1992) e ó seu pequeno tamaño –apenas unhas micras de lonxitude–, fan que os ooquistes de *Cryptosporidium* sexan moi difíciles de eliminar mediante técnicas físicas (FAYER *et al.* 2000). Tamén contribúe a elo o feito de que sexan resistentes ó tratamento coa maioría dos desinfectantes, polo que non é doado retiralos cos tratamentos rutineiros utilizados nos procesos de depuración de augas (KORICH *et al.* 1990; CARMENA *et al.* 2007; SHORE GARCÍA 2007; CASTRO-HERMIDA *et al.* 2008a; FAYER e XIAO 2008; ORTEGA-PIERRES 2009; BAJER *et al.* 2012).

Tanto os seres humanos como os animais infectados excretan grandes cantidades de ooquistes nas súas deposicións (entre 10^6 e 10^{11} ooquistes/gramo de feces) (GOODGAME *et al.* 1993; ORTEGA-MORA e WRIGHT 1994; CHAPPELL *et al.* 1996; NYDAM *et al.* 2001; PARAUD *et al.* 2014), que presentan capacidade infectiva dende o mesmo momento en que son expulsados (SMITH e ROSE 1998). Deste xeito, nas zonas rurais tipicamente gandeiras a contribución dos animais infectados á contaminación ambiental é moi importante, en particular na contaminación das augas superficiais e das augas destinadas ó consumo humano e ó regadío (FARIZAWATI *et al.* 2005; CASTRO-HERMIDA *et al.* 2008b; CASTRO-HERMIDA *et al.* 2008a; CASTRO-HERMIDA *et al.* 2009; BUDU-AMOAKO *et al.* 2012; CASTRO-HERMIDA *et al.* 2015).

Un entendemento máis profundo da epidemioloxía deste parasito permitiría reduci-los custes sanitarios que causan os andazos de criptosporidiose en humanos, calculados en torno a uns 32 millóns de dólares anuais en custos médicos e de case 100 millóns de dólares en perdas de produtividade para países como os Estados Unidos (ZARDI *et al.* 2005; HUANG e WHITE 2006; YODER *et al.* 2012; RYAN *et al.* 2014). Do mesmo xeito, as infeccións provocadas por *Cryptosporidium* xeran importantes gastos veterinarios e elevadas perdas económicas nas explotacións gandeiras ó ser un dos principais axentes causais do síndrome da diarrea neonatal en ruminantes de interese comercial (gando vacún, ovino e caprino), a cal pode ocasionar unha forte deshidratación que retrase o normal crecemento destes animais ou mesmo provoca-la súa morte (HEINE *et al.* 1984; DE GRAAF *et al.* 1999; O'HANDLEY e OLSON 2006). Do mesmo xeito, a análise moleculares da criptosporidiose no gando contribuirá ao estudo dos patróns de transmisión desta enfermidade debido a que este é un dos seus grandes reservorios, podendo así desenrolar novas medidas de prevención destinadas a minimizar a exposición ao parasito e a mellorar o tratamento de augas destinadas ó consumo humano (CORSO *et al.* 2003; ZHOU *et al.* 2003a; XIAO *et al.* 2004b). Porén, un maior coñecemento da bioloxía e epidemioloxía deste parasito ten unha grande importancia sanitaria e económica, especialmente cando aínda non existe ningunha vacina ou tratamento farmacolóxico específico completamente eficaz que serva para deter dita infección (AMADI *et al.* 2002; ARMSON *et al.* 2003; SHORE GARCÍA 2007; FAYER e XIAO 2008; AMADI *et al.* 2009; CABADA e WHITE 2010; STRIEPEN 2013; CHECKLEY *et al.* 2015).

7.1 Taxas de incidencia e outros aspectos epidemiolóxicos xerais da criptosporidiose en humanos

A incidencia da criptosporidiose está asociada sobre todo á idade e ao estado inmunolóxico dos doentes así como ao seu entorno medioambiental e estatus socioeconómico. Aínda que afecta a tódalas franxas de idade, amosa maiores taxas de incidencia e síntomas máis severos no grupo formado polos rapaces menores de dez anos (MOSIER e OBERST 2000; KHAN *et al.* 2004; CHALMERS e DAVIES 2010). Logo da infancia a incidencia diminúe de xeito continuo a medida que avanzan os tramos de idade, aínda que se detectaron algúns casos de criptosporidiose en persoas anciáns (CHALMERS *et al.* 2009; CHALMERS *et al.* 2011; ELWIN *et al.* 2012a).

Esta enfermidade diagnostícase en todo o mundo, o que demostra que *Cryptosporidium* é un parasito cosmopolita e cunha grande ubicuidade ambiental. Nos países desenvolvidos presenta xeralmente prevalenzas menores ó 4%, tendo en conta a persoas de tódalas idades (FAYER e UNGAR 1986; SHORE GARCÍA 2007), cunha taxa de incidencia de 2,9 casos por 100.000 habitantes nos EEUU (YODER *et al.* 2012) e de 2,29 casos por 100.000 habitantes de media nos países europeos (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2013) no ano 2010. Por contra, nos países en vías de desenvolvemento a prevalenza é maior –principalmente en nenos con idades comprendidas entre os 0 e 5 anos–, podendo chegar ó 20% nalgunhas rexións de África e Sudamérica (SOAVE *et al*. 1989; CHACÍN-BONILLA *et al*. 1997; ESTEBAN *et al*. 1998; NCHITO *et al*. 1998; SHORE GARCÍA 2007; SHIRLEY *et al*. 2012). Isto débese principalmente á maior exposición a auga non potabilizada e a alimentos contaminados por ooquistes de *Cryptosporidium* spp., ó contacto con animais infectados, ás malas condicións sanitarias e á malnutrición crónica que padece a poboación infantil destes países (KOTLOFF *et al*. 2013).

A prevalenza da criptosporidiose nos doentes inmunodeprimidos tamén é maior naqueles países que presentan unha renda mais baixa (ASPOCK e HASSL 1990; GÓMEZ MORALES *et al*. 1992; POZIO *et al*. 1997; MATOS *et al*. 1998). Así, en países desenvolvidos atópase ó redor do 10–15% mentres que en países en vías de desenvolvemento dita cifra sitúase entre o 30 e 50% (PETERSEN 1992; GUERRANT 1997; SHORE GARCÍA 2007). Con respecto a este grupo de doentes cabe destacar que, se ben é certo que durante os anos oitenta e principios dos noventa a criptosporidiose asociouse cun aumento da taxa de mortalidade dos doentes inmunocomprometidos tanto nos países desenvolvidos como nos países en vías de desenvolvemento (GOLDSTEIN *et al*. 1996), esta diminuíu drásticamente a partires de finais dos anos noventa grazas á introdución das novas terapias antiretrovirais (MANABE *et al*. 1998; POZIO e MORALES 2005; NISSAPATORN e SAWANGJAROEN 2011).

Nembargante, hai varias circunstancias que dificultan o coñecemento preciso das taxas de incidencia e prevalenza reais da criptosporidiose na poboación xeral. En primeiro lugar, dado que a grande maioría das infeccións causadas por *Cryptosporidum* spp. son asintomáticas ou provocan diarreas autolimitadas, esta enfermidade non se declara de xeito habitual ás autoridades sanitarias nacionais. Unido a isto, outra característica que dificulta o estudo epidemiolóxico desta enfermidade débese a que a lexislación que regula a notificación dos casos positivos de criptosporidiose varía de maneira considerable en distintos países. Así, por exemplo, a criptosporidiose é unha enfermidade de declaración obrigatoria tanto nos

Estados Unidos (YODER *et al.* 2012) como no Reino Unido –o cal conta mesmo cun centro de referencia adicado integramente o estudo deste parasito (*Cryptosporidium* Reference Unit CRU, Swansea, Reino Unido; http://www.wales.nhs.uk/sites3/page.cfm?orgId=457&pid=25284)– dende os anos noventa. Sen embargo, e a pesares de que os casos de criptosporidiose deben ser notificados en toda Europa dende a pasada década (SEMENZA e NICHOLS 2007), existen aínda un bo número de países (maioritariamente do Sur e do Leste de Europa) que ou ben non vixían de xeito efectivo a criptosporidiose ou só declaran unha fracción reducida dos casos –principalmente cando son debidos a un andazo ou á contaminación de alimentos ou reservorios de auga–, ocasionando así que as taxas de incidencia nacionais para dita enfermidade sexan particularmente baixas (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2007; EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2013). Dadas estas circunstancias, cabe supoñer que a incidencia real de dita enfermidade na poboación xeral é moito maior que a que amosan os datos oficias e os diversos estudos epidemiolóxicos publicados ata a actualidade.

8. A criptosporidiose en Europa e en España

A resolución dos traballos epidemiolóxicos que existen a nivel europeo sobre a criptosporidiose varían segundo a rexión. Así, os países do norte de Europa levan varios lustros estudando a presenza destes parasitos no seu medio ambiente, nas súas explotacións gandeiras e na súa poboación por medio de traballos epidemiolóxicos de grande envergadura, nos cales se engloban un bo número de casos/mostras e se avalían un grande número de variables que permiten obter unha panorámica clara sobre a incidencia, periodicidade e patoxenicidade desta infección (ENEMARK *et al.* 2002; SEMENZA e NICHOLS 2007; CHALMERS *et al.* 2009; GARVEY e MCKEOWN 2009; CHALMERS *et al.* 2011; FOURNET *et al.* 2013). Por contra, o estudo da criptosporidiose nos países da cunca mediterránea e do sur de Europa está moito menos desenvolvido, existindo un menor número de publicacións sobre a enfermidade que, xeralmente, avalían un número menor de mostras ou ben só casos puntuais (ALVES *et al.* 2003a; ALVES *et al.* 2003b; ALVES *et al.* 2006; DRUMO *et al.* 2012; SPANAKOS *et al.* 2015). De feito, o primeiro estudo epidemiolóxico da criptosporidiose a nivel estatal para un país do sur de Europa levouse a cabo en datas relativamente recentes en Francia (THE ANOFEL *CRYPTOSPORIDIUM* NATIONAL NETWORK 2010), o cal se puido desenrolar grazas á creación no

citado país dunha rede hospitalaria publica de vixilancia desta enfermidade de adscrición voluntaria.

O coñecemento epidemiolóxico da criptosporidiose en España é limitado. Trátase dunha enfermidade pouco notificada e suxeita a vixilancia por parte das autoridades sanitarias españolas dende o ano 2009 (NAVARRO-I-MARTÍNEZ *et al.* 2011), sendo de declaración obrigatoria só dende o ano 2013 (RED NACIONAL DE VIGILANCIA EPIDEMIOLÓGICA 2013). Os rexistros oficiais dispoñibles para esta enfermidade anteriores a dita data débense á notificación semanal dos casos positivos detectados que efectuaban algúns laboratorios dos servizos de microbioloxía clínica dos hospitais adscritos o sistema nacional de saúde, aínda que dito procedemento era voluntario e só participaban no mesmo unhas poucas Comunidades Autónomas (CENTRO NACIONAL DE EPIDEMIOLOGÍA 2003). Grazas a estes datos sábese que na pasada década producíanse unha media duns 100 casos ó ano (CENTRO NACIONAL DE EPIDEMIOLOGÍA 2003) e que a enfermidade amosaba unha incidencia menor a 1 caso por 100.000 habitantes/ano (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2007; EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2013).

En España existen relativamente poucos traballos que avalíen mostras humana –a maioría dos estudos da criptosporidiose realízanse en mostras procedentes de explotacións gandeiras ou acuícolas (GÓMEZ-BAUTISTA *et al.* 2000; NAVARRO-I-MARTÍNEZ *et al.* 2003; GÓMEZ-COUSO *et al.* 2004a; SUÁREZ-LUENGAS *et al.* 2007; QUÍLEZ *et al.* 2013), así como en mostras de orixe ambiental (CARMENA *et al.* 2007; ALTZIBAR *et al.* 2015)– os cales ademais non reflicten necesariamente a realidade epidemiolóxica da enfermidade na poboación xeral xa que se centran no estudo de andazos puntuais (ARTIEDA *et al.* 2012), na notificación de casos observados en doentes inmunocomprometidos (LLORENTE *et al.* 2007), nos casos detectados nalgúns hospitais (DE LUCIO *et al.* 2015; SEGURA *et al.* 2015) ou ben se tratan de revisións onde se recompilan os casos detectados ó longo dos anos (SOLER 2004).

9. A criptosporidiose en Galicia

Nos últimos anos hai un número crecente de estudos da criptosporidiose no ámbito galego, que se fundamentan sobre todo no estudo da presenza de ooquistes viables en mostras ambientais ou en mostras procedentes de animais de interese gandeiro ou acuícola.

Con respecto ás primeiras, destacan aquelas referidas a análise da contaminación das augas dalgúns ríos galegos –como sería o caso do Tambre e os seus afluentes (CASTRO-

HERMIDA *et al.* 2009)– e os traballos realizados sobre a presenza de ooquistes viables tanto en augas destinadas ó consumo humano como nas plantas de depuración e tratamento de augas, nos cales se constatou que as principais especies presentes nas nosas augas son *C. parvum*, *C. hominis* e *C. andersoni*, e que os métodos dos que dispoñen actualmente as plantas de tratamento de augas en Galicia non son totalmente efectivos á hora de inactiva-la capacidade infectiva destes parasitos (VILLACORTA-MARTÍNEZ DE MATURANA *et al.* 1992; CASTRO-HERMIDA *et al.* 2008b; CASTRO-HERMIDA *et al.* 2008a; CASTRO-HERMIDA *et al.* 2010; CASTRO-HERMIDA *et al.* 2011a; CASTRO-HERMIDA *et al.* 2015). Na maioría destes proxectos destinados a determina-la presenza de ooquistes viables nas plantas de tratamentos de augas participan o Departamento de Produción Animal do Centro de Investigacións Agrarias de Mabegondo (adscrito á Xunta de Galicia) e o departamento de Microbioloxía e Parasitoloxía da Universidade de Santiago de Compostela.

En Galicia existe un censo de gando bovino que chega case ó millón de reses –segundo datos do Instituto Galego de Estatística (www.ige.eu)–, así como un grande número de cabezas de gando ovino, caprino e porcino. Dada a importancia económica de dito sector na nosa comunidade e a que a criptosporidiose no gando pode ocasionar grandes perdas económicas, nos últimos anos aumentou de xeito notable o número de publicacións adicadas a analiza-lo impacto desta enfermidade nas granxas galegas e en animais salvaxes (VILLACORTA *et al.* 1991; LORENZO *et al.* 1995; ARES-MAZÁS *et al.* 1999; CASTRO-HERMIDA *et al.* 2005; GÓMEZ-COUSO *et al.* 2005; CASTRO-HERMIDA *et al.* 2006a; CASTRO-HERMIDA *et al.* 2006b; CASTRO-HERMIDA *et al.* 2007; GÓMEZ-COUSO *et al.* 2007; DÍAZ *et al.* 2010a; DÍAZ *et al.* 2010b; CASTRO-HERMIDA *et al.* 2011b; GARCÍA-PRESEDO *et al.* 2013b; GARCÍA-PRESEDO *et al.* 2013a; REBOREDO-FERNÁNDEZ *et al.* 2015a). Os resultados destes traballos amosaron que a principal especie infectante do gando galego é *C. parvum*, aínda que tamén se observou a presenza de *C. xiaoi* e o xenotipo cervino en gando ovino e caprino, cuxos ooquistes poderían chegar a contamina-las augas superficiais destinadas ó consumo humano ou ó regadío (CASTRO-HERMIDA *et al.* 2008b; CASTRO-HERMIDA *et al.* 2009; CASTRO-HERMIDA *et al.* 2011a). Nestes procesos de monitorización da enfermidade na cabana gandeira galega participa tamén o Laboratorio de Sanidade e Produción Animal que a Xunta de Galicia ten en Lugo (http://www.medioruralemar.xunta.es/areas/gandaria/sanidade_animal/laboratorio/).

Outro sector cunha grande implantación en Galicia é o da acuicultura. O feito de que se demostrara a contaminación das augas superficiais galegas con ooquistes viables de

Cryptosporidium spp., unido a que algunhas das especies de interese acuícola en Galicia son moluscos filtradores, levou á que se realizasen estudos para determina-la presenza de ooquistes nestes organismos –principalmente en mexillóns, ostras, ameixas ou berberechos– e avalia-la súa capacidade infectiva unha vez consumidos. As principais especies atopadas en moluscos foron *C. parvum* e *C. hominis*, procedentes con toda probabilidade dos cursos de auga que desembocan nas rías (FREIRE-SANTOS *et al.* 2000; GÓMEZ-BAUTISTA *et al.* 2000; GÓMEZ-COUSO *et al.* 2003; GÓMEZ-COUSO *et al.* 2004a; GÓMEZ-COUSO *et al.* 2006b). É particularmente salientable que os seus ooquistes manteñen intacta a capacidade para infectar, incluso despois da súa preparación e cociñado (FREIRE-SANTOS *et al.* 2001; FREIRE-SANTOS *et al.* 2002; GÓMEZ-COUSO *et al.* 2006a).

Finalmente, a pesares de que en Galicia existen poucos datos sobre a incidencia da criptosporidiose en humanos –non foi incluída como enfermidade de declaración obrigatoria rutineira ante a Rede Galega de Vixilancia en Saúde Pública (RGVSP) ata finais do ano 2013 (CONSELLARÍA DE SANIDADE 2013; BOLETÍN EPIDEMIOLÓXICO DE GALICIA 2014)–, si que hai estudos sobre a incidencia da enfermidade en doentes infantís e afectados pola SIDA (VILLACORTA MARTÍNEZ DE MATURANA 1989; LLOVO TABOADA 1990) e sobre algún andazo epidémico (GÓMEZ-COUSO *et al.* 2004b). Nembargante, todos estas análises teñen en común a avaliación dun número reducido de casos que non serven para determina-la incidencia real de *Cryptosporidium* spp. na poboación nin para inferi-las súas variacións espaciais, temporais ou estacionais desta enfermidade na nosa Comunidade Autónoma.

XUSTIFICACIÓN e OBXECTIVOS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Xustificación

Os ooquistes das diversas especies de *Cryptosporidium* presentan diferenzas morfolóxicas moi sutís, o cal dificulta a identificación precisa das especies e subtipos do parasito en mostras clínicas ou medioambientais. A metodoloxía clásica empregada para a súa detección baséase fundamentalmente na microscopía ou en técnicas de inmunoanálise. Nembargante, estas amosan un baixo nivel de resolución, pois unicamente permiten detecta-la presenza de ooquistes de criptosporidios nas mostras analizadas. Así, a correcta identificación das especies e subtipos infectantes só pode facerse recorrendo ó uso de técnicas moleculares que exploran a variación xenética dos parasitos. Mais o coñecemento dos padróns de diversidade xenética e da estrutura poboacional deste grupo de protozoos –incluídas as daquelas liñaxes que parasitan humanos– é aínda incipiente, xa que a maioría dos estudos céntranse na análise dun número reducido de *loci*. Isto redunda nunha limitada resolución dos estudos epidemiolóxicos existentes tanto na súa dimensión xeográfica como temporal, nun coñecemento superficial das propiedades xenéticas das poboacións naturais do parasito e nun descoñecemento case absoluto das posibles asociacións entre a constitución xenética das distintas especies e subtipos do parasito e as súas propiedades patoxénicas. Neste contexto faise evidente a necesidade de desenvolver iniciativas ambiciosas para a análise xenética de múltiples *loci* en grandes coleccións de mostras que sexan representativas destas parasitacións en distintas áreas xeográficas do mundo e recollidas ó longo do tempo, especialmente agora que a criptosporidiose é recoñecida como unha enfermidade de declaración obrigatoria en tódolos países europeos. Nembargante, as técnicas moleculares dispoñibles actualmente –baseadas na amplificación por PCR e secuenciación dun número reducido de *loci* marcadores– esixen unha inversión de tempo e recursos inabordables para a maioría dos laboratorios clínicos.

Con estes antecedentes, na presente tese plantexámonos a necesidade de describi-los padróns de diversidade multilocus das principais especies de *Cryptosporidium* que afectan a humanos. Esta análise permitirá determinar se a clasificación das especies destes parasitos en subtipos de acordo á secuencia do *locus gp60* representa fielmente a variación xenética no resto do xenoma do parasito. Esta información serviría como punto de partida para o desenrolo dun protocolo de xenotipado de alto rendemento da criptosporidiose rápido, fiable, automatizado e económico, e baixo uns estándares e criterios sólidos de identificación da especie e do subtipo infectante de aplicación sobre grandes coleccións de mostras. Dita

ferramenta diagnóstica sería empregada sobre mostras fecais positivas para *Cryptosporidium* spp. procedentes tanto de doentes da área sanitaria de Santiago de Compostela como de gando vacún, cuxos resultados servirían para realiza-lo primeiro estudo epidemiolóxico a grande escala desta enfermidade en humanos nun país do Sur de Europa.

Na actualidade existe unha plétora de ferramentas de xenotipado de alto rendemento deseñadas para a determinación da secuencia dun elevado número variable de marcadores moleculares en grandes colecciones de muestras. Entre elas destacan: (i) *Taqman SNP Genotyping Assays*, (ii) *SNaPshot Multiplex System*, e (iii) *SNPlex Genotyping System*, todas elas comercializadas por Applied Biosystems, Inc.; (iv) *MassARRAY System* (Agena Bioscience, Inc.; antes Sequenom) e as plataformas de secuenciación mediante microarrays (v) *Illumina SNP Genotyping* (Illumina, Inc.) e (vi) *Affimetrix Microarrays* (Affymetrix, Inc.).

Para o deseño dunha ferramenta xenotipado masivo de *Cryptosporidium* seleccionamo-lo *MassARRAY System* (Agena Bioscience, Inc.), unha metodoloxía baseada na microamplificación por PCR dos marcadores seleccionados e a secuenciación mediante espectrometría de masas MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight). Esta decisión fundamentouse en que esta técnica é a que mellor se axusta ás dimensións dos estudos epidemiolóxicos neste parasito xa que cada ensaio permite a análise simultánea de ata 50 posicións variantes dun só nucleótido (SNVs) en lotes de 384 mostras. Os rangos de análise doutros métodos alternativos son ou ben demasiado limitados –*e.g.* *Taqman* e *SNaPshot* foron deseñados para a análise de números reducidos de SNVs (<15) en mostras individuais–, ou exceden as necesidades do noso deseño –*e.g.* os microarrays permiten o xenotipado simultáneo de centos de miles de marcadores. Este mellor axuste supón que a tecnoloxía seleccionada presenta unha esixencia de recursos materiais, humanos temporais e económicos moi inferiores ós que amosan outros métodos alternativos. Unha segunda vantaxe moi importante é que o catálogo de SNVs analizados pódese modificar antes de cada ensaio, de xeito que o protocolo se pode adaptar ós intereses mudables dos estudos. Por exemplo, permitindo a detección de novas linaxes do parasito en función do hóspede analizado ou da súa procedencia xeográfica.

2. Obxectivos

A seguir relaciónanse os obxectivos principais desta tese, así como os obxectivos específicos deseñados para acadalos.

1. **Caracteriza-los padróns de variación xenética e identifica-las principais especies e subtipos na poboación de *Cryptosporidium* que afecta a humanos na área sanitaria de Santiago de Compostela.**

    1.1. Obter unha colección de illados representativos das principais especies e familias de subtipos de *Cryptosporidium* que afectan a humanos en todo o mundo. Para elo seleccionaranse ó azar mostras de *C. parvum* e *C. hominis* recollidas en feces de orixe humana no Servizo de Microbioloxía e Parasitoloxía do Complexo Hospitalario Universitario de Santiago, previamente identificadas no CDC mediante a análise dos *locus SSU rRNA* e *COWP1*. Este panel completarase con mostras de *C. meleagridis* e *C. felis* procedentes de bancos de mostras públicos, por seren estas especies tamén patóxenos habituais de humanos, e con mostras recollidas polo Laboratorio de Sanidade e Produción Animal de Galicia, en Lugo (Consellería de Medio Rural, Xunta de Galicia), representativas da poboación de *Cryptosporidium* que parasita a cabana gandeira de Galicia.

    1.2. Obter entre 8-10 secuencias nucleotídicas en cada aillado de 10 *loci* de copia única no xenoma de *Cryptosporidium*. Analizaranse *COWP1* e *gp60* por seren os *loci* máis empregados en estudos análogos e outros oito *loci* seleccionados en distintos grupos de ligamento, representativos do xenoma do parasito.

    1.3. Realizar unha análise xenético-poboacional dos niveis de diversidade multilocus intra e interespecífica na mostra.

2. **Deseñar un método analítico de alto rendemento para o xenotipado de criptosporidios en grandes coleccións de illados de orixe humana e gandeira, baseado na identificación simultánea de variantes nucleotídicas que permitan o diagnóstico das distintas especies e subtipos.**

2.1. Partindo dos datos de variación obtidos no experimento anterior, seleccionar variantes xenéticas (SNVs) informativas para a identificación da especies e subtipos de *Cryptosporidium* máis comúns en mostras humanas e bovinas.

2.2. Deseñar un protocolo diagnóstico de alto rendemento, baseado no sistema de xenotipado MassARRAY, para a identificación precisa e inequívoca das variantes nucleotídicas en illados de orixe humana e animal.

2.3. Aplicar dito protocolo a mostras representativas das especies e subtipos identificados nun panel de illados de orixe humana e animal previamente xenotipados mediante PCR e secuenciación Sanger dos *loci* diagnóstico do obxectivo 1.

2.4. Compara-lo rendemento do método –en termos de sensibilidade e especificidade– coas técnicas alternativas máis comunmente utilizadas para o mesmo fin.

3. Realizar un estudo epidemiolóxico das infeccións causadas por *Cryptosporidium* en doentes humanos na área sanitaria de Santiago de Compostela.

3.1. Determina-la especie e o subtipo infectante nun panel de mostras fecais procedentes de doentes humanos da área sanitaria de Santiago de Compostela recollidas entre os anos 2000 e 2008, empregando o método diagnóstico anteriormente deseñado.

3.2. Determina-la incidencia da enfermidade na área sanitaria de Santiago.

3.3. Avalia-los datos epidemiolóxicos no contexto de diversas variables demográficas, clínicas e medio ambientais durante o período de tempo para o que existen mostras analizadas.

3.4. Estuda-los padróns espaciais, temporais e estacionais da criptosporidiose na mostra.

3.5. Determina-la existencia de características patoxénicas diferenciais entre especies e/ou subtipos.

PUBLICACIÓNS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Multilocus patterns of genetic variation across *Cryptosporidium* species suggest balancing selection at the *gp60* locus

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

MOLECULAR ECOLOGY

Multilocus patterns of genetic variation across *Cryptosporidium* species suggest balancing selection at the *gp60* locus

J. L. ABAL-FABEIRO,*†‡ X. MASIDE,*†‡ X. BELLO,*†‡ J. LLOVO§ and C. BARTOLOMÉ *†‡
**Departamento de Anatomía Patolóxica e Ciencias Forenses, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain, †Medicina Xenómica, CIMUS, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain, ‡Xenómica Comparada de Parasitos Humanos, IDIS, 15782 Santiago de Compostela, Spain, §Servizo de Microbioloxía e Parasitoloxía, CHUS, 15706 Santiago de Compostela, Spain*

Abstract

Cryptosporidium is an apicomplexan protozoan that lives in most vertebrates, including humans. Its *gp60* gene is functionally involved in its attachment to host cells, and its high level of genetic variation has made it the reference marker for sample typing in epidemiological studies. To understand the origin of such high diversity and to determine the extent to which this classification applies to the rest of the genome, we analysed the patterns of variation at *gp60* and nine other nuclear loci in isolates of three *Cryptosporidium* species. Most loci showed low genetic polymorphism (π_S <1%) and similar levels of between-species divergence. Contrastingly, *gp60* exhibited very different characteristics: (i) it was nearly ten times more variable than the other loci; (ii) it displayed a significant excess of polymorphisms relative to between-species differences in a maximum-likelihood Hudson–Kreitman–Aguadé test; (iii) *gp60* subtypes turned out to be much older than the species they were found in; and (iv) showed a significant excess of polymorphic variants shared across species from random expectations. These observations suggest that this locus evolves under balancing selection and specifically under negative frequency-dependent selection (FDS). Interestingly, genetic variation at the other loci clusters very well within the groups of isolates defined by *gp60* subtypes, which may provide new tools to understand the genome-wide patterns of genetic variation of the parasite in the wild. These results suggest that *gp60* plays an active and essential role in the life cycle of the parasite and that genetic variation at this locus might be essential for the parasite's long-term success.

Keywords: balancing selection, *Cryptosporidium*, frequency-dependent selection, *gp60*, trench warfare model

Received 21 March 2013; revision received 8 June 2013; accepted 18 June 2013

Introduction

In host–pathogen interactions, natural selection is expected to increase hosts' resistance to disease and pathogens' ability to infect hosts. Reciprocal selective pressures from both antagonists drive this co-evolution, such that the fitness of genotypes in one of them is a function of the genotype frequencies in the other. In principle, this can lead to an evolutionary 'arms race', in which recurrent selective sweeps produce rapid turnovers of virulence and resistance alleles in hosts and pathogens (Bergelson *et al*. 2001; Charlesworth 2006; Aguileta *et al*. 2009) as illustrated by several pathogen surface protein genes (reviewed in Aguileta *et al*. 2009). But in certain conditions, involving different forms of frequency-dependent selection (FDS), this system could also reach an equilibrium state (e.g. Tellier & Brown 2007), where balanced polymorphisms at the host's and parasite's relevant loci are maintained over long period of time, thus favouring local high long-term allelic diversity (Bergelson *et al*. 2001; Brown & Tellier 2011).

Correspondence: Xulio Maside, Fax: +34 881815403;
E-mail: xulio.maside@usc.es

Some classical examples of this kind of selection are loci involved in pathogen recognition and immune response (Leffler *et al*. 2013), like plant resistance (*R*) genes (Bergelson *et al*. 2001), blood group antigens (Fumagalli *et al*. 2009; Segurel *et al*. 2012), human innate immunity genes (Cagliani *et al*. 2008; Ferrer-Admetlla *et al*. 2008; Andres *et al*. 2010) or the extensively studied major histocompatibility complex (reviewed in Garrigan & Hedrick 2003; Klein *et al*. 2007; Spurgin & Richardson 2010).

Contrastingly, loci under balancing selection have been less frequently reported in pathogens, like those encoding the outer membrane proteins of *Neisseria* (Urwin *et al*. 2004; Buckee *et al*. 2008), the *flaA* flagellin gene of *Campylobacter* (Dingle *et al*. 2005) or several antigen genes of *Plasmodium falciparum*, the only eukaryote parasite model system in which this selective regime has been thoroughly investigated (Tetteh *et al*. 2009; Ochola *et al*. 2010; Amambua-Ngwa *et al*. 2012).

This could also be the case for *gp60*, one of the most studied genes in *Cryptosporidium* (Apicomplexa, Eucoccidiorida), an intracellular protozoan parasite that produces gastrointestinal disease in humans and all classes of vertebrates worldwide (Fayer 2010) and is a major cause of persistent diarrhoea in more than 60 million children and immunocompromised patients, where it may be life-threatening (Xiao 2010).

The *gp60* gene displays higher than genome-average levels of nucleotide variation, and its alleles (haplotypes) are used to define intraspecific groups (subtypes or haplogroups). These are named with a species-specific prefix—I for *Cryptosporidium hominis*, II for *Cryptosporidium parvum* and III for *Cryptosporidium meleagridis*—followed by a letter assigned to each subtype, which corresponds to different *gp60* alleles (Strong *et al*. 2000). This high genetic diversity, together with estimated K_A/K_S ratios close to 1, has been attributed to the action of positive selection in response to the pressure exerted by the host's immune system (Widmer 2009; Bouzid *et al*. 2010; Widmer & Lee 2010), although these observations could also be explained by weak selective constraints.

Considering that a vast number of *gp60* haplotypes are known from different *Cryptosporidium* species (Feng *et al*. 2000; Xiao 2010) and that this gene encodes the precursor protein of two sporozoite surface glycoproteins (gp40 and gp15) involved in sporozoite motility and parasite adhesion and host cell invasion, which are recognized as members of two antigen families by neutralizing antibodies (Strong *et al*. 2000; Cevallos *et al*. 2000), it is a likely candidate for being subject to balancing selection. This is important because the detection of sites under this kind of selection has been used to identify potential vaccine targets (Mu *et al*. 2007). However, the nature of the evolutionary forces responsible for the high diversity of this locus and the extent to which *gp60* subtyping is a good descriptor of the population structure at other genomic loci have yet to be determined.

Here, we used a multilocus approach to compare the patterns of nucleotide variation at *gp60* with those of nine other loci in isolates from the three *Cryptosporidium* species most often found in humans. We used population genetic tools to describe the intra- and between-species levels of genetic diversity and argue that these patterns are consistent with a long-term stable regime of balancing selection operating at this locus. Our results also bring new light on the genetic structure of *Cryptosporidium* populations at *gp60* and at the genome-wide level.

Material and methods

Cryptosporidium samples

Stool samples usually contain large numbers of *Cryptosporidium* oocysts that harbour four haploid sporozoites each. Sporozoites invade the intestinal epithelial cells, where they experience several rounds of asexual reproduction until they eventually enter a sexual cycle, which probably involves self-fertilization, which results in the production of new haploid oocysts that may re-infect the host or be excreted with the faeces.

In this work, *C. parvum* and *C. hominis* samples were obtained from stools of diarrhoeal patients at the Complexo Hospitalario Universitario de Santiago (Spain), which were collected from different locations within the Santiago de Compostela's health area between 2000 and 2003 (Table S1, Supporting information).

DNA extractions from *C. meleagridis* of human origin were obtained from the *Cryptosporidium* Reference Unit (Swansea, UK).

Gene selection and primers design

Sequences for *COWP1* and *gp60* were retrieved from GenBank. Eight additional genes (*ACoAs*, *dyn4*, *eIF4a*, *gtub*, *gtRNAl*, *ISWIr*, *KhRNAb* and *RNaseLi*) were chosen from conserved regions of the three *Cryptosporidium* genome projects available in CryptoDB (http://cryptodb.org/cryptodb/). We searched for single copy, orthologous genes using the symmetrical best match approach (Koonin 2005) with the aid of the WU-BLAST-2.0 (BLASTN), using as query the annotated genes of *C. parvum*. Hits with ≥70% nucleotide homology between either *C. parvum* or *C. hominis* and *C. muris* over a sequence ≥1 kb were extracted, aligned with MUSCLE v 3.6 (Edgar 2004) and used to design primers. The eight selected loci were randomly chosen of the 73 coding regions that met these requirements.

Genomic DNA was extracted from stool samples using the QIAamp DNA Stool Mini kit (QIAgen), and PCRs were performed using FastStart High Fidelity PCR System (Roche Diagnostics). Primer sequences and annealing temperatures are provided in Table S2 (Supporting information).

Sequence analyses

Direct sequencing of the PCR products was performed on an ABI3730XL sequencer using Big Dye (Applied Biosystems). Sequences were checked for accurate base calling using CodonCode Aligner (CodonCode Corporation); alignments were performed with MUSCLE (Edgar 2004) and manually corrected with BioEdit (Hall 1999). Sequence accession numbers are provided in Table S1 (Supporting information).

The levels of nucleotide diversity were described using the statistics π (Nei 1987) and θ_W (Watterson 1975). Genetic differentiation between groups of sequences was quantified as the average number of nonsynonymous substitutions per site (K), using the Nei–Gojobori method. The Jukes and Cantor correction for multiple hits was used in all calculations. These analyses were performed with the aid of DnaSP v5.10.02 (Librado & Rozas 2009) and MEGA5 (Tamura *et al*. 2011). The number of shared synonymous and nonsynonymous polymorphisms was calculated by hand, using DnaSP v5.10.02 outputs.

Maximum-likelihood estimates of the population recombination rate parameter ($4N_er$) for each locus were obtained using LDhat (McVean *et al*. 2002).

Results

Genetic diversity

Ten single-exon protein-coding loci (Table S2, Supporting information) were amplified and sequenced in *C. hominis* and *C. parvum* and eight in *C. meleagridis* (in 10, 13 and 7 samples, respectively). Genetic variation was generally low (Table 1). However, in *C. hominis*

Table 1 Average pairwise nucleotide diversity (expressed as percentage)

			Synonymous				Nonsynonymous			
Species	*Locus*	*N*	*L*	*S*	π_S	θ_{WS}	*L*	*S*	π_A	θ_{WA}
Cryptosporidium hominis	*ACoAs*	10	214.2	1	0.09	0.17	691.8	0	0	0
	COWP1	10	114.8	0	0	0	380.2	0	0	0
	dyn4	4	204.0	0	0	0	690.0	0	0	0
	eIF4a	10	166.2	0	0	0	586.8	0	0	0
	gp60	10	86.4	23	5.52	9.41	279.6	34	2.47	4.30
	gtRNAl	10	225.5	0	0	0	803.5	0	0	0
	gtub	10	215.0	1	0.09	0.16	712.0	1	0.03	0.05
	ISWIr	10	138.8	0	0	0	497.2	0	0	0
	KhRNAb	10	121.7	0	0	0	415.3	0	0	0
	RNaseLi	8	239.2	0	0	0	840.8	0	0	0
Cryptosporidium parvum	*ACoAs*	12	214.2	0	0	0	691.8	1	0.06	0.05
	COWP1	13	114.8	2	0.27	0.56	380.2	0	0	0
	dyn4	8	203.7	0	0	0	690.3	0	0	0
	eIF4a	12	166.2	1	0.10	0.20	586.8	0	0	0
	gp60	12	89.5	0	0	0	276.5	0	0	0
	gtRNAl	12	225.8	1	0.07	0.15	803.2	0	0	0
	gtub	13	213.5	0	0	0	713.5	0	0	0
	ISWIr	13	139.8	0	0	0	499.2	0	0	0
	KhRNAb	10	121.8	1	0.17	0.29	415.2	0	0	0
	RNaseLi	11	239.8	0	0	0	840.2	0	0	0
Cryptosporidium meleagridis	*ACoAs*	2	215.8	0	0	0	690.2	0	0	0
	COWP1	7	114.8	0	0	0	380.2	0	0	0
	gp60	5	90.4	12	5.51	6.37	281.6	13	1.94	2.22
	gtub	5	214.8	4	1.03	0.89	712.2	0	0	0
	ISWIr	7	140.8	0	0	0	501.2	0	0	0
	KhRNAb	4	121.8	0	0	0	415.2	0	0	0

N, sample size; *L*, number of sites; *S*, number of polymorphic sites; π_S and π_A, pairwise nucleotide diversity at synonymous and nonsynonymous sites, respectively (Nei 1987); θ_{WS} and θ_{WA}, nucleotide site variability based on the number of synonymous and nonsynonymous segregating sites, respectively (Watterson 1975).

and *C. meleagridis*, synonymous diversity at *gp60* (π_S ~ 5.50%) was over one order of magnitude greater than at the other loci (weighted average π_S = 0.02 ± 0.02% and 0.27 ± 0.27%, respectively). In fact, high sequence differentiation among some *gp60* haplotypes (even from the same species) prevented the unambiguous alignment of the 5′ ends of the sequences, so this part was excluded from the analyses. The trimmed sequence corresponds to positions 1 to 357 of the cgd6_1080 CryptoDB reference sequence and includes the microsatellite sequence (a polyserine tract) commonly used for sample typing (Xiao 2010). Thus, the levels of polymorphism and divergence at this locus might have been underestimated.

Nonsynonymous diversity was much lower than at synonymous sites (Table 1). Again, *gp60* had the most variants, 47 of 49 in the whole sample.

Genetic divergence

The *gp60* locus also stood out by having higher synonymous differentiation in all between-species comparisons (average K_S = 36.43 ± 3.74%; Table 2). Contrastingly, the weighted average K_S at the other loci was significantly lower: 6.63 ± 0.79% between *C. hominis* and *C. parvum* and slightly higher between these and *C. meleagridis* (weighted average K_S = 16.17 ± 3.14% and 16.19 ± 2.99%, respectively). At nonsynonymous sites, K_A for *gp60* (average K_A = 17.97 ± 0.21%) was again significantly larger than at the control loci (weighted average K_A between *C. hominis* and *C. parvum* = 0.03 ± 0.02% and 0.61 ± 0.19% between these and *C. meleagridis*).

The *C. hominis gp60* samples could be grouped into two highly divergent subtypes: Ib (N = 9) and Id (N = 1; K_S = 34.59 ± 8.46%, Table 3). Again, the average synonymous divergence between these subtypes was two orders of magnitude lower at the other nine loci (average K_S = 0.10 ± 0.07%), as only three fixed substitutions were detected, at *ACoAs* and *gtub* (Table 4).

The *C. parvum* samples included two different *gp60* subtypes: the most common and widely distributed IIa (N = 10) and a subtype we called IIn (N = 3), which presents a single fixed substitution at position 278 of the cdg6_1080 sequence (outside the alignable region) that causes the replacement of an aspartic acid by a glutamic acid in the coded protein. This variant was found in 25 of 252 *C. parvum* sequences, typed as IIa, randomly retrieved from GenBank. Its relatively high frequency among *C. parvum* isolates and the observed genetic differentiation at other loci examined (e.g. one fixed change in *ACoAs* and five unshared polymorphisms; Table 4) support the notion that IIa and IIn are genetically isolated from each other and could be considered as different subtypes.

Table 2 Average pairwise nucleotide divergence at 10 loci (expressed as percentage), at synonymous (K_S, below diagonal) and nonsynonymous sites (K_A, above)

Locus	*Ch*	*Cp*	*Cm*
gp60			
Ch	—	13.20	21.00
		2.73	*3.46*
Cp	31.00	—	19.70
	7.40		*3.16*
Cm	43.60	34.70	—
	9.20	*7.49*	
Other loci*			
Ch	—	0.03	0.61
		0.02	*0.19*
Cp	6.63	—	0.61
	0.79		*0.19*
Cm	16.17	16.19	—
	3.14	*2.99*	

Ch, *C. hominis*; *Cp*, *C. parvum*; and *Cm*, *C. meleagridis*. Standard errors are in italics. Standard errors of the divergence estimates at *gp60* were obtained by bootstrap (1000 replicates). Divergence between *C. meleagridis* and the other species was estimated on the basis of sequence data from seven genes: *ACoAs*, *COWP1*, *eIF4a*, *gtRNAl*, *gtub*, *ISWIr* and *KhRNAb*.
*Average divergence values were weighted by the relative length of the sequences at each locus.

Table 3 Average pairwise nucleotide divergence at synonymous sites (expressed as percentage) between *Cryptosporidium* subtypes

	Subtype			
Locus	Ib	Id	II	III
gp60				
Ib	—	*8.46*	*8.06*	*9.29*
Id	34.59	—	*7.23*	*8.23*
II	31.30	28.58	—	***7.35***
III	44.10	38.39	34.69	—
Average across 9 loci				
Ib	—	*0.07*	*0.00*	*0.91*
Id	0.10	—	*0.00*	*0.89*
II	6.50	6.39	—	*0.39*
III*	19.23	19.05	17.47	—

Bootstrap estimates of the standard errors are in italics. Given the low diversity observed, the values of the comparisons involving subtypes from *C. parvum* and *C. meleagridis* were pooled into groups II and III, respectively.
*Divergence estimates with *C. meleagridis* subtypes were obtained using sequence data from seven genes: *ACoAs*, *COWP1*, *eIF4a*, *gtRNAl*, *gtub*, *ISWIr* and *KhRNAb*.

The five *C. meleagridis gp60* sequences were all distinct. One was identical to subtype IIIb (Feng *et al.* 2011) and the other four were novel subtypes (here

Table 4 Nucleotide variants at six genomic loci found to be segregating within any of the three *Cryptosporidium* species

			Locus												
			eIF4a	*KhRNAb*	*ACoAs*		*COWP1*		*gtub*						*gtRNAl*
Species	Subtype	Isolate	483	807	690	1120	834	915	150	171	175	177	507	795	273
			C	A	T	G	C	T	G	C	C	T	C	A	C
Ch	Ib	25	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	57	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	67	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	107	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	124	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	141	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	182	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	351	.	.	C	.	T	.	.	.	A	.	T	.	.
	Ib	395	.	.	C	.	T	.	.	.	A	.	T	.	.
	Id	77	.	.	.	.	T	.	.	.	.	.	.	.	.
Cp	IIa	1	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIa	5	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIa	15	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIa	33	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIa	73	.	—	.	A	.	A	.	.	.	.	.	.	T
	IIa	93	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIa	138	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIa	153	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIa	160	—	—	—	—	.	A	.	.	.	.	.	.	—
	IIa	167	.	.	.	A	.	A	.	.	.	.	.	.	.
	IIn	43	T	.	.	.	.	A	.	.	.	.	.	.	.
	IIn	48	.	—	.	.	T	.	.	.	.	.	.	.	.
	IIn	155	.	T	.	.	.	A	.	.	.	.	.	.	.
Cm	IIIb	10357	—	.	—	—	.	.	—	—	—	—	—	—	—
	IIIf	95	.	.	.	.	.	.	.	.	.	C	.	.	.
	IIIg	8716	—	.	—	—	.	.	.	T	.	.	.	.	—
	—	9067	—	.	—	—	.	.	A	T	.	.	.	.	—
	IIIh	1848	—	.	.	.	.	.	.	.	.	C	.	G	—
	—	9077	—	.	—	—	.	.	.	.	.	C	.	G	—
	IIIi	3549	—	.	—	—	.	.	—	—	—	—	—	—	—

Fixed differences between the species are not included. *Ch*, *C. hominis*; *Cp*, *C. parvum*; *Cm*, *C. meleagridis*. Numbers in the table header are nucleotide positions in the reference sequence of each locus; nonsynonymous sites are in bold case; the first line indicates the ancestral state, as inferred from the genotype at *C. meleagridis*; dots indicate that the nucleotide is the same as the ancestral one (ancestry was inferred by parsimony); —, genotype not available.

named IIIf, IIIg, IIIh and IIIi). No variation was detected in *ACoAs*, *COWP1* and *KhRNAb*. Contrastingly, *gtub* displayed substantial synonymous polymorphism (π_S = 1.03%, Table 1), the largest for any locus other than *gp60*, and *gtub* haplotypes were not shared between samples with different subtypes (Table 4), which suggests that the population structure defined by variation at *gp60* may extend to other loci. At any rate, most within-species diversity at *gp60* corresponds to differences between rather than within subtypes. To check whether our sample was misleading, we compiled a larger data set with *gp60* sequences of subtypes Ib, Id, IIa and IIn extracted from GenBank (N = 61, 34, 120 and 17, respectively). Interestingly, we detected some diversity within subtypes (π_S and θ_{WS} estimates of the order of 0.21% and 1.31%, respectively, Table S3, Supporting information), which means that *gp60* haplotypes define groups of sequences that have accumulated a non-negligible amount of diversity (Strong *et al*. 2000).

Genetic differentiation between subtypes presented interesting features. Silent differentiation between the two *C. hominis gp60* subtypes Ib and Id (K_S = 34.59 ± 8.46%) was much larger than at the control loci in the same groups of isolates, even in between-species comparisons (Table 3). Furthermore, K_S values at *gp60* did not vary significantly across subtypes from different species (average K_S = 35.41 ± 2.72%), whereas at the control loci, synonymous divergence between *C. hominis*

and *C. parvum* was nearly three times lower than that between any of these species and *C. meleagridis* (Table 3).

Natural selection at gp60

Average K_A/K_S between *gp60* subtypes was high (0.47 ± 0.03, from Table S4, Supporting information), in good agreement with previous data (Widmer 2009). Given that the more rapidly evolving regions of the gene were excluded from the analysis, this is probably an underestimate of the true ratio. This is in sharp contrast with the low K_A/K_S observed at the control loci (average 0.02 ± 0.005). In fact, average K_A between isolates with different subtypes was more than one hundred times greater at *gp60* than at the control loci, whereas K_S values only increased by a factor of 3. Thus, *gp60* displayed not only a higher overall differentiation but also a significantly higher K_A/K_S ratio than the control loci.

To test the hypothesis that *gp60* variants might have been the target of long-term balancing selection, we applied the Hudson–Kreitman–Aguadé test (Hudson *et al*. 1987), which assesses the fit of the patterns of within- and between-species variation, at two or more loci, to the neutral theory prediction that the levels of neutral polymorphism and divergence should be proportional to the mutation rate and constant across loci (Kimura 1983). We used a maximum-likelihood version of the test that allows for explicit tests for selection in a multilocus framework (Wright & Charlesworth 2004). This enabled the comparison of selection and neutral models by means of a likelihood-ratio test. The selection model produced significantly better fits to the data in all comparisons, regardless of the species used to estimate divergence (using polymorphism data from *C. hominis* and *C. meleagridis*; Table S5, Supporting information). This method also provides maximum-likelihood estimates of the selection parameter *k*, a measure of the degree to which diversity is increased by selection; *k* values indicated a significant excess of synonymous polymorphism at *gp60* in these species. The lack of polymorphism at the alignable region of the *gp60* locus in our *C. parvum* sample explains the negative results when using this species.

gp60 trans-specific polymorphisms

If we assume that genetic diversity at *gp60* has been maintained by some sort of long-term balancing selection since before the split of the sampled species, some of the observed polymorphisms should be shared between them. To test this hypothesis, we retrieved from GenBank the sequences of other *gp60* subtypes from the three *Cryptosporidium* species included in our study, aligned them along with our own data and compared the numbers of between-species shared polymorphisms with random expectations by means of the hypergeometric distribution (Hasselmann *et al*. 2008). We found a significant excess of shared synonymous and nonsynonymous polymorphisms in all species comparisons (Table 5), which is in sharp contrast with the observation that only one of the polymorphisms at *COWP1* (the only control locus with sufficient variation data available for this analysis) was shared between species and/or groups of isolates with the same subtype ($P < 0.05$; sequence data retrieved from GenBank, not shown).

Recombination rate

Our results suggest that a significant fraction (nearly half) of the nucleotide substitutions at the control loci were fixed within isolates sharing the same subtype (Table 4), pointing towards the existence of barriers to genetic exchange between parasites with different *gp60* allelic groups. This is further supported by the lack of evidence for meiotic recombination at any locus in our sample (maximum-likelihood estimates of the population recombination rate, $4N_er$, were not significantly different from zero, in a permutation test, McVean *et al*. 2002; data not shown). To obtain a broader representation of variation at *gp60* worldwide, we analysed a larger sample of 60 distinct alleles, including all subtypes described to date in *C. hominis*, *C. parvum* and *C. meleagridis*, as well as several from other species (sequence data were retrieved from Feng *et al*. 2011). The resulting estimate of $4N_er$ was 25 ($P = 0.048$ in a permutation test), which is a very low value, considering that most haplotypes have persisted for very long period of time in these populations. This is in good agreement with the scarce evidence for recombination in these parasites (e.g. Tanriverdi *et al*. 2008; Widmer & Lee 2010; who analyse patterns of variation at multiple microsatellite loci), which is mostly restricted to experimental crosses in the laboratory (Tanriverdi *et al*. 2007).

Discussion

Gp60 presents significantly higher within-species genetic variation than other nine loci used as genomic controls. This can be largely attributed to differences between rather than within *gp60* allelic classes. The only exception is *C. parvum*, because IIa and IIn differ by just one nonsynonymous change (located outside the alignable region). However, the overall genetic diversity of this locus does not seem to be lower in *C. parvum* than in other *Cryptosporidium* species, as it also presents many different subtypes, most of which are rare in humans (Xiao 2010). On the other hand, within-subtype variation

Table 5 Shared polymorphisms at the *gp60* locus across three *Cryptosporidium* species

	S	*P*	NS	*P*
Number of sites (x3)	243		792	
C. hominis polymorphisms	28		35	
C. parvum polymorphisms	29		35	
Shared between species	18	3.9×10^{-13}	25	0
Number of sites (x3)	252		801	
C. hominis polymorphisms	30		33	
C. meleagridis polymorphisms	22		24	
Shared between species	7	7.0×10^{-3}	9	9.3×10^{-8}
Number of sites (x3)	254		799	
C. parvum polymorphisms	37		38	
C. meleagridis polymorphisms	27		34	
Shared between species	11	3.6×10^{-4}	11	8.3×10^{-8}

NS, nonsynonymous; S, synonymous. Accession numbers of additional sequences from GenBank (most of them referenced in Feng *et al*. (2011)): *C. hominis* (AF164502, DQ665688, AY262031, DQ665692, AY738184, AF440638, EF208067, FJ971716), *C. parvum* (AY262034, DQ192501, AF164491, AF440636, AF164501, HM234171-HM234172, AY738194, AY873780-AY873782, AM937006) and *C. meleagridis* (AB539717-AB539721, AF401498-AF401501, DQ067570); Codons that display several changes that prevent the unambiguous inference of the evolutionary paths were excluded from the analysis. *P*, cumulative probability in a hypergeometrical distribution ($X \geq$ number of observed between-species shared polymorphisms).

at *gp60* is much smaller and does not differ from that observed at the control loci (Tables 1 and S3, Supporting information). In principle, this high within-species genetic variation could be potentially explained if *gp60* was present in multiple, highly diverged copies in the parasite's genome. However, a large body of evidence supports the notion that *gp60* is a single copy gene: (i) genetic analyses with restriction enzymes and Southern blot (Strong *et al*. 2000; Cevallos 2000); (ii) the genome sequence assemblies of three different *Cryptosporidium* species (*C. hominis*, *C. parvum* and *C. muris*; Accession nos: AAEL00000000.1, AAEE00000000.1 and AAZY02000000, respectively); and (iii) the extremely low fraction of samples with more than one haplotype reported, which is usually attributed to co-infection with more than one *Cryptosporidium* lineage (Tanriverdi *et al*. 2008; Chalmers *et al*. 2009, 2010).

Under the null hypothesis of a genome-wide homogeneous nucleotide mutation rate, the large between-subtype neutral divergence observed at *gp60*, as compared with the control loci (Tables 3 and S4, Supporting information), suggests that most *gp60* alleles are older than the species that harbour them. This could also explain previous puzzling observations such as higher differentiation between subtypes from the same species than between alleles from different species (e.g. *C. hominis* Ib and Id in our data set or *C. cuniculus* Va and Vb; from Figure 1 of Feng *et al*. (2011)). In this scenario, highly divergent conspecific haplotypes would correspond to ancient alleles that were maintained segregating in the same species, or population, since their origin. On the other hand, those haplotypes that originated at the time of the split of the species they are found in are expected to present a genome-average level of neutral divergence. Overall, these results are consistent with a long-term structured variation at this locus across the whole genus *Cryptosporidium* and question the use of *gp60* to study the phylogenetic relationships of *Cryptosporidium* isolates.

Alternatively, the higher differentiation at *gp60* could be explained by an increased mutation rate at this locus (as proposed in Waldron & Power 2011), which would explain the higher rate of neutral evolution, K_S, coupled with a relaxation of purifying selection, consistent with the observed high K_A/K_S ratio. However, this scenario cannot be reconciled with the observed significant excess of shared variants across species in *gp60* only.

The MLHKA test strongly suggests a significant excess of synonymous polymorphism at *gp60*, which is consistent with the hypothesis that this locus is subject to a different selective regime than the other loci. Such observations are commonly used as 'footprints' of long-term balancing selection (Kreitman 2000). Given that the surface proteins encoded by *gp60* are detected in sporozoites and merozoites (Cevallos *et al*. 2000; Strong *et al*. 2000), infective forms of the haploid stages of the parasite's life cycle (Tanriverdi *et al*. 2007), variability is likely to be maintained by FDS rather than by overdominance (heterozygote advantage; Charlesworth 2006). FDS is a form of selection in which the fitness of a phenotype varies over time, depending on the frequencies of other phenotypes in the parasite population (direct FDS) and/or the genotypic frequencies of the host (indirect FDS; Brown & Tellier 2011). In the case of a patho-

gen, rare alleles are likely to have an advantage due to their greater chance of evading the host immune system. Two processes have been proposed to act in such cases. In the 'arms race' model, the successive sweep to fixation of new positively selected virulence alleles in the pathogen population is expected to cause a reduction in variability at linked sites. In the 'trench warfare' model, alleles are maintained in the population, although periods of lower frequency can cause less extreme bottleneck episodes for different allelic lineages, generating populations with numerous allelic classes at the selected loci (subtypes) within which diversity is low (Bergelson *et al*. 2001; Charlesworth 2006; Aguileta *et al*. 2009; Brown & Tellier 2011). The large allelic diversity shown by the gp60 locus does not seem compatible with the first model. On the contrary, the long-term subtype persistence, the low diversity exhibited worldwide within the *gp60* allelic lineages and the excess of shared synonymous and nonsynonymous polymorphic variants among distantly related species are consistent with the maintenance of longstanding polymorphism through the trench warfare model.

As mentioned above, the excess of diversity was not observed in our *C. parvum* sample, as genotypes IIa and IIn differ in just one nucleotide position outside the alignable region. In fact, the vast majority of *C. parvum gp60* sequences isolated from humans present either of these two genotypes (120 and 17, respectively in our sample from GenBank, Table S3, Supporting information). Contrastingly, *C. parvum* haplotypes from different hosts display high levels of differentiation (average K_S and K_A in a sample including subtypes IIa, IIc, IId, IId, IIf, IIg, IIh and Iii are 22.4% and 10.7%, respectively; data from Feng *et al*. 2011), which suggests that the same selective regime operates in the three *Cryptosporidium* species studied. The reduced level of variation of *C. parvum* infecting humans could be interpreted as evidence for a recent selective sweep or a recent adaptation of the parasite to the human host. Although these results may be modulated as new sequence data become available, they highlight the importance of the presence of this and other zoonotic parasite species in different hosts, which could serve as genetic reservoirs from which new variants can be horizontally transmitted to humans.

Finally, we did not detect an excess of shared variants among isolates of different subtypes (or species) at the control loci, which suggests that genetic variation of the parasite's genome is to some extent clustered within groups defined by the *gp60* subtypes. This could be explained as a by-product of recurrent selective episodes at *gp60* that in the short term may cause sweeps of the variation nearby the selected sites. If meiotic recombination rates are low, as suggested by our data, this sweeps may have a genome-wide effect. However, the fact that the control loci do not display the same patterns of diversity as *gp60* suggests that this effect fades with time and that recombination occurs in natural populations, at least rarely, as suggested by our analysis of *gp60* diversity worldwide. Alternatively, demographic events such as a recent population admixture could also result in a structuring of the genetic variation, but this phenomenon cannot explain the comparatively higher levels of variation observed at *gp60* only. In either case, these observations suggest that the artificial clustering of *Cryptosporidium* variation in *gp60* subtypes for epidemiological purposes does seem to reflect a real pattern of genetic variation in natural populations of the parasite.

Acknowledgements

We thank Deborah Charlesworth for her critical reading and constructive comments on an earlier version of the manuscript, as well as three anonymous reviewers for their contribution to improve the manuscript. We are also indebted to Dr. A. Campos and Dr. M.L. Pérez del Molino (CHUS, Santiago de Compostela, Spain) and the Fundación Pública Galega de Medicina Xenómica (SERGAS, Spain) for the use of their laboratory facilities, as well as to Kristin Elwin (*Cryptosporidium* Reference Unit, Swansea, UK) for providing information on the C. meleagridis samples. This work was financed by grant PGIDIT06PXIB228073PR from Xunta de Galicia, Spain, to CB. JLA-F was supported by a student fellowship cofinanced by Xunta de Galicia, Spain, and the European Social Fund; XM was supported by a Programa Ramón y Cajal contract (Ministerio de Ciencia e Innovación, Spain); XB was supported by grant PGIDIT06PXIB228073PR (Xunta de Galicia. Spain) and CB was supported by a Programa Isidro Parga Pondal contract (Xunta de Galicia, Spain).

References

Aguileta G, Refregier G, Yockteng R, Fournier E, Giraud T (2009) Rapidly evolving genes in pathogens: methods for detecting positive selection and examples among fungi, bacteria, viruses and protists. *Infection, Genetics and Evolution*, 9, 656–670.

Amambua-Ngwa A, Tetteh KK, Manske M *et al*. (2012) Population genomic scan for candidate signatures of balancing selection to guide antigen characterization in malaria parasites. *PLoS Genetics*, 8, e1002992.

Andres AM, Dennis MY, Kretzschmar WW *et al*. (2010) Balancing selection maintains a form of ERAP2 that undergoes nonsense-mediated decay and affects antigen presentation. *PLoS Genetics*, 6, e1001157.

Bergelson J, Kreitman M, Stahl EA, Tian D (2001) Evolutionary dynamics of plant *R*-genes. *Science*, 292, 2281–2285.

Bouzid M, Tyler KM, Christen R *et al*. (2010) Multi-locus analysis of human infective *Cryptosporidium* species and subtypes using ten novel genetic loci. *BMC Microbiology*, 10, 213.

Brown JK, Tellier A (2011) Plant-parasite coevolution: bridging the gap between genetics and ecology. *Annual Review of Phytopathology*, 49, 345–367.

Buckee CO, Jolley KA, Recker M *et al*. (2008) Role of selection in the emergence of lineages and the evolution of virulence in *Neisseria meningitidis*. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 15082–15087.

Cagliani R, Fumagalli M, Riva S *et al*. (2008) The signature of long-standing balancing selection at the human defensin beta-1 promoter. *Genome Biology*, 9, R143.

Cevallos AM, Zhang X, Waldor MK et al. (2000) Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins *gp40* and *gp15*. *Infection and Immunity*, 68, 4108–4116.

Chalmers RM, Elwin K, Thomas AL, Guy EC, Mason B (2009) Long-term *Cryptosporidium* typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. *Eurosurveillance*, 14 pii:19086.

Chalmers RM, Smith R, Elwin K, Clifton-Hadley FA, Giles M (2010) Epidemiology of anthroponotic and zoonotic human cryptosporidiosis in England and Wales, 2004-2006. *Epidemiology and Infection*, 139, 700–712.

Charlesworth D (2006) Balancing selection and its effects on sequences in nearby genome regions. *PLoS Genetics*, 2, e64.

Dingle KE, Colles FM, Falush D, Maiden MC (2005) Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. *Journal of Clinical Microbiology*, 43, 340–347.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32, 1792–1797.

Fayer R (2010) Taxonomy and species delimitation in *Cryptosporidium*. *Experimental Parasitology*, 124, 90–97.

Feng X, Rich SM, Akiyoshi D *et al*. (2000) Extensive polymorphism in *Cryptosporidium parvum* identified by multilocus microsatellite analysis. *Applied and Environmental Microbiology*, 66, 3344–3349.

Feng Y, Lal AA, Li N, Xiao L (2011) Subtypes of *Cryptosporidium* spp. in mice and other small mammals. *Experimental Parasitology*, 127, 238–242.

Ferrer-Admetlla A, Bosch E, Sikora M *et al*. (2008) Balancing selection is the main force shaping the evolution of innate immunity genes. *Journal of Immunology*, 181, 1315–1322.

Fumagalli M, Cagliani R, Pozzoli U *et al*. (2009) Widespread balancing selection and pathogen-driven selection at blood group antigen genes. *Genome Research*, 19, 199–212.

Garrigan D, Hedrick PW (2003) Perspective: detecting adaptive molecular polymorphism: lessons from the MHC. *Evolution*, 57, 1707–1722.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.

Hasselmann M, Vekemans X, Pflugfelder J *et al*. (2008) Evidence for convergent nucleotide evolution and high allelic turnover rates at the complementary sex determiner gene of Western and Asian honeybees. *Molecular Biology and Evolution*, 25, 696–708.

Hudson RR, Kreitman M, Aguadé M (1987) A test of neutral molecular evolution based on nucleotide data. *Genetics*, 116, 153–159.

Kimura M (1983) *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge.

Klein J, Sato A, Nikolaidis N (2007) MHC, TSP, and the origin of species: from immunogenetics to evolutionary genetics. *Annual Review of Genetics*, 41, 281–304.

Koonin EV (2005) Orthologs, paralogs, and evolutionary genomics. *Annual Review of Genetics*, 39, 309–338.

Kreitman M (2000) Methods to detect selection in populations with applications to the human. *Annual Review of Genomics and Human Genetics*, 1, 539–559.

Leffler EM, Gao Z, Pfeifer S *et al*. (2013) Multiple Instances of ancient balancing selection shared between humans and chimpanzees. *Science*, 339, 1578–1582.

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451–1452.

McVean G, Awadalla P, Fearnhead P (2002) A coalescent-based method for detecting and estimating recombination from gene sequences. *Genetics*, 160, 1231–1241.

Mu J, Awadalla P, Duan J *et al*. (2007) Genome-wide variation and identification of vaccine targets in the *Plasmodium falciparum* genome. *Nature Genetics*, 39, 126–130.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Ochola LI, Tetteh KK, Stewart LB *et al*. (2010) Allele frequency-based and polymorphism-versus-divergence indices of balancing selection in a new filtered set of polymorphic genes in *Plasmodium falciparum*. *Molecular Biology and Evolution*, 27, 2344–2351.

Segurel L, Thompson EE, Flutre T *et al*. (2012) The ABO blood group is a trans-species polymorphism in primates. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 18493–18498.

Spurgin LG, Richardson DS (2010) How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proceedings of the Royal Society - Biological Sciences*, 277, 979–988.

Strong WB, Gut J, Nelson RG (2000) Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. *Infection and Immunity*, 68, 4117–4134.

Tamura K, Peterson D, Peterson N *et al*. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.

Tanriverdi S, Blain JC, Deng B, Ferdig MT, Widmer G (2007) Genetic crosses in the apicomplexan parasite *Cryptosporidium parvum* define recombination parameters. *Molecular Microbiol ogy*, 63, 1432–1439.

Tanriverdi S, Grinberg A, Chalmers RM *et al*. (2008) Inferences about the global population structures of *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Applied and Environmental Microbiology*, 74, 7227–7234.

Tellier A, Brown JK (2007) Stability of genetic polymorphism in host-parasite interactions. *Proceedings of the Royal Society - Biological Sciences*, 274, 809–817.

Tetteh KK, Stewart LB, Ochola LI *et al*. (2009) Prospective identification of malaria parasite genes under balancing selection. *PLoS ONE*, 4, e5568.

Urwin R, Russell JE, Thompson EA *et al*. (2004) Distribution of surface protein variants among hyperinvasive meningococci: implications for vaccine design. *Infection and Immunity*, 72, 5955–5962.

Waldron LS, Power ML (2011) Fluorescence analysis detects *gp60* subtype diversity in *Cryptosporidium* infections. Infection, *Genetics and Evolution*, 11, 1388–1395.

Watterson GA (1975) On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology*, 7, 256–276.

Widmer G (2009) Meta-analysis of a polymorphic surface glycoprotein of the parasitic protozoa *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Epidemiology and Infection*, 137, 1800–1808.

Widmer G, Lee Y (2010) Comparison of single- and multilocus genetic diversity in the protozoan parasites *Cryptosporidium parvum* and *C. hominis*. *Applied and Environmental Microbiology*, 76, 6639–6644.

Wright SI, Charlesworth B (2004) The HKA test revisited: a maximum-likelihood-ratio test of the standard neutral model. *Genetics*, 168, 1071–1076.

Xiao L (2010) Molecular epidemiology of cryptosporidiosis: an update. *Experimental Parasitology*, 124, 80–89.

J.L. Abal-Fabeiro performed research and analysed data; X. Maside designed and performed research, analysed data and wrote the article; X. Bello performed research; J. Llovo designed research and provided samples; and C. Bartolomé designed and performed research, analysed data and wrote the article.

Data accessibility

DNA sequences: GenBank Accession nos JQ349161-JQ349402.

Sequence alignments: Dryad doi:10.5061/dryad.k8vr8.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sampling details and sequence accession numbers.

Table S2 Primers used for the PCR amplification of *Cryptosporidium* nuclear genes.

Table S3 Nucleotide diversity at *gp60* within *Cryptosporidium*
subtypes, expressed as % (GenBank data).

Table S4 Estimates of pairwise nucleotide divergence at synonymous (%; below the diagonal) and nonsynonymous sites (above) between *C. hominis*, *C. parvum* and *C. meleagridis* subtypes.

Table S5 Maximum likelihood Hudson-Kreitman-Aguadé test on variation data at *gp60* synonymous sites.

1.1 Material suplementario

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table S1. Sampling details and sequence accession numbers.

Species	Sample ID	Collection place	Date	Accession number									
				ACoAs	*COWP1*	*dyn4*	*eIF4a*	*gp60*	*gtRNAl*	*gtub*	*ISWIr*	*KhRNAb*	*RNaseLi*
C. hominis	25	Negreira, Galicia, Spain	28/11/2000	JQ349379	JQ349360	-	JQ349324	JQ349234	JQ349261	JQ349297	JQ349161	JQ349220	JQ349202
	57	Santiago , Galicia, Spain	04/06/2001	JQ349380	JQ349361	JQ349335	JQ349325	JQ349235	JQ349262	JQ349298	JQ349162	JQ349221	JQ349203
	67	Brion, Galicia, Spain	18/07/2001	JQ349381	JQ349362	JQ349336	JQ349326	JQ349236	JQ349263	JQ349299	JQ349163	JQ349222	JQ349204
	77	Santiago, Galicia, Spain	30/08/2001	JQ349382	JQ349363	JQ349337	JQ349327	JQ349243	JQ349264	JQ349300	JQ349164	JQ349223	JQ349205
	107	Ames, Galicia, Spain	10/11/2001	JQ349383	JQ349365	JQ349338	JQ349328	JQ349237	JQ349265	JQ349301	JQ349165	JQ349224	-
	124	Rianxo, Galicia, Spain	07/03/2002	JQ349384	JQ349366	-	JQ349329	JQ349238	JQ349266	JQ349302	JQ349166	JQ349225	JQ349206
	141	Teo, Galicia, Spain	04/04/2002	JQ349385	JQ349367	-	JQ349330	JQ349239	JQ349267	JQ349303	JQ349167	JQ349226	-
	182	Santiago, Galicia, Spain	15/10/2002	JQ349386	JQ349368	-	JQ349331	JQ349240	JQ349268	JQ349304	JQ349168	JQ349227	JQ349207
	351	Santiago, Galicia, Spain	28/10/2003	JQ349387	JQ349369	-	JQ349332	JQ349241	JQ349269	JQ349305	JQ349169	JQ349228	JQ349208
	395	A Estrada, Galicia, Spain	02/12/2003	JQ349388	JQ349364	-	JQ349333	JQ349242	JQ349270	JQ349306	JQ349170	JQ349229	JQ349209
C. parvum	1	Santiago, Galicia, Spain	16/03/2000	JQ349389	JQ349347	JQ349339	JQ349312	JQ349244	JQ349271	JQ349284	JQ349171	JQ349210	JQ349191
	5	Val do Dubra, Galicia, Spain	13/06/2000	JQ349390	JQ349348	JQ349340	JQ349313	JQ349245	JQ349272	JQ349285	JQ349172	JQ349211	JQ349192
	15	Rodeiro, Galicia, Spain	26/09/2000	JQ349391	JQ349349	JQ349341	JQ349314	JQ349246	JQ349273	JQ349286	JQ349173	JQ349212	JQ349193
	33	Rianxo, Galicia, Spain	26/01/2001	JQ349392	JQ349350	JQ349342	JQ349315	JQ349247	JQ349274	JQ349287	JQ349174	JQ349213	JQ349194
	43	Pobra do Caramiñal, Galicia, Spain	22/02/2001	JQ349399	JQ349357	JQ349345	JQ349321	JQ349253	JQ349280	JQ349294	JQ349181	JQ349218	JQ349199
	48	Ribeira, Galicia, Spain	12/03/2001	JQ349398	JQ349358	-	JQ349322	JQ349254	JQ349281	JQ349295	JQ349182	-	JQ349200
	73	Vedra, Galicia, Spain	31/07/2001	JQ349393	JQ349351	JQ349343	JQ349316	JQ349248	JQ349275	JQ349288	JQ349175	-	-
	93	Padrón, Galicia, Spain	08/10/2001	JQ349394	JQ349352	JQ349344	JQ349317	JQ349252	JQ349276	JQ349289	JQ349176	JQ349214	JQ349195
	138	Santiso, Galicia, Spain	25/03/2002	JQ349395	JQ349353	-	JQ349318	JQ349249	JQ349277	JQ349290	JQ349177	JQ349215	JQ349196
	153	Tordoia, Galicia, Spain	31/05/2002	JQ349396	JQ349354	-	JQ349319	-	JQ349278	JQ349291	JQ349178	JQ349216	JQ349197
	155	Rianxo, Galicia, Spain	13/06/2002	JQ349400	JQ349359	JQ349346	JQ349323	JQ349255	JQ349282	JQ349296	JQ349183	JQ349219	JQ349201
	160	Trazo, Galicia, Spain	12/07/2002	-	JQ349355	-	-	JQ349250	-	JQ349292	JQ349179	-	-
	167	Arzúa, Galicia, Spain	02/09/2002	JQ349397	JQ349356	-	JQ349320	JQ349251	JQ349279	JQ349293	JQ349180	JQ349217	JQ349198
C. meleagridis	95	Santa Comba, Galicia, Spain	11/10/2001	JQ349401	JQ349370	-	JQ349334	JQ349257	JQ349283	JQ349307	JQ349184	JQ349230	-
	1848	Wales, UK	26/09/2000	JQ349402	JQ349371	-	-	JQ349259	-	JQ349308	JQ349185	JQ349231	-

3549	Yorkshire and the Humber, UK	20/08/2001	-	JQ349372	-	-	JQ349260	-	-	JQ349186	-	-
8716	West Midlands, UK	06/02/2004	-	JQ349373	-	-	JQ349258	-	JQ349309	JQ349187	JQ349232	-
9067	South West, UK	25/05/2004	-	JQ349374	-	-	-	-	JQ349310	JQ349188	JQ349233	-
9077	East, UK	27/05/2004	-	JQ349375	-	-	-	-	JQ349311	JQ349189	-	-
10357	South West, UK	25/05/2005	-	JQ349376	-	-	JQ349256	-	-	JQ349190	-	-

Table S2. Primers used for the PCR amplification of *Cryptosporidium* nuclear genes.

			primers			
locus tag [a]	gene ID	symbol	name	sequence 5'-3'	T_a (ºC)	size (bp)
cgd1_580	3371500	*dyn4*	Dynactin 4-F	GGTATWGTAGATGATCCA	48	957
			Dynactin 4-R	AGCTTGAATTTTYCTTAA		
cgd1_880	3371494	*eIF4a*	Euk-factor-F	AACCATCWGCAATTCAAC	49	778
			Euk-factor-R	ATGACTTCACGRTCCTTT		
cgd1_980	3371492	*RNaseLi*	RNaseLi-F	TGTGTTAAGAAATGTCCA	48	1161
			RNaseLi-R	RAATTGAACATCCATAAA		
cgd1_1280	3371592	*KhRNAb*	Kh-F1 [b]	GTTGGATATATWACAGGAA	48	614
			Kh-R1 [b]	GTTTCATCTTCAACACG		
			Khn-F [c]	GTWCAGCAAAARCATGG	48	823
			Khn-R [c]	TTTCRTACATTCTSCKTTC		
			Khns-F [d]	CAGGAAARAARGGCCARGG	51	568
			Khns-R [d]	TTTGCWCCAGTYACATACCC		
cgd1_3710	3371515	*ACoAs*	ACoAs-F	TGCATTAATTTGGGAAG	48	963
			ACoAs-R	GTTTGCCAATAWGTATCA		
cgd6_1080	3376025	*gp60*	gp60-F3	ATAGTCTCCGCTGTATTC	50	750
			gp60-R3	TGCAACCAAACTGTAC		
			gp60-F2 [e]	CCAGCCGTTCCACTCAG	52	729
			gp60-R2 [e]	GTYTGCAACCAAACTGTAC		

cgd6_2090	3375880	*COWP1*	COWP-F4 COWP-R4	GTAGATAATGGAAGAGATTGTG GGACTGAAATACAGGCATTATCTTG	51	553
cgd7_1980	3371797	*gtub*	gtub-F gtub-R	AATAGGCATGGARTTTTGG GGGCCCCACTGAATAAAT	51	1008
cgd8_790	3374640	*gtRNAl*	gtRNAl-F gtRNAl-R	CCTTCAGGTTACTTACATA CATTWCCCCACTTCATAA	48	1108
cgd8_4620	3374446	*ISWIr*	SNF2-F SNF2-R	GCAATGATGGAYCCTGTT ATGWGAACAACAAATCCT	48	663

T_a, annealing temperature.

[a] Locus tag assigned in the *C. parvum* genome annotation; cgd1 to cgd8 indicate the linkage group (chromosome).

[b] Used to amplify *KhRNAb* from *C. parvum* and *C. hominis*.

[c] Used to amplify *KhRNAb* from *C. meleagridis*.

[d] Used to re-amplify the PCR product obtained with Khn-F and Khn-R (nested PCR). [e] Used to re-amplify the PCR product obtained with gp60-F3 and gp60-R3 (nested PCR).

Table S3. Nucleotide diversity at *gp60* within *Cryptosporidium* subtypes, expressed as % (GenBank data).

		synonymous				nonsynonymous			
subtype	*N*	*L*	*S*	π_S	θ_{WS}	*L*	*S*	π_A	θ_{WA}
Ib	61	156.0	11	0.23	1.50	513.0	26	0.41	1.08
Id	34	161.3	8	0.29	1.21	525.7	8	0.09	0.37
IIa	120	136.1	16	0.20	2.19	424.9	44	0.19	1.19
IIn	17	172.2	2	0.14	0.34	538.8	1	0.02	0.06

N, sample size; *L*, number of sites; *S*, number of polymorphic sites; π_S and π_A, pairwise nucleotide diversity at synonymous and nonsynonymous sites, respectively; θ_{WS} and θ_{WA}, nucleotide site variability based on the number of synonymous and nonsynonymous segregating sites, respectively. The polyserine tract was excluded from the analysis.

Table S4. Estimates of pairwise nucleotide divergence at synonymous (%; below the diagonal) and nonsynonymous sites (above) between *C. hominis*, *C. parvum* and *C. meleagridis* subtypes.

locus		Ib	Id	IIa	IIn	IIIb	IIIf	IIIg	IIIh	IIIi
gp60	Ib		12.88	13.58	13.58	20.63	21.24	21.75	20.75	21.24
	Id	34.59		9.38	9.38	18.11	20.77	21.27	20.29	20.77
	IIa	31.30	28.58		0.00	17.75	20.16	20.67	19.68	20.16
	IIn	31.30	28.58	0.00		17.75	20.16	20.67	19.68	20.16
	IIIb	37.67	46.32	38.95	38.95		4.35	3.94	3.95	3.54
	IIIf	45.78	37.42	33.65	33.65	12.87		0.38	1.16	0.77
	IIIg	48.01	35.46	35.52	35.52	14.28	1.20		0.77	0.38
	IIIh	43.43	35.33	31.71	31.71	14.24	1.20	2.42		0.38
	IIIi	45.78	37.42	33.65	33.65	12.87	0.00	1.20	1.20	
other loci	Ib		0.02	0.03	0.02	0.00	0.57	0.61	0.71	0.00
	Id	0.10		0.02	0.00	0.00	0.55	0.56	0.67	0.00
	IIa	6.50	6.39		0.02	0.00	0.57	0.56	0.71	0.00
	IIn	6.50	6.39	0.14		0.00	0.55	0.56	0.67	0.00
	IIIb	17.58	17.58	16.49	16.49		0.00	0.00	0.00	0.00
	IIIf	16.19	16.02	15.78	15.87	0.00		0.00	0.00	0.00
	IIIg	21.03	20.82	18.69	18.87	0.00	0.31		0.00	0.00
	IIIh	20.08	19.78	18.18	18.31	0.00	0.12	0.47		0.00
	IIIi	17.58	17.58	16.49	16.49	0.00	0.00	0.00	0.00	

Table S5. Maximum likelihood Hudson-Kreitman-Aguade test on variation data at *gp60* synonymous sites.

source data							
polymorphism	divergence with	*N*	model	ln*L*	LRT	*p*	*k*
C. hominis	*C. parvum*	10	I	-37.2	24.3	8.4×10^{-7}	35.0
			II	-25.1			
C. hominis	*C. meleagridis*	8	I	-45.0	36.3	1.6×10^{-9}	55.8
			II	-26.8			
C. meleagridis	*C. hominis*	6	I	-29.3	11.1	8.5×10^{-4}	13.5
			II	-23.7			
C. meleagridis	*C. parvum*	6	I	-28.5	10.5	1.2×10^{-3}	13.7
			II	-23.2			
C. parvum	*C. hominis*	10	I	-32.2	1.8	0.174	0.0
			II	-31.3			
C. parvum	*C. meleagridis*	8	I	-26.6	2.5	0.117	0.0
			II	-25.4			

N, Number of loci; Model I: all loci evolve neutrally; Model II: *gp60* evolves under selection. LTR is the value of the likelihood ratio test; *k* is the ML estimate of selection parameter for *gp60*, the locus putatively under selection. The lack of evidence for selection when *C. parvum* was used as the source of polymorphism was predictable since the aligned fraction of the two g*p60* haplotypes was identical.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

2. High-throughput genotyping assay for the large-scale genetic characterization of *Cryptosporidium* parasites from human and bovine samples

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

High-throughput genotyping assay for the large-scale genetic characterization of *Cryptosporidium* parasites from human and bovine samples

J. L. ABAL-FABEIRO[1,2,3], X. MASIDE[1,2,3], J. LLOVO[3,4], X. BELLO[1,2,3], M. TORRES[5], M. TREVIÑO[4], L. MOLDES[4], A. MUÑÓZ[6], A. CARRACEDO[1,7,8] *and* C. BARTOLOMÉ[1,2,3]*

[1] *Departamento de Anatomía Patolóxica e Ciencias Forenses, Universidade de Santiago de Compostela,15782 Santiago de Compostela, Spain*
[2] *Medicina Xenómica, CIMUS, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[3] *Xenómica Comparada de Parasitos Humanos, IDIS, Santiago de Compostela, Spain*
[4] *Servizo de Microbioloxía e Parasitoloxía, Complexo Hospitalario Universitario de Santiago de Compostela,15706 Santiago de Compostela, Spain*
[5] *Centro Nacional de Genotipado, Universidade de Santiago de Cmpostela, Spain*
[6] *Departamento de Microbioloxía e Parasitoloxía, Facultade de Bioloxía, Universidade de Santiago de Compostela,15782 Santiago de Compostela, Spain*
[7] *Fundación Pública Galega de Medicina Xenómica (SERGAS)-CIBERER (USC), Complexo Hospitalario Universitario de Santiago de Compostela, 15706 Santiago de Compostela, Spain*
[8] *Center of Excellence in Genomic Medicine Research, King Abdulaziz University, 21589 Jeddah, Kingdom of Saudi Arabia*

(*Received 26 June 2013; revised 6 September 2013; accepted 8 September 2013; first published online 15 November 2013*)

SUMMARY

The epidemiological study of human cryptosporidiosis requires the characterization of species and subtypes involved in human disease in large sample collections. Molecular genotyping is costly and time-consuming, making the implementation of low-cost, highly efficient technologies increasingly necessary. Here, we designed a protocol based on MALDI-TOF mass spectrometry for the high-throughput genotyping of a panel of 55 single nucleotide variants (SNVs) selected as markers for the identification of common *gp60* subtypes of four *Cryptosporidium* species that infect humans. The method was applied to a panel of 608 human and 63 bovine isolates and the results were compared with control samples typed by Sanger sequencing. The method allowed the identification of species in 610 specimens (90·9%) and gp60 subtype in 605 (90·2%). It displayed excellent performance, with sensitivity and specificity values of 87·3 and 98·0%, respectively. Up to nine genotypes from four different *Cryptosporidium* species (*C. hominis*, *C. parvum*, *C. meleagridis* and *C. felis*) were detected in humans; the most common ones were *C. hominis* subtype Ib, and *C. parvum* IIa (61·3 and 28·3%, respectively). 96·5% of the bovine samples were typed as IIa. The method performs as well as the widely used Sanger sequencing and is more cost-effective and less time consuming.

Key words: *Cryptosporidium* epidemiology, high-throughput SNV genotyping, MALDI-TOF mass spectrometry.

INTRODUCTION

Discrimination between *Cryptosporidium* species and subtypes is crucial for epidemiological studies and for the prevention and control of cryptosporidiosis. These require the screening of vast numbers of samples to determine the genotypes involved in human disease and the possible sources of contamination. Nowadays, Sanger DNA sequencing at a reduced number of genes is the most common tool used for molecular determination (Strong *et al.* 2000; Xiao *et al.* 2004). However, when large numbers of samples are analysed, this method is expensive and unpractical (Chalmers, 2008). Another limitation of the technique is that sometimes it is difficult to obtain high-quality sequences from stool DNA extractions containing inhibitors and/or DNA degrading substances (Chalmers, 2008; Jex *et al.* 2008).

There is a range of high-throughput cost-effective tools available to identify *Cryptosporidium* species and subtypes (Jex *et al.* 2008). The most widely used is the electrophoretic display of genetic variants using single-stranded conformation polymorphism (SSCP), based on changes in the mobility of single-stranded DNA in a non-denaturing polyacrylamide gel (Gasser *et al.* 2006; Jex *et al.* 2007b). This method is highly efficient, although very dependent on the genes used to discriminate polymorphisms (Chalmers *et al.* 2005; Jex *et al.* 2007b, 2008), so the selection of adequate loci is essential to obtain accurate results. A caveat to this technique is that it does not identify the nature of the variants detected and further sequencing is needed to determine it.

The aim of our study was to test and describe a new method for genotyping *Cryptosporidium* isolates

* Corresponding author: CIMUS, Despacho P2D2, Avda. Barcelona s/n, 15782 Santiago de Compostela, Galicia, Spain. E-mail: carolina.bartolome@usc.es

Parasitology (2014), 141, 491–500.
doi:10.1017/S0031182013001807

based on the combined use of single base extension (SBE) of a panel of selected single nucleotide variants (SNVs), that includes both polymorphic positions within a species (single nucleotide polymorphisms, SNPs) and fixed changes between species, followed by sequencing by mass spectrometry (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight, MALDI-TOF). Its performance was tested in a total of 608 human isolates representative of community cases seeking medical assistance and 63 bovine samples. The results were compared with Sanger sequencing data from a panel of control samples.

MATERIALS AND METHODS

Cryptosporidium samples

The samples of human origin were routinely collected from diarrhoeal patients between years 2000 and 2008 at the Complexo Hospitalario Universitario de Santiago (CHUS, Santiago de Compostela, Spain) and diagnosed by staining oocysts with the auramine phenol method (Casemore *et al*. 1984). *Cryptosporidium* positive samples were stored at −20 °C in 1·5 mL plastic vials. The bovine stool samples were collected from calves (< 1 month of age) in 2007 and diagnosed as *Cryptosporidium*-positive at the Laboratorio de Sanidade e Producción Animal da Xunta de Galicia (Lugo, Spain) using immunochromatography.

DNA extraction

DNA was isolated from 180–200 mg of stool samples using the QIAamp DNA Stool Mini kit (QIAgen, Izasa, Barcelona, Spain) with slight modifications of the manufacturer's protocol to improve the disruption of *Cryptosporidium* oocysts. Three freeze-thawing cycles were performed after the addition of Buffer ASL by immersion in liquid nitrogen for 1 min and thawing in a water-bath at 95 °C for 2 min.

Genotyping

Samples were genotyped at single nucleotide variants from five different loci (*ACoAs*, *COWP1*, *gp60*, *gtub* and *ISWIr*), allowing us to distinguish between *Cryptosporidium* species and subtypes infecting humans using the MassARRAY system (Sequenom), which combines the iPLEX Gold genotyping technology with MALDI-TOF mass spectrometry and allows for the automated analysis of large numbers of samples. This assay is based on a primer extension aimed to detect sequence differences at the nucleotide level (Fig. 1). The protocol includes an initial PCR amplification of the region surrounding the sequence variation of interest, followed by the addition of a primer with mass-modified terminators that anneals

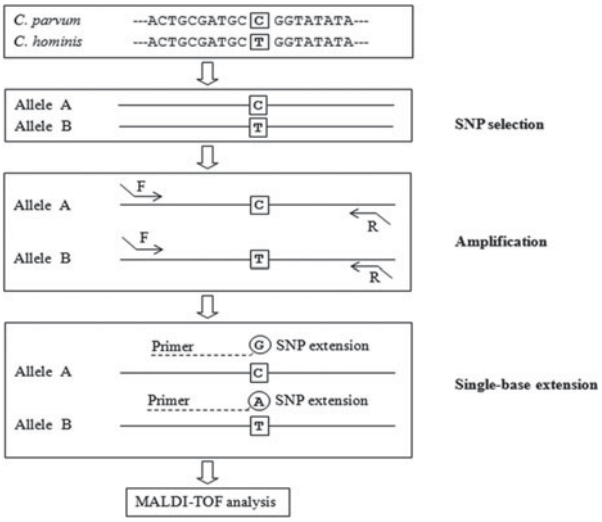


Fig. 1. Schematic diagram of the iPLEX™ Gold genotyping process. SNV markers are selected. The region around each SNV is PCR amplified in each sample. Specific oligonucleotides are added for a single-base extension. SNV alleles are identified by allele-specific differences in mass between alternative extension products.

immediately upstream of the polymorphic site and produces a specific single-base extension of the product complementary to the SNV (Gabriel *et al*. 2009). The mass difference of the single-base extension products enables allelic discrimination, which is performed by MALDI-TOF mass spectrometry (Sauer and Gut, 2002). Several SNVs of interest can be amplified simultaneously, a process known as 'multiplexing'.

The protocol was initially developed in two plexes of 20 and 15 SNVs. These were applied to 671 samples distributed into eight plates containing 90 samples and six appropriate controls each. However, given that some of the SNVs produced low-quality results (e.g. low call rates or dubious genotypes), 20 additional SNVs were also genotyped (Tables 1 and 2). These were arranged in three plexes of ten, six and four SNVs each that were applied to a single plate with 83 samples with dubious genotypes and the corresponding positive and negative controls. The designs of amplification and extension primers, as well as allele calling, were performed at the Universidade de Santiago de Compostela node of the Centro Nacional de Genotipado (CEGEN).

Diagnostic SNVs were chosen among those described in a multilocus variation study (Abal-Fabeiro *et al*. 2013) and from comparison of selected representative sequences retrieved from GenBank (Supplemental Table 1S – in Online version only). All SNVs were located in the coding sequences of five genes: *ACoAs*, *COWP1*, *gp60*, *gtub* and *ISWIr*, which have been mapped to four different chromosomes (1, 6, 6, 7 and 8 respectively; footnote 'a' of Supplemental Table S2 – in Online version only in

Table 1. Sensitivity and specificity assessment (%) of the 19 single nucleotide variant (SNV) markers used to identify *Cryptosporidium* species

SNV name	Allele	Identifies	*TP*	*FN*	*TN*	*FP*	*Sn*	*Sp*
ACOAS_235	C	*m*	1	0	81	0	100·0	100·0
	T	*p, h*	78	3	1	0	96·3	100·0
COWP1_111	G	*s*	0	0	82	0	–	100·0
COWP1_114	C	*p, m*	39	1	42	0	97·5	100·0
	T	*h, c, u, f*	40	2	40	0	95·2	100·0
COWP1_142	C	*f*	0	0	82	0	–	100·0
COWP1_189	G	*c*	0	0	82	0	–	100·0
COWP1_255	C	*u*	0	0	82	0	–	100·0
	A	*s*	0	0	82	0	–	100·0
COWP1_291	G	*f*	0	0	82	0	–	100·0
COWP1_333	C	*m*	1	0	81	0	100·0	100·0
	T	*p, h, f*	79	2	1	0	97·5	100·0
COWP1_336	G	*u*	0	0	82	0	–	100·0
	A	*s*	0	0	82	0	–	100·0
COWP1_399	T	*m*	0	1	81	0	0·0	100·0
	A	*p, h*	32	49	1	0	39·5	100·0
COWP1_435	C	*c*	0	0	82	0	–	100·0
GP60_73	G	*f*	0	0	82	0	–	100·0
	T	*p*	36	3	6	37	92·3	14·0
GTUB_ 96	A	*mu*	0	0	82	0	–	100·0
GTUB_360	G	*p*	36	3	42	1	92·3	97·7
	A	*h*	38	4	39	1	90·5	97·5
GTUB_735	T	*p*	34	5	43	0	87·2	100·0
	C	*h*	39	3	40	0	92·9	100·0
GTUB_804	G	*m*	1	0	81	0	100·0	100·0
	A	*p, h*	79	2	1	0	97·5	100·0
ISWIR_104	T	*mu*	0	0	82	0	–	100·0
ISWIR_120	C	*h*	39	3	40	0	92·9	100·0
	T	*p, m*	38	2	42	0	95·0	100·0
ISWIR_249	G	*m*	1	0	81	0	100·0	100·0
	A	*p, h*	72	9	1	0	88·9	100·0
Pooled			683	92	1728	39	88·1	97·8

m: *C. meleagridis*; *p*: *C. parvum*; *h*: *C. hominis*; *s*: *C. suis*; *f*: *C. felis*; *c*: *C. canis*; *u*: *C. ubiquitum*; *mu*: *C. muris*. *Sn*: *sensitivity*; *Sp*: *specificity* (both expressed as percentage). The number of true positive (*TP*), false negatives (*FN*), true negatives (*TN*) and false positives (*FP*) were estimated by comparing the iPLEX typing results with those obtained by Sanger sequencing (considered the gold standard, see Materials and Methods). Additional SNVs selected to reinforce the accuracy of base calling in a reduced number of samples with uncertain genotypes are typed in bold.

Abal-Fabeiro *et al.* 2013). Nineteen SNVs were selected for species identification and 36 for subtype assignment. The subtype of each sample was determined on the basis of the genotypes of various SNVs at *gp60*. Two other loci, *ACoAs* and *gtub*, were also used for verification purposes, taking advantage of the fact that the patterns of genetic variation in *Cryptosporidium* at these loci are structured according to the *gp60* haplotypes (Abal-Fabeiro *et al.* 2013).

Four types of internal quality-controls of the genotyping protocol were included: (i) 91 samples previously sent to the Center for Disease Control and Prevention (CDC, Atlanta, USA) for identification at the species level by Sanger sequencing of both the *COWP1* and *SSU rRNA* (82 of which were successfully characterized). (ii) A subset of 24 of these 82 samples typed by Sanger sequencing of *gp60* (Abal-Fabeiro *et al.* 2013). (iii) Repeated samples collected from the same individual at different stages of the infection, which were analysed blindly (i.e. 38 patients and 7 cows were sampled twice, and 5 other patients were sampled three times). (iv) Human blood DNA samples (*Cryptosporidium* free) and reagent blanks (negative controls).

Results were analysed in a sequential way; in the first round only the SNVs determining the species were evaluated, while in the second round the genotypes of a different group of SNVs were used to assign the subtype of isolates successfully characterized at the species level.

Given the high levels of within-species diversity described in *C. meleagridis* (Abal-Fabeiro *et al.* 2013) all the samples infected with this species were PCR amplified and sequenced at the *gp60* locus to check if the genotypes matched the Sanger sequencing results using primers and protocols previously described (Abal-Fabeiro *et al.* 2013). Other *Cryptosporidium* samples showing iPLEX inconclusive results were also sequenced at the *gp60* locus for species or subtype identification.

Table 2. Sensitivity and specificity assessment (%) of the 36 single nucleotide variant (SNV) markers used to identify *Cryptosporidium gp60* subtypes

SNV name	Allele	Identifies	*TP*	*FN*	*TN*	*FP*	*Sn*	*Sp*
ACOAS_256	C	Ib	8	1	1	0	88·9	100·0
	T	Id	1	0	9	0	100·0	100·0
ACOAS_686	A	IIa	9	1	3	0	90·0	100·0
	G	IIn	3	0	10	0	100·0	100·0
GP60_33_1	C	Ia	0	0	10	0	–	100·0
GP60_33_2	A	Ig	0	0	10	0	–	100·0
GP60_45-1	G	Ia	0	0	10	0	–	100·0
GP60_45-2	T	Ig	0	0	9	1	–	90·0
GP60_110	G	IIIb	0	0	1	0	–	100·0
	A	IIIf, IIIi	0	1	0	0	0·0	–
GP60_111	T	IIIg	0	0	1	0	–	100·0
	C	IIIf, IIIh, IIIi	1	0	0	0	100·0	–
GP60_131	G	IIIh	0	0	1	0	–	100·0
GP60−144	C	IIb	0	0	13	0	–	100·0
	T	IIa, IId, IIf, IIg, IIj,IIn	0	10	3	0	0·0	–
GP60_162	T	If	0	0	10	0	–	100·0
GP60_171_1	T	IIf	0	0	13	0	–	100·0
GP60_171_2	A	IIIb	0	0	1	0	–	100·0
	C	IIIf, IIIg,IIIh, IIIi	1	0	0	0	100·0	–
GP60_181	G	IIj	0	0	13	0	–	100·0
GP60_183	C	IId	0	0	13	0	–	100·0
GP60_215	C	IIb	0	0	13	0	–	100·0
GP60_219	T	If	0	0	10	0	–	100·0
GP60_228	T	IId	0	0	13	0	–	100·0
	C	IIb, IIf	0	0	13	0	–	100·0
GP60_241	G	IIh	0	0	13	0	–	100·0
GP60_243	C	IIc	0	0	13	0	–	100·0
GP60_259	A	IIIf, g	1	0	0	0	100·0	–
	G	IIIb, IIIh, IIIi	0	0	1	0	–	100·0
GP60_260	C	IIj	0	0	13	0	–	100·0
GP60_261	C	Ia, Ig	0	0	10	0	–	100·0
GP60_270	C	IIg	0	0	13	0	–	100·0
GP60_274	A	IIc	0	0	13	0	–	100·0
GP60_276	A	IId	0	0	13	0	–	100·0
GP60_281	C	IIh	0	0	13	0	–	100·0
GP60_305	G	IIIb	0	0	0	0	–	–
GP60_318	T	Ie	0	0	10	0	–	100·0
GP60_335	C	IIIf	1	0	0	0	100·0	–
	G	IIIb, IIIg, IIIh, IIIi	0	0	1	0	–	100·0
GP60_349	A	Ie	0	0	10	0	–	100·0
GTUB_60	A	IIIj	0	0	1	0	–	100·0
	G	IIIf, IIIg, IIIh	1	0	0	0	100·0	–
GTUB_85	A	Ib	9	0	1	0	100·0	100·0
	C	Id	1	0	9	0	100·0	100·0
GTUB_87	C	IIIf, IIIh	1	0	0	0	100·0	–
	T	IIIg, IIIj	0	0	1	0	–	100·0
GTUB_417	T	Ib	8	1	1	0	88·9	100·0
	C	Id	1	0	9	0	100·0	100·0
GTUB_705	G	IIIh	0	0	1	0	–	100·0
	A	IIIf, IIIg, IIIj	1	0	0	0	100·0	–
Pooled			47	14	326	1	77·0	99·7

TP, *FN*, *TN* and *FP* were estimated by comparing the iPlex typing results with those obtained by Sanger sequencing (considered the gold standard) of *gp60* (Abal-Fabeiro *et al*. 2013). Additional SNVs selected to reinforce the accuracy of base calling in a reduced number of samples with uncertain genotypes are typed in bold.

To describe the diagnostic performance of each SNV allele, two widely used parameters were estimated: *sensitivity* (*Sn*), defined as the proportion of cases with the investigated attribute (i.e. a particular genotype) which are correctly identified and *specificity* (*Sp*), which measures the proportion of correct negative calls of the marker. $Sn = TP/(TP + FN)$, where *TP* is the number of true positive (TP) calls and *FN* the number of false negatives (FN). $Sp = TN/(TN + FP)$, where *TN* and *FP* stand for the

numbers of true negative and false positive calls, respectively. The number of TP, FN, true negatives (TN) and false positives (FP) were estimated by comparing the iPLEX typing results with those obtained by Sanger sequencing of diagnostic loci in a subset of samples (see above).

RESULTS

Identification of the species

Nineteen SNVs at five different genomic loci were selected to identify *Cryptosporidium* species (Table 1). Seven of the SNVs were monoallelic (i.e. the genotyping reaction is expected to work only if a particular allele is present) and 12 biallelic (two alternative results are expected). The calling performance of each SNV allele was determined by comparing their typing results with those obtained by successful Sanger sequencing of 82 out of 91 samples previously sent to the CDC for species identification. iPLEX genotyping permitted the identification of the *Cryptosporidium* species in 86 out of these 91 control isolates (94·5%), a slightly larger fraction than with the Sanger sequencing method, which was successful for 82 samples (90·1%; the difference is not statistically significant, $P = 0{\cdot}40$, in a two-tailed Fisher's exact test). The two methods produced coincident species determinations across the 91 control samples. The only discrepancies corresponded to two samples that were not typed by the iPLEX method, six by Sanger sequencing and three samples that were not typed by any of the two approaches.

Redundant SNVs were used to ensure the correct classification of the samples and to prevent against sporadic failures of the genotyping assays. This can be illustrated with the case of the two alleles of COWP1_399: the first one (nucleotide T), failed to detect the single *C. meleagridis* positive control (Table 1), as determined by Sanger sequencing and by other redundant SNVs such as ACOAS_235, COWP1_333, GT_804 or ISWIR_249, that correctly identified this species. The second allele (nucleotide A), only detected 32 out of the 81 *Cryptosporidium parvum* or *Cryptosporidium hominis* samples among the controls, which were also identified by alternative markers.

Despite the failure of some SNV callings, pooled sensitivity (*Sn*) and specificity (*Sp*) values across all markers (based on samples where Sanger sequencing produced a result) were high: 88·1 and 97·8%, respectively.

Identification of gp60 subtypes

Once the species was identified for each isolate, a new set of 36 SNVs selected for subtype determination were genotyped (22 monoallelic and 14 biallelic, Table 2). The results of the subtyping matched precisely those obtained by Sanger sequencing of the *gp60* gene in a subset of 24 human isolates that were used as positive controls for the subtyping process: Ib (n = 9), IIa (n = 10), IIn (n = 3), Id (n = 1) and IIIf (n = 1) (Abal-Fabeiro *et al*. 2013). Again, there was large redundancy between many of the markers and their performance varied significantly. Pooled *Sn* and *Sp* parameters values were high: 77·0 and 99·7, respectively. The lower sensitivity can be attributed to the performance of one marker (GP60_144; nucleotide T), which displayed a high proportion of false negative results.

Considering the two-step genotyping process as a whole (including the species and subtype determination), the overall *Sn* and *Sp* values for the combined set of markers were 87·3 and 98·0, respectively. These parameters would reach even higher values if only a selection of those SNVs with the best performance in terms of sensitivity and specificity was considered (pooled *Sn* = 94·5 and *Sp* = 99·8; Supplemental Table 2S – in Online version only).

Table 3. Subtyping results obtained by SNV-marker high-throughput genotyping in a panel of 671 human and bovine samples

Species	gp60 subtype	Human	Cattle
C. hominis	Ia	3	0
	Ib	336	0
	Id	27	0
	Ie	4	0
	U	0	1
C. parvum	IIa	155	56
	IId	3	2
	IIn	16	0
	U	2	1
C. meleagridis	IIIb	1	0
	IIIf	2	0
C. felis		1	0
Not typed		58	3
Total		608	63

U: undetermined.

Analysis of a panel of 671 human and bovine samples

The analysis of the full panel of samples allowed species identification in 550 of the 608 human isolates (90·5%), 370 of which corresponded to *C. hominis* (60·8%), 176 to *C. parvum* (28·9%), three to *C. meleagridis* (0·5%) and one to *C. felis* (0·1%) (Table 3). Ambiguous results with genotype calls corresponding to *C. hominis* and *C. parvum* were obtained in two additional samples. Isolate 578 produced, among other base calls common to both species, a *C. parvum* specific G at GTUB_360 and a *C. hominis* specific C at GTUB_735 and ISWIR_120

(Supplemental Table 3S – in Online version only). The other one, sample 744, showed a heterozygous C/T at COWP1_114 compatible with an infection with several species (Supplemental Table 3S – in Online version only), although the remaining SNV calls were specific to *C. hominis*. To further investigate the existence of multiple infections, the *gp60* locus in these samples was sequenced by the Sanger method to determine not only the species but also the subtype/s causing the cryptosporidiosis. We found no evidence for variation in the length of the PCR amplicons and there were no double peaks in the sequencing electropherograms, as would be expected in the case of a mixed infection with two different *gp60* subtypes. The nucleotide sequences revealed that sample 578 contained *C. parvum* IIa and sample 744 *C. hominis* Ib.

Sixty (95·2%) of the 63 bovine isolates were successfully characterized and *C. parvum* was found in 59 cases (Table 3 and Supplemental Table 3S – in Online version only). One of the bovine samples (G27) showed a C call at ISWIR_120 compatible with the presence of *C. hominis*, something rarely reported to date (Smith *et al.* 2005). We failed to PCR amplify the *gp60* product for this allele.

The *gp60* subtype was determined in 605 out of the 671 isolates and the results obtained were consistent across samples collected from the same individual at different stages of the infection, except in three of them where one of the repeats failed to produce signal at any SNV (data not shown).

In humans the most frequent *C. hominis* subtype was Ib (n = 336; Table 3), followed by Id (n = 27), while the presence of Ia and Ie was uncommon (3 and 4 isolates, respectively). The predominant variant in *C. parvum* was IIa (n = 155), while other subtypes (IIn and IId) were found at much lower frequencies (n = 16 and 3, respectively). Two *C. parvum* isolates (10 and 572, Supplemental Table 4S – in Online version only) showed inconclusive results (i.e. lack of genotype call at the relevant SNVs) and only after sequencing their *gp60* gene they could be unambiguously assigned to IIa.

Two *C. meleagridis* isolates (95 and 451, Supplemental Table 4S – in Online version only) were also genotyped by Sanger sequencing of the *gp60* locus and subsequently assigned to subtype IIIf (Abal-Fabeiro *et al.* 2013) in good agreement with the iPLEX™ method. The third *C. meleagridis* isolate (161), typed as IIIb, could be not verified by Sanger sequencing.

Subtype was identified in 58 (92·1%) of the 63 bovine samples. The most prevalent subtype was IIa (n = 56), followed by IId (n = 2) (Table 3). One additional sample (G39) exhibited inconclusive results (Supplemental Table 4S – in Online version only), being classified as IIa only after its *gp60* nucleotide sequence was determined by Sanger sequencing.

DISCUSSION

The accurate diagnosis of cryptosporidiosis relies on the correct identification of *Cryptosporidium* species and subtypes, and it is essential for the study of the population structure, epidemiology and evolution of the parasite (Gasser *et al.* 2006; Jex *et al.* 2007b). The precise classification of the different genetic variants of this pathogen is central to detect potential sources of contamination and therefore to the prevention and control of these parasites (Jex *et al.* 2007a; Chalmers, 2008). To estimate the disease burden, identify outbreaks or understand population trends in surveillance data (Gasser, 2006; Chalmers, 2008; The ANOFEL *Cryptosporidium* National Network, 2010; Yoder *et al.* 2010) the genetic characterization of hundreds of samples is required, making increasingly necessary the application of efficient high-throughput technologies capable of genotyping large sample panels at low cost. Here we present one such method for the simultaneous genotype determination of a moderate number of SNVs at different loci and apply it to a large sample.

Marker selection

One major difficulty for the use of high-throughput technologies for molecular typing of non-model organisms is the lack of a panel of variants (preferably single nucleotide substitutions) that can be used as a reference. Here, we took advantage of the increasing number of nucleotide sequences of marker loci from different *Cryptosporidium* isolates that are available in public databases (GenBank, CryptoDB) and our own work (Abal-Fabeiro *et al.* 2013) to compile a panel of SNVs for the identification of *Cryptosporidium* genotypes commonly found in humans.

The ability of a technique to classify the different *Cryptosporidium* species depends on the choice of suitable genetic markers, which should combine low levels of polymorphism within species with a considerable degree of variation among them (Gasser, 2006). One of the most widely used genes for the classification of *Cryptosporidium* isolates is the small ribosomal subunit (SSU rRNA), which has been sequenced for all species (Jex *et al.* 2008). However, as found in the *C. parvum* and *C. hominis* genomes, this locus is present in several paralogous copies not necessarily identical (Strong and Nelson, 2000; Abrahamsen *et al.* 2004; Xu *et al.* 2004). In most *Cryptosporidium* species the number and sequences of the *SSU rRNA* copies are still ignored and the finding of any new genetic variant could be erroneously assigned to a novel species, when it could be a previously unidentified copy from a known species (Navarro-i-Martinez *et al.* 2003). The use of single copy genes as molecular markers is therefore highly recommended, since they facilitate the

sequence analyses and avoid misinterpretations of the data. Consequently, all marker SNVs selected for the multiplex study correspond to single copy nuclear loci (Abal-Fabeiro *et al.* 2013): *COWP1* and *gp60* had been extensively used for typing the main species involved in human and bovine infections (McLauchlin *et al.* 2000; Pedraza-Diaz *et al.* 2001; Kato *et al.* 2003; Leoni *et al.* 2006; Hunter *et al.* 2007; Geurden *et al.* 2009; Widmer and Lee, 2010) and the other three loci, (*ACoAs*, *gtub* and *ISWIr*) also present variants that are fixed between species (Abal-Fabeiro *et al.* 2013).

Furthermore, the use of a single locus to classify the isolates in different groups of alleles (subtyping) can be problematic (Widmer, 2009). The analysis of the microsatellite length polymorphism in *gp60* (Strong *et al.* 2000) as the main tool to identify *Cryptosporidium* variants exhibited inconsistencies in *C. hominis* and *C. parvum*, where sequences with the same microsatellite size carried different serine trinucleotides (Sulaiman et al. 2005; Gatei et al. 2007). As a consequence, many authors identified subtypes according to length polymorphism and nucleotide variation by counting the number of TCA, TCG or TCT repeats present in the *gp60* microsatellite region (Sulaiman *et al.* 2005; Jex and Gasser, 2010; Xiao, 2010). However, multilocus studies comparing the subtype classification obtained with *gp60* and other loci showed discrepancies; for example, samples sharing a *gp60* allele exhibited differences when other genetic markers, particularly micro and mini-satellites, were included. These observations were made after scoring isolates either according to amplicon lengths (Mallon *et al.* 2003; Tanriverdi and Widmer, 2006) or combining both amplicon lengths and SNVs (Gatei *et al.* 2007, 2008; Widmer and Lee, 2010). Considering that microsatellites are highly unstable and have some of the highest mutation rates observed at molecular loci (Goldstein and Pollock, 1997; Ellegren, 2004), polymorphisms either in length or in nucleotide composition are not excessively surprising. The finding of genetic differences at distinct microsatellite loci, is also predictable. Therefore, and to avoid such conflicts, we selected SNVs in *gp60* (Strong *et al.* 2000), in a region which did not include the microsatellite, and two additional single copy nuclear genes, *gtub* and *ACoAs*, which proved to be useful to classify samples into groups that can be associated with distinct *gp60* subtypes (Abal-Fabeiro et al. 2013).

Performance

We designed a two-step genotyping protocol based on the iPLEX methodology, consisting of an initial determination of the species, followed by the identification of the *gp60* subtype. It displayed very good performance at the two phases of the analysis, particularly at the identification of the species. However, two kinds of problems arose at this stage in some samples: the failure to produce an extension at the targeted SNVs and the presence of ambiguous genotype calls. Indeed, 49 out of the 61 samples that were not assigned to a particular species (Table 3) failed to produce an extension at all targeted SNVs (Supplemental Table 3S – in Online version only). This probably reflects lack of success in the single-base extension step due to below-requirements template DNA quantity or quality. In this respect it must be noted that the iPLEX methodology produced genotype data for six samples where Sanger sequencing failed. This performance is consistent with the observation that the former method has lower DNA quality restrictions and works comparatively better for samples with low DNA concentration or partially degraded DNA (Mendisco *et al.* 2011).

In samples with ambiguous calls corresponding to *C. hominis* and *C. parvum*, the lack of supporting evidence from the Sanger sequencing data does not rule out the possibility of mixed infections. Given the smaller amplicon sizes needed by the mass spectrometry method, the mixed results might reflect the presence of degraded DNA templates of a hypothetical second genotype, which would be missed by Sanger sequencing. A similar pattern would be observed if the parasite load of one of the species was much larger than the second one. To address the issue of mixed infections in which small amounts of DNA from a minor species could be involved, alternative methods like PCR-RFLP, SSCP or the use of species-specific primers have been proposed (Chalmers *et al.* 2005).

Genotyping results

MALDI-TOF genotyping performed as well as Sanger sequencing in our internal controls. In fact, the most informative SNVs (those selected because of their best performance in terms of sensitivity and specificity) showed mean sensitivity and specificity results comparable to those obtained with other molecular techniques such as real-time PCR (Stroup *et al.* 2006; Hadfield *et al.* 2011), RFLP analysis of *COWP1* and *SSU rRNA* (Chalmers *et al.* 2009), Sanger sequencing of *gp60* (Chalmers *et al.* 2008; Hijjawi *et al.* 2010) or fragment-size-analysis of three microsatellite markers (*gp60*, *ML1* and *ML2*) (Hunter *et al.* 2007).

We identified nine different *gp60* subtypes from four *Cryptosporidium* species in our panel of human-derived isolates (Table 3). The most common subtypes were Ib and IIa, which represent 61·3 and 28·3% of the typed human samples, respectively.

Subtypes Id and IIn were also abundant while the others were found in just a few isolates. The performance of probes designed to identify the less frequent *gp60* subtypes, like *C. hominis* If, Ig or *C. parvum* IIb, IIc, IIf, IIg, IIh, IIj could not be determined because none of them were detected in our sample panel despite the use of multiple SNVs.

Most of the markers that failed the primer extension were designed for the identification of *C. meleagridis* subtypes. Given the particularly high levels of within-species *gp60* diversity described in this species (Abal-Fabeiro *et al*. 2013), the possibility that these failures reflect undetected variation in the priming sites cannot be excluded.

The bovine sample group was not so diverse because most animals harboured *C. parvum*. The only exception was one calf putatively infected with *C. hominis*. Nevertheless, this result has to be taken with caution, since this isolate only produced base calls at three SNVs and all attempts to sequence the *gp60* gene in this sample were unsuccessful, probably due to the lack of good-quality DNA.

Although mass spectrometry has been widely used for the genotyping of SNVs for population surveys, often with medical purposes, as far as we know this is the first time this technology was used for the genotyping of *Cryptosporidium* samples. In our view, this method has the following advantages over Sanger sequencing: (i) it allows the simultaneous analysis of a large number of markers. This is particularly interesting because it allows the scrutiny of multiple genomic regions. (ii) The multiplexing design allows an easy update of the SNVs panel to be analysed. Further studies with the same purpose as this one could be limited to the sets of most informative SNVs (Supplemental Table 2S – in Online version only), greatly improving the method's performance indicators (sensitivity and specificity) without a noticeable loss of information. In addition, other SNVs of interest, for example to identify other species or *gp60* subtypes of interest, could also be included in multiplex designs. (iii) The method is particularly suitable for the genotyping of large numbers of samples, with high automatization and limited handling. (iv) It is much faster than other sequencing techniques; the full analysis of our panel of 55 SNVs in the 671 samples can be accomplished in less than 3 weeks, as compared with the several months necessary if other methods are used. (v) We also estimated that this technology is around 40 times cheaper than Sanger sequencing, which is now commonly used in *Cryptosporidium* studies, with 0·1 € per genotype as compared with over 4·0 € per Sanger sequencing reaction. The cost benefit increases with the sample size to be analysed. (vi) The small size of the amplicons means that the method can be used even with degraded template DNA. Finally (vii), the genotyping results are very robust, as demonstrated by the high repeatability in our replicated samples.

Overall, here we presented a method for the high-throughput genotyping of *Cryptosporidium* isolates in large sample panels. The method is based on the multiplex analysis of a collection of SNVs used as species and *gp60* subtype markers, by means of mass spectrometry. Compared to Sanger sequencing this method is practical, efficient, cheaper and less time consuming and it can be progressively adapted for the diagnosis of new species or subtypes as soon as their sequences are characterized.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182013001807.

ACKNOWLEDGEMENTS

We are grateful to Alfredo Rúa and David Fernández-García (Laboratorio de Sanidade e Producción Animal, Lugo, Spain) who kindly provided Cryptosporidium-positive bovine samples. We thank Dr A. Campos (Servicio de Hematoloxía-CHUS, Santiago de Compostela, Spain) for lending us laboratory equipment, as well as the Fundación Pública Galega de Medicina Xenómica (SERGAS, Spain) for the use of their laboratory facilities, and Santiago Méndez-Torres (Servicio de Microbioloxía e Parasitoloxía-CHUS, Santiago de Compostela, Spain) for technical assistance, and Luis Navarro-i-Martinez, Fernando Bornay-Llinares (Universidad Miguel Hernández, Alicante, Spain) and Norman J. Pieniazek (CDC, Atlanta, USA) for the Sanger sequencing of samples used as positive controls. We also thank the anonymous reviewers whose careful review and suggestions helped to improve an earlier version of this manuscript.

FINANCIAL SUPPORT

This work was financed by a grant from Xunta de Galicia, Spain to CB (PGIDIT06PXIB228073PR). JLA-F was supported by a graduate student fellowship co-financed by Xunta de Galicia, Spain and the European Social Fund; XM was supported by a Programa Ramón y Cajal contract (Ministerio de Ciencia e Innovación, Spain); XB was supported by grant PGIDIT06PXIB228073PR (Xunta de Galicia. Spain) and CB was supported by a Programa Isidro Parga Pondal contract (Xunta de Galicia, Spain).

REFERENCES

Abal-Fabeiro, J. L., Maside, X., Bello, X., Llovo, J. and Bartolomé, C. (2013). Multilocus patterns of genetic variation across *Cryptosporidium* species suggest balancing selection at the *gp60* locus. *Molecular Ecology* 22, 4723–4732. doi: 10.1111/mec.12425.

Abrahamsen, M. S., Templeton, T. J., Enomoto, S., Abrahante, J. E., Zhu, G., Lancto, C. A., Deng, M., Liu, C., Widmer, G., Tzipori, S., Buck, G. A., Xu, P., Bankier, A. T., Dear, P. H., Konfortov, B. A., Spriggs, H. F., Iyer, L., Anantharaman, V., Aravind, L. and Kapur, V. (2004). Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science* 304, 441–445. doi: 10.1126/science.1094786.

Casemore, D. P., Amstrong, M., Jackson, B., Nichols, G. and Thorn, B. T. (1984). Screening for *Cryptosporidium* in stools. *Lancet* 323, 734–735. doi: 10.1016/S0140-6736(84)92245-1.

Chalmers, R. M. (2008). *Cryptosporidium*: from laboratory diagnosis to surveillance and outbreaks. *Parasite* 15, 372–378. doi: 10.1051/parasite/2008153372.

Chalmers, R. M., Ferguson, C., Caccio, S., Gasser, R. B., Abs, E. L. O. Y. G., Heijnen, L., Xiao, L., Elwin, K., Hadfield, S., Sinclair, M. and Stevens, M. (2005). Direct comparison of selected methods for genetic categorisation of *Cryptosporidium parvum* and *Cryptosporidium hominis* species. *International Journal for Parasitology* 35, 397–410. doi: 10.1016/j.ijpara.2005.01.001.

Chalmers, R. M., Hadfield, S. J., Jackson, C. J., Elwin, K., Xiao, L. and Hunter, P. (2008). Geographic linkage and variation in *Cryptosporidium hominis*. *Emerging Infectious Diseases* 14, 496–498. doi: 10.3201/eid1403.071320.

Chalmers, R. M., Elwin, K., Thomas, A. L., Guy, E. C. and Mason, B. (2009). Long-term *Cryptosporidium* typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. *Eurosurveillance* 14, pii: 19086.

Ellegren, H. (2004). Microsatellites: simple sequences with complex evolution. *Nature Reviews of Genetics* 5, 435–445. doi: 10.1038/nrg1348.

Gabriel, S., Ziaugra, L. and Tabbaa, D. (2009). SNP genotyping using the Sequenom MassARRAY iPLEX platform. *Current Protocols in Human Genetics*, Chapter 2, Unit 2 12. doi: 10.1002/0471142905.hg0212s60.

Gasser, R. B. (2006). Molecular tools – advances, opportunities and prospects. Veterinary *Parasitology* 136, 69–89. doi: 10.1016/j.vetpar.2005.12.002.

Gasser, R. B., Hu, M., Chilton, N. B., Campbell, B. E., Jex, A. J., Otranto, D., Cafarchia, C., Beveridge, I. and Zhu, X. (2006). Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. *Nature Protocols* 1, 3121–3128. doi: 10.1038/nprot.2006.485.

Gatei, W., Das, P., Dutta, P., Sen, A., Cama, V., Lal, A. A. and Xiao, L. (2007). Multilocus sequence typing and genetic structure of *Cryptosporidium hominis* from children in Kolkata, India. *Infection, Genetics and Evolution* 7, 197–205. doi: 10.1016/j.meegid.2006.08.006.

Gatei, W., Barrett, D., Lindo, J. F., Eldemire-Shearer, D., Cama, V. and Xiao, L. (2008). Unique *Cryptosporidium* population in HIV-infected persons, Jamaica. *Emerging Infectious Diseases* 14, 841–843. doi: 10.3201/eid1405.071277.

Geurden, T., Levecke, B., Caccio, S. M., Visser, A., De Groote, G., Casaert, S., Vercruysse, J. and Claerebout, E. (2009). Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhoea in human patients in Belgium. *Parasitology* 136, 1161–1168. doi: 10.1017/S0031182009990436.

Goldstein, D. B. and Pollock, D. D. (1997). Launching microsatellites: a review of mutation processes and methods of phylogenetic interference. *Journal of Heredity* 88, 335–342.

Hadfield, S. J., Robinson, G., Elwin, K. and Chalmers, R. M. (2011). Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of real-time PCR. *Journal of Clinical Microbiology* 49, 918–924. doi: 10.1128/JCM.01733-10.

Hijjawi, N., Ng, J., Yang, R., Atoum, M. F. and Ryan, U. (2010). Identification of rare and novel *Cryptosporidium GP60* subtypes in human isolates from Jordan. *Experimental Parasitology* 125, 161–164. doi: 10.1016/j.exppara.2010.01.011.

Hunter, P. R., Hadfield, S. J., Wilkinson, D., Lake, I. R., Harrison, F. C. and Chalmers, R. M. (2007). Subtypes of *Cryptosporidium parvum* in humans and disease risk. *Emerging Infectious Diseases* 13, 82–88. doi: 10.3201/eid1301.060481.

Jex, A. R. and Gasser, R. B. (2010). Genetic richness and diversity in *Cryptosporidium hominis* and *C. parvum* reveals major knowledge gaps and a need for the application of ‘‘next generation’’ technologies – research review. *Biotechnology Advances* 28, 17–26. doi: 10.1016/j.biotechadv.2009.08.003.

Jex, A. R., Ryan, U. M., Ng, J., Campbell, B. E., Xiao, L., Stevens, M. and Gasser, R. B. (2007a). Specific and genotypic identification of *Cryptosporidium* from a broad range of host species by nonisotopic SSCP analysis of nuclear ribosomal DNA. *Electrophoresis* 28, 2818–2825. doi: 10.1002/elps.200600772.

Jex, A. R., Whipp, M., Campbell, B. E., Caccio, S. M., Stevens, M., Hogg, G. and Gasser, R. B. (2007b). A practical and cost-effective mutation scanning-based approach for investigating genetic variation in *Cryptosporidium*. *Electrophoresis* 28, 3875–3883. doi: 10.1002/elps.200700279.

Jex, A. R., Smith, H. V., Monis, P. T., Campbell, B. E. and Gasser, R. B. (2008). *Cryptosporidium* – biotechnological advances in the detection, diagnosis and analysis of genetic variation. *Biotechnology Advances* 26, 304–317. doi: 10.1016/j.biotechadv.2008.02.003.

Kato, S., Lindergard, G. and Mohammed, H. O. (2003). Utility of the *Cryptosporidium* oocyst wall protein (*COWP*) gene in a nested PCR approach for detection infection in cattle. *Veterinary Parasitology* 111, 153–159. doi: 10.1016/S0304-4017(02)00353-9.

Leoni, F., Amar, C., Nichols, G., Pedraza-Diaz, S. and McLauchlin, J. (2006). Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000. *Journal of Medical Microbiology* 55, 703–707. doi: 10.1099/jmm.0.46251-0.

Mallon, M. E., MacLeod, A., Wastling, J. M., Smith, H. and Tait, A. (2003). Multilocus genotyping of *Cryptosporidium parvum* Type 2: population genetics and sub-structuring. Infection, *Genetics and Evolution* 3, 207–218. doi: 10.1016/S1567-1348(03)00089-3.

McLauchlin, J., Amar, C., Pedraza-Diaz, S. and Nichols, G. L. (2000). Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. *Journal of Clinical Microbiology* 38, 3984–3990.

Mendisco, F., Keyser, C., Hollard, C., Seldes, V., Nielsen, A. E., Crubezy, E. and Ludes, B. (2011). Application of the iPLEX Gold SNP genotyping method for the analysis of Amerindian ancient DNA samples: benefits for ancient population studies. *Electrophoresis* 32, 386–393. doi: 10.1002/elps.201000483.

Navarro-i-Martinez, L., Bornay-Llinares, F. J., Rueda, C., del Aguila, C., da Silva, A. J., Oleaga, A., Ramajo, V., Fenoy, S. and Pieniazek, N. J. (2003). Molecular characterization of *Cryptosporidium* sp. from animals in Spain. *Journal of Eukaryotic Microbiology* 50(Suppl.), 553–554. doi: 10.1111/j.1550-7408.2003.tb00626.x.

Pedraza-Diaz, S., Amar, C. F., McLauchlin, J., Nichols, G. L., Cotton, K. M., Godwin, P., Iversen, A. M., Milne, L., Mulla, J. R., Nye, K., Panigrahl, H., Venn, S. R., Wiggins, R., Williams, M. and Youngs, E. R. (2001). *Cryptosporidium meleagridis* from humans: molecular analysis and description of affected patients. *Journal of Infection* 42, 243–250. doi: 10.1053/jinf.2001.0839.

Sauer, S. and Gut, I. G. (2002). Genotyping single-nucleotide polymorphisms by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. *Journal of Chromatography B. Analytical Technologies in Biomedical and Life Sciences* 782, 73–87. doi: 10.1016/S1570-0232(02)00692-X.

Smith, H. V., Nichols, R. A., Mallon, M., Macleod, A., Tait, A., Reilly, W. J., Browning, L. M., Gray, D., Reid, S. W. and Wastling, J. M. (2005). Natural *Cryptosporidium hominis* infections in Scottish cattle. *Veterinary Record* 156, 710–711. doi: 10.1136/vr.156.22.710.

Strong, W. B. and Nelson, R. G. (2000). Preliminary profile of the *Cryptosporidium parvum* genome: an expressed sequence tag and genome survey sequence analysis. *Molecular and Biochemical Parasitology* 107, 1–32. doi: 10.1016/S0166-6851(99)00225-X.

Strong, W. B., Gut, J. and Nelson, R. G. (2000). Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. *Infection and Immunity* 68, 4117–4134. doi: 10.1128/IAI.68.7.4117-4134.2000.

Stroup, S. E., Roy, S., McHele, J., Maro, V., Ntabaguzi, S., Siddique, A., Kang, G., Guerrant, R. L., Kirkpatrick, B. D., Fayer, R., Herbein, J., Ward, H., Haque, R. and Houpt, E. R. (2006). Real-time PCR detection and speciation of *Cryptosporidium* infection using Scorpion probes. *Journal of Medical Microbiology* 55, 1217–1222. doi: 10.1099/jmm.0.46678-0.

Sulaiman, I. M., Hira, P. R., Zhou, L., Al-Ali, F. M., Al-Shelahi, F. A., Shweiki, H. M., Iqbal, J., Khalid, N. and Xiao, L. (2005). Unique endemicity of cryptosporidiosis in children in Kuwait. *Journal of Clinical Microbiology* 43, 2805–2809. doi: 10.1128/JCM.43.6.2805-2809.2005.

Tanriverdi, S. and Widmer, G. (2006). Differential evolution of repetitive sequences in *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Infection, Genetics and Evolution* 6, 113–122. doi: 10.1016/j.meegid.2005.02.002.

The ANOFEL *Cryptosporidium* National Network (2010). Laboratory-based surveillance for *Cryptosporidium* in France, 2006–2009. *Eurosurveillance* 15, 19642.

Widmer, G. (2009). Meta-analysis of a polymorphic surface glycoprotein of the parasitic protozoa *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Epidemiology and Infection* 137, 1800–1808. doi: 10.1017/S0950268809990215.

Widmer, G. and Lee, Y. (2010). Comparison of single- and multilocus genetic diversity in the protozoan parasites *Cryptosporidium parvum* and *C. hominis*. *Applied and Environmental Microbiology* 76, 6639–6644. doi: 10.1128/AEM.01268-10.

Xiao, L. (2010). Molecular epidemiology of cryptosporidiosis: an update. *Experimental Parasitology* 124, 80-89. doi: 10.1016jj.exppara.2009.03.018.

Xiao, L., Fayer, R., Ryan, U. and Upton, S. J. (2004). *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clínica{ Microbiology Reviews* 17, 72-97. doi: 10.1128jCMR.17.1.72-97.2004.

Xu, P., Widmer, G., Wang, Y., Ozaki, L.S., Alves, J.M., Serrano, M. G., Puiu, D., Manque, P., Akiyoshi, D., Mackey, A. J.,, Pearson, W. R., Dear, P. H., Bankier, A. T., Peterson, D. L.Abrahamsen, M. S., Kapur, V., Tzipori, S. and Buck, G.A. (2004). The genome of *Cryptosporidium hominis*. *Nature* 431, 1107-1112. doi: 10.1038fnature02977.

Yoder, J.S., Harral, C. and Beach, M. J. (2010). C¡yptosporidiosis surveillance- United States, 2006-2008. *MMWR. Surveillance Summaries* 59, 1-14.

2.1 Material suplementario

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Supplemental Table 1. Sequences retrieved from GenBank and used as a reference for the classification of *Cryptosporidium* samples

Accesion No.	Locus	Species	Subtype
AF411632.1	*COWP1*	*C. ubiquitum*	
AF266270.1	*COWP1*	*C. suis*	
AF266274.1	*COWP1*	*C. canis*	
AY700394.1	*gp60*	*C. felis*	
EU161648.1	*gp60*	*C. hominis*	Ia
EU146131.1	*gp60*	*C. hominis*	Ie
EU161655.1	*gp60*	*C. hominis*	If
EF208067.1	*gp60*	*C. hominis*	Ig
AY166805.1	*gp60*	*C. parvum*	IIb
AF440636.1	*gp60*	*C. parvum*	IIc
EU140508.1	*gp60*	*C. parvum*	IIc
AY738195.1	*gp60*	*C. parvum*	IIc
AY166806.1	*gp60*	*C. parvum*	IId
AY382675.1	*gp60*	*C. parvum*	IIe
AY738188.1	*gp60*	*C. parvum*	IIf
AY873780.1	*gp60*	*C. parvum*	IIg
AY873781.1	*gp60*	*C. parvum*	IIh
AY700385.1	*gp60*	*C. parvum*	IIj

Supplemental Table 2. Most informative single nucleotide variants (SNVs) used to identify *Cryptosporidium* species and subtypes represented in our dataset of human and bovine samples.

Species	Subtype	ACOAS_235	COWP1_114	COWP1_333	GTUB_360	GTUB_735	GTUB_804	ISWIR_120	COWP1_142	COWP1_291	ACOAS_256	ACOAS_686	GP60_33_1	GP60_110	GP60_111	GP60_183	GP60_228	GP60_259	GP60_261	GP60_276	GP60_318	GP60_335	GP60_349	GTUB_85	GTUB_87	GTUB_417	GTUB_705
C. parvum		T	C	T	G	T	A	T																			
	IIa										T	A												C		C	
	IId										T	G				C	T			A				C		C	
	IIn										T	G												C		C	
C. hominis		T	T	T	A	C	A	C																			
	Ia										T	G	C						C					C		C	
	Ib										C	G												A		T	
	Id										T	G												C		C	
	Ie										T	G								T		A	A		T		
C. meleagridis		C	C	C			G	T																			
	IIIb													G							G						A
	IIIf										T	G			C			A			C				C		A
C. felis									C	G																	

Grey cells indicate iPLEX results that have not yet been confirmed by Sanger sequencing.

Supplemental Table 3. Genotyping results for the 19 single nucleotide variants (SNVs) used as species' markers in a panel of 671 human and bovine samples.

Number of occurrences	Sample code name	Diagnostic	ACOAS_235	COWP1_111	COWP1_114	**COWP1_142**	COWP1_189	COWP1_255	**COWP1_291**	COWP1_333	COWP1_336	COWP1_399	COWP1_435	GP60_73	GTUB_96	GTUB_360	GTUB_735	GTUB_804	ISWIR_104	ISWIR_120	ISWIR_249
147		*h*	T	A	T		A	A		T	A	A		T		A	C	A		C	A
88		*p*	T	A	C		A	A		T	A	A		T		G	T	A		T	A
53		*p*	T		C			A		T				T		G	T	A		T	A
50		*h*	T		T		A	A		T	A	A		T		A	C	A		C	A
49		x																			
37		*h*	T		T			A		T				T		A	C	A		C	A
35		*h*	T		T		A	A		T		A		T		A	C	A		C	A
25		*p*	T		C		A	A		T	A	A		T		G	T	A		T	A
15		*p*	T		C		A	A		T		A		T		G	T	A		T	A
13		*h*	T	A	T		A	A		T	A	A				A	C	A		C	A
8		*h*	T		T			A		T		A		T		A	C	A		C	A
7		*h*	T		T		A	A		T	A	A				A	C	A		C	A
7		*h*	T		T		A	A		T		A				A	C	A		C	A
7		*p*	T		C			A		T		A		T		G	T	A		T	A
7		x												T							
5		*h*	T	A	T	T	A	A		T	A	A		T		A	C	A		C	A
5		*h*	T	A	T		A	A		T	A	A		T		A	C	A		C	
5		*p*	T		C		A	A		T				T		G	T	A		T	A
5		*p*	T		C			A		T								A			
4		*h*		A			A				A	A		T		A	C			C	A
3		*h*	T	A	T		A	A		T	A	A		T	C	A	C	A		C	A
3		*h*	T		T		A	A		T		A				A	C	A		C	
3		*h*	T		T			A		T						A	C	A		C	A
3		*p*	T		C		A	A		T	A			T		G	T	A		T	A
3		*p*	T		C			A		T	A	A		T		G	T	A		T	A
2		*h*	T	A	T		A	A		T	A	A		T		A		A		C	A
2		*h*	T	A			A	A		T	A	A		T		A	C	A		C	A
2		*h*	T		T		A	A		T	A	A		T			C	A		C	A
2		*h*	T		T		A	A		T	A			T		A	C	A		C	A
2		*h*	T		T		A	A		T				T		A	C	A		C	A
2		*h*	T		T			A		T	A	A		T		A	C	A		C	A
2		*h*	T		T			A		T				T		A	C	A		C	
2		*h*	T		T			A		T				T				A			
2		*h*	T		T			A		T								A			
2		*h*					A				A	A		T		A	C			C	A
2		*p*	T		C		A	A		T		A		T		G	T	A		T	A
2		*p*		A	C		A	A		T	A	A		T		G	T	A		T	A
1	75	x												T				A			
1	92	*p*	T		C			A		T						G		A		T	
1	95	*m*	C		C			A		C				T	C	A		G		T	G
1	99	*h*	T											T		A	C	A		C	A
1	121	*p*	T		C			A		T				T		G		A		T	
1	133	*h*	T											T		A	C	A		C	
1	135	*p*	T		C			A		T				T				A		T	A
1	144	*p*			C			A		T				T		G	T	A		T	A
1	161	*m*	C		C			A		C				T				G		T	
1	169	*h*	T		T			A		T						A	C	A		C	
1	174	*h*	T											T			C			C	
1	178	*p*			C			A						T							
1	179	*p*					A					A		T		G	T			T	
1	220	*p*	T		C			A		T		A				G	T	A		T	A
1	269	*p*	T		C	T	A	A		T	A	A		T		G	T	A		T	A
1	289	*p*	T		C			A		T				T			T	A		T	A

Supplemental Table 3. Continuation.

Number of occurrences	Sample code name	Diagnostic	ACOAS_235	COWP1_111	COWP1_114	**COWP1_142**	COWP1_189	COWP1_255	**COWP1_291**	COWP1_333	COWP1_336	COWP1_399	COWP1_435	GP60_73	GTUB_96	GTUB_360	GTUB_735	GTUB_804	ISWIR_104	ISWIR_120	ISWIR_249
1	297	*h*					A					A		T		A	C			C	A
1	339	*h*	T		T		A	A		T		A		T		G	C	A		C	A
1	423	*h*	T	A	T		A	A		T	A	A				A	C	A		C	
1	443	*p*	T	A	C			A		T	A	A				G	T	A		T	A
1	451	*m*	C	A	C		A	A		C	A	T		T	C	A	C	G		T	G
1	476	*h*	T	A	T		A	A		T	A	A		T		A	C			C	A
1	494	*p*	T				A	A		T	A	A		T		G	T	A		T	A
1	504	*h*	T				A	A		T	A	A		T		A	C	A		C	A
1	562	*p*		A			A				A	A		T		G	T			T	A
1	578	*h + p?*					A				A	A				G	C			C	A
1	579	*p*	T		C			A		T	A					G	T	A		T	A
1	612	*p*	T	A	C		A	A			A	A		T		G	T	A		T	A
1	615	*p*	T		C		A	A		T	A	A		T	C	G	T	A		T	A
1	631	*h*	T	A	T		A			T	A	A		T		A	C	A		C	A
1	645	*h*	T	A	T		A	A		T		A		T		A	C	A		C	A
1	655	*p*	T	A	C		A	A		T	A	A				G	T	A		T	A
1	682	*h*	T		T		A	A	T	T	A	A		T		A	C	A		C	A
1	712	*h*					A				A	A		T			C			C	A
1	714	*h*	T		T		A	A		T		A		T			C	A		C	A
1	744	*h + p?*	T		C/T			A		T				T		A		A		C	A
1	777	*h*	T				A	A		T		A		T		A	C	A		C	A
1	792	*p*	T		C		A	A		T	A	A		T		G	C/T	A		T	A
1	838	*h*	T		T		A	A		T	A	A		T		A	C	A		C	
1	844	*h*	T	A	T		A	A		T		A				A	C	A		C	
1	852	*h*	T		T			A		T	A			T		A	C	A		C	A
1	855	*p*	T		C		A	A	T	T	A	A		T		G		A		C/T	A
1	865	*h*	T		T			A						T		A	C	A		C	A
1	968	*p*	T		C		A	A		T						G	T	A		T	A
1	980	*f*				C			G					T			T				
1	A144	*h*			T			A		T		A		T		A	C	A		C	A
1	A147	*h*												T		A	C	A		C	
1	A52	*p*	T	A	C		A	A		T	A			T		G	T	A		T	A
1	C2	*h*	T		T		A	A		T				T			C	A		C	A
1	C23	*h*	T		T	T	A	A		T		A		T		A	C	A		C	A
1	G20	x						A						T							
1	G25	*p*	T					A		T	A	A		T		G/A	T			T	A
1	G26	x	T					A										A			
1	G27	*h*	T											T						C	
1	G30	*p*	T							T				T			T	A		T	A
1	G31	*p*	T		C			A		T				T	C	G	T	A			A
1	G36	*p*	T					A						T		G	T	A		T	A
1	G52	*p*	T		C		A	A		T	A	A		T		G	T	A			A
1	G54	*p*	T											T		G	T			T	A
1	G56	*p*	T		C			A		T						G	T	A		T	A

h= C. hominis; p= C. parvum, m= C. meleagridis, f = C. felis and x= not typed. Bovine samples are labelled with an alphanumeric code starting with a "G" (G1-G65B). Underlined results represent moderate quality of base calling. Heterozygous calls show both alternative alleles separated by a slash. Additional SNVs selected to reinforce the accuracy of base calling in a subset of 83 samples with uncertain genotypes are typed in bold.

Supplemental Table 4. Genotyping results for the 36 single nucleotide variants (SNVs) used as subtype markers in a panel of 671 human and bovine samples

Number of occurrences	Sample code name	Diagnostic	ACOAS_256	ACOAS_686	**GP60_33_1**	**GP60_33_2**	GP60_45_1	GP60_45_2	GP60_110	GP60_111	**GP60_131**	**GP60_144**	**GP60_162**	**GP60_171_1**	**GP60_171_2**	GP60_181	**GP60_183**	GP60_215	**GP60_219**	**GP60_228**	**GP60_241**	GP60_243	GP60_259	**GP60_260**	**GP60_261**	**GP60_270**	**GP60_274**	GP60_276	GP60_281	**GP60_305**	GP60_318	GP60_335	**GP60_349**	**GTUB_60**	GTUB_85	GTUB_87	GTUB_417	GTUB_705
135		Ib	C	G					A									A				T												A	T	T	
49		IIa	T	A																								G						C	T	C	
48		x																																			
44		IIa	T	A					A																			G						C	T	C	
19		Ib	C	G			T		A									A				T												A	T	T	
13		Ib	C	G					A									A				T												A		T	
12		Ib	C	G					A									A				T												A	T	T	A
11		IIa	T	A					A									A									G							C	T	C	
10		IIa	T	A					A																		G				C			C	T	C	
9		Ib	C	G				G	A									A				T												A	T	T	
9		Ib	C	G					A									A				T					G							A	T	T	
8		Ib	C	G			G		A									A				T					G				C			A	T	T	
8		IIa	T	A			T		A																		G							C	T	C	
8		IIa	T	A																							G							C		C	
7		IIa	T	A			T																				G							C	T	C	
7		x					T																														
6		Id	T	G			G	G																										C	T	C	
6		IIa	T	A			T																				G							C		C	
5		Ib	C	G			T		A									A				T												A		T	
5		Ib	C	G				G	A							G		A				T					G							A		T	A
5		Ib	C	G					A									A																	T	T	
5		IIa	T	A																							G				C			C	T	C	
5		IIa	T	A																														C	T	C	
4		Ib	C	G			T		A									A				T									C			A	T	T	
4		Ib	C	G			T		A									A				T												A	T	T	A
4		Ib	C	G					A									A				T									C			A	T	T	
4		IIa	T	A			T		A																		G							C		C	
4		IIn	T	G																							G							C	T	C	
3		Id	T	G			G	G														T												C	T	C	
3		IIa	T	A					A																		G							C		C	
3		IIn	T	G					A																		G				C			C	T	C	
2		Ib	C	G			G	G	A									A				T												A	T	T	
2		Ib	C	G			T		A									A																A	T	T	
2		Ib	C	G			T		A													T												A		T	
2		Ib	C	G			T															T												A		T	
2		Ib	C	G				T	A							A		A				T												A	T	T	
2		Ib	C	G				T								A						T												A	T	T	
2		Ib	C	G					A									A				T					G								T	T	
2		Ib	C	G					A									A				T												A	T/C	T	
2		Ib	C	G					A													T												A	T	T	
2		Ib	C	G														A				T												A	T	T	
2		Ib	C						A									A				T												A	T	T	
2		Ib	C						A									A				T												A		T	
2		Id	T	G			G	G	A									A																C	T	C	
2		Id	T	G				T								A						T												C	T	C	
2		IIa	T	A				G																			G							C	T	C	
2		IIa	T	A					A									A									G							C	T/C	C	
2		IIa	T	A																															T	C	
2		IIa		A																							G							C	T	C	
2		IIn	T	G					A																		G							C	T	C	
1	10	*p*			A	A					G									C	A				C		G		G				G	C			
1	11	Ib			A		T					T		C						G		T			C		G		G				G	A			
1	48	IIn	T	G			T		A																		G							C	T	C	
1	54	IIn	T	G			T		A																		G							C		C	
1	56	IIa	T	A			T		A									A									G							C	T	C	
1	70	Ib	C	G																		T												A		T	
1	74	Id	T															A																C	T	C	
1	76	IId	T	A	A				A								C	A		T		T			C		A		G			T	G	C	T	C	
1	86	IIn	T	G					A																		G							C		C	
1	87	IIn		G			T																				A		G				G	C		C	
1	92	IIa		A			T																				G							C		C	
1	95	IIIf	T	G						C	A	T		C				A					A				G		A/G		C		G	C	C	C	A
1	99	Ib	C	G			T																											A	T	T	
1	102	IIn	T	G		A														C	A						A/G		G				G	C	T	C	
1	124	Ib	C	G																		T												A	T	T	
1	135	IIa		A			T		A																		G							C			
1	138	IIa																									G							C			
1	141	Ib																				T												A			
1	144	IIa		A			T		A																		G							C		C	
1	154	IIa	T	A			T		A									C/A									G							C		C	
1	157	IIa	T	A																							G								T	C	
1	158	Ib	C	G	A	A			A								T	A			A	T			A		G		G				G	A	T	T	
1	161	IIIb					T		G		A																		G		G		G				A
1	168	x																									G										
1	169	Ib	C	G/A					A																									A	T	T	
1	174	Ib		G			T																											A			
1	175	x					T															T															
1	178	IIa					T																											C			
1	183	Ib	C	G																C	A	T					G		G				G	A		T	
1	203	Ib	C	G	A				A					A			T	A		C	A	T			A				G		G		G	A	T	T	
1	208	IIa	T	A	A	A														C	A					C	G	G		G		G		G	C	T	C	
1	217	IId	T	G	T				A								C	A		T							A		G				G	C	T	C	
1	230	Ib	C	G	A				A									A		C	A	T				C	G		G				G	A	T	T	
1	255	Ib	C	G					A									A				T							G		G		G	A	T	T	
1	269	IIa	T	A	A	A					G	T								C	A			A			G		G		G		G	C	T	C	
1	274	Ib	C	G	A				A			T		C				A		G		T				C	A		G				G	A	T	T	
1	278	Id	T	G							G								C			T		A					G				G			C	
1	287	IIa	T	A	A													A		C	A					C	G		G		G			C	T	C	
1	289	IIa	T	A																														C			
1	297	Id	T	G	A	A			A		G	T						A	C	C			A			C	G		G		C		G		T	C	

Supplemental Table 4. Continuation.

Number of occurrences	Sample code name	Diagnostic	ΔCOAS_256	ΔCOAS_686	**GP60_33_1**	**GP60_33_2**	GP60_45_1	GP60_45_2	GP60_110	GP60_111	**GP60_131**	**GP60_144**	**GP60_162**	**GP60_171_1**	**GP60_171_2**	GP60_181	**GP60_183**	GP60_215	**GP60_219**	**GP60_228**	**GP60_241**	GP60_243	GP60_259	**GP60_260**	**GP60_261**	**GP60_270**	**GP60_274**	GP60_276	GP60_281	**GP60_305**	GP60_318	GP60_335	**GP60_349**	**GTUB_60**	GTUB_85	GTUB_87	GTUB_417	GTUB_705
1	309	Id	T	G												A														G			C	T	C	
1	315	Id	T	G				T								A					T										G			C	T	C	
1	317	Id	T	G				T								A					T													C		C	
1	318	IIa	T	A		A													C	A						G	G		G		G		G	C		C	
1	332	Ib	C	G	A				A									A			T				C		G		G				G	C/A		T	
1	339	Ia	T	G	C		G	G										A			T			C					G				G	C	T	C	
1	340	Ib	C	G	A				A									A	G		T				C		G		G				G	C/A	T	T	
1	341	Ib	C	G					A									A	C	A	T										G		G			T	
1	350	Id	T	G			G																											C		C	
1	363	Ib	C	G	A				A		C/T		A				T	A	C	A	T			A	C		G		G				G	A		T	
1	373	Id	T	G			G																								G				T	C	
1	376	Id	T	G			G	G	A												T													C	T	C	
1	379	Id	T	G			G	G																							G			C	T	C	
1	383	Id	T	G			G	G	A									A			T													C	T	C	
1	403	Ia	T	G			G	G			G	T													C	C			G				G	C	T	C	
1	406	x			A	A					G								C	A						C	G		G				G				
1	408	IIa	T	A	A	A													C	A				A		C	G	G	G		T		G	C	T	C	
1	420	Ib	C/T	G																						C			G					A		T	
1	432	IId	T	G	A				A				A				C		T		T					C	T	A	G				G	C	T	C	
1	447	IIa	T	A				G													C/T							G						C		C	
1	451	IIIf	T	G	A					C	A			C				A					A						A/G		C		G	C	C	C	A
1	457	Ib	C	G						C								A			T													A		T	
1	464	IIn	T	G					A									A									G							C	T	C	
1	477	Ie	T	G	A	A						T						A	C	A	T		A				G		G	T		A	G	A	C	T	
1	478	Ie	T	G	C	A			G/A			T		A				A	C	A	T		A		C	G	G		G	T		A	G	A	T/C	T	
1	483	Ie	T	G																	T						G			T				A	T	T	
1	486	Id	T	G				T								A		A			T													C	T	C	
1	488	Ie		G	A	A			A			T						A	C	A	T		A		C		G		G	T			G	A	T	T	
1	500	IIa	T	A	A	A			G										C	A					C	G	G		G		G		G	C	T	C	
1	504	Ib	C	G	A				A									A	C	A						G/A	G		G				G	C	T	T	
1	529	Ib	C						A									A																	T	T	
1	537	Ib	C				T		A									A			T						G				C		G	A	T	T	
1	557	IIa	T	A					A									A									G							C		C	
1	560	Ib	C	G					A									A	C	A	T				C		G		G			T/A	G	C/A	T	T	
1	563	Ia	T	G	C		G	G	A		G							A	C		T		C	C			G	T				G	C	T	C	A
1	572	*p*			A														C	A	T				C	T	G		G				G	C			
1	573	IIa	T	A			G														T						G							C	T	C	
1	578	*h+p*?	C	G															C	A									G							C	
1	606	Ib	C	G			T	G	A												T														T	T	
1	614	IIa	T	A	A	A	G		A										C	A					C/A	G	G		G		G		G	C	T	C	
1	615	IIa	T	A		A			A										C	A					C	G	G		G				G	C	C	C	
1	621	Ib	C	G	A				A			T					T	A	C	A	T			A	C		G		G				G	A	T	T	A
1	631	Ib	C	G					A												T														A		T	
1	657	Ib	C	G					A								T	A	C	A	T			A			G		G				G	A	T	T	
1	659	Ib	C	G			T	G	A									A			T						G							A	T	T	
1	665	Ib	C	G	A				A									A		A	T				C		G		G				G	C/A	T	T	
1	667	Ib		G	A		T	T											G						C		G		G				G	A	T	T	
1	679	Ib	C	G														A			T						G							A	T	T	
1	682	Ib	C	G	A				A							G		A	G		T				C	G	G		G						T	T	
1	691	IIa	T	A														A									G							C	T	C	
1	698	Ib	C	G				G	A			T					T	A	C	A	T						G		G				G	A	T	T	
1	724	IIn	T	G	A	A			A		G								C	A				A	C	G	G				G	T		C	T	C	
1	726	IIa	T	A	A	A			A									A	C	A		A			C	G/A	G		G		C			C	T	C	
1	744	*h+p*?		A	A		T		A									A	G		T				C		G		G				G	A	T	T	
1	750	IIa	T	A				G																			G							C	T/C	C	
1	777	Ib	C						A									A			T						G							A	T	T	
1	814	Ib			A														G		T				C				G					A			
1	832	Ib	C	G					A									A			T										G			A	T	T	
1	844	Ib	C	G					A									A			T								G		G	T	G	A	T	T	
1	851	Ib	C	G	A		T		A								T	A	C	A	T			A	C				G	T	C		G	A	T	T	
1	852	Ib	C	G			G/T		A									A			T													A	T	T	
1	853	Ib	C	G			T		A			T						A	C	A	T						G		G		C		G	A	T	T	
1	854	Ib	C	G	A				A					A				A	C	A	T			A	C				G	T			G	A	T	T	
1	855	IIa	C/T		A	A			A									A	C	A					C		G		G				G	C	T	C	
1	856	Ib	C	G	A		T		A								T	A	C	A	T			A	C		A/G		G	T			G	A	T	T	
1	857	Ib	C	G			T		A									A			T						G			T	C			A		T	
1	858	Ib	C	G	A				A									A		A	T				C		G		G	T			G	A	T	T	
1	859	Ib	C	G					A								T	A			T			A			G			T			G	A	T	T	
1	861	Ib	C	G	A				A									A	G		T			A	C				G	T			G	A	T	T	
1	863	Ib	C	G	A			G	A			T					T	A	C	A	T			A	C	C	G		G			T	G	A	T	T	
1	865	Ib	C	G/A	A		T											A			T				C		G		G		C		G	A	T	T	
1	870	IIa	T	A			G	G	A									A									G							C	T	C	A
1	871	Ib	C	G				G	A	C		T					T	A	C	A	T					C/T	G		A/G			T	G	A	T	T	A
1	874	Ib		G																											C			A		T	
1	875	Ib			A		T					T							G		T				C				G					A			
1	883	Ib	C	G					A									A			T										C			A	T	T	A
1	886	Ib	C	G	A	A	G		A			T					T	A	C	A	T			A	C	G	G		G		C	T	G	A	T	T	
1	889	Ib	C	G			G	G	A								T	A			T			A			G		G				G	A	T	T	
1	892	Ib		G			T		A									A													C			A		T	
1	893	Ib	C	G				G	A									A			T										C			A	T	T	
1	901	Ib	C	G	A		G	G	A					C				A	G		T				C		G		G				G	A	T	T	
1	926	IIa	T	A				G	A												T						G							C	T	C	
1	928	IIa	T	A				G	A																		G							C	T	C	
1	968	IIa		A				G													T						G							C	T	C	
1	980	*f*	C	G	A		T															G			C				G					C	C	T	
1	989	IIa	T	A		A			A									A	C	A		A			C	G	G		G				G	C	T	C	
1	A100	IIa	T						A																		G							C	T	C	
1	A102	Ib	C	G	A				A			T					T	A	C	A	T				C		G		G	T			G	A	T	T	
1	A109	Ib	C	G	A				A			T					T	A	C	A	T			A	C				G	T		T	G	A	T	T	
1	A110	Ib	C	G	A				A			T					T	A	C	A	T			A	C		G		G	T		T	G	A	T	T	A

Supplemental Table 4. Continuation.

Number of occurrences	Sample code name	Diagnostic	ACOAS_256	ACOAS_686	GP60_33_1	GP60_33_2	GP60_45_1	GP60_45_2	GP60_110	GP60_111	GP60_131	GP60_144	GP60_162	GP60_171_1	GP60_171_2	GP60_181	GP60_183	GP60_215	GP60_219	GP60_228	GP60_241	GP60_243	GP60_259	GP60_260	GP60_261	GP60_270	GP60_274	GP60_276	GP60_281	GP60_305	GP60_318	GP60_335	GP60_349	GTUB_60	QTUB_85	QTUB_87	QTUB_417	QTUB_705
1	A111	Ib	C	G	A				A								T	A		C	A	T			A	C	G	G		G				G	A	T	T	
1	A112	Ib	C	G					A									A		G		T				C		G		G		G			A	T	T	
1	A118	Ib	C	G	A	A			A									A			A	T				C		G		G				G	A	T	T	
1	A121	Ib	C	G					A			C/T					T	A		C	A	T			A	C		G		G	T			G	A	T	T	
1	A124	Ib	C	G					A									A		C	A	T									T			G	A	T	T	
1	A127	Ib	C	G					A									A				T						G				G			A	T	T	
1	A134	Ib	C	G			T		A							A		A				T													A	T	T	
1	A142	Ib		G			T	T														T													A		T	
1	A144	Ib	C	G			T		A													T					A	A							A	T	T	
1	A147	Ib					T	T																											A	T	T	
1	A148	Ib	C	G	A		T		A	C							T	A		C	A	T			A	C		A		G	T			G	A	T	T	
1	A52	IIa	T	A			T																					G			G/C				C	T	C	
1	A55	Ib	C	G			T		A									A		G		T						G		G					A	T	T	
1	A62	IIa	T	A			G		A																			G							C	T	C	
1	A65	IIa	T	A					A									A										G			C				C	T	C	
1	A67	Id	T	G	A	A	T											A		G		T						G		G	C		G	C	T	C	
1	A79	Id	T	G					A									A				T									C			C	T	C	
1	A82	IIa	T	A	A	A	T		A							G				C	A					C	G	G		G			G	C	T	C	
1	A95	Ib	C	G	A				A	T								A				T	G/A					G		G		G		G	A	T	T	
1	A98	Ib	C	G	A			G	A			T					T	A		C	A	T			A	C	A	G		G			T	G	A	T	T	
1	C2	Ib	C	G				T								A						T						G							A	T	T	
1	C21	Ib	T	G	A		T	T								A				C	A	T				C	G			G				G	A		T	
1	C23	Ib	C	G	A				A		G	T					T	A	C	C	A	T			A	C		G		G			T	G	A	T	T	
1	C4	Ib	C	G	A			T	A		G					A			C			T			A	C		A		G					A	T	T	
1	C47	Ib	C	G	A	A	G	T	A		G	T				A		A	C	C		T	A		A	C				G				G	A	T	T	
1	C5	Ib	C	G				T	A							A		A				T													A		T	
1	G20	x					T																												C	T		
1	G25	IId		A	A													A		T	A	T				C		A		G			G	C		C/T	
1	G27	*h*		G	A		T																		C					G							
1	G29	IIa	T	A	A	A			A		G							A		C	A					C	G	G		G	T			G	C	T	C	
1	G30	IIa					T		A									A																				
1	G34	IIa	T	A			T	T																				G							C	T	C	
1	G36	IIa	T	A																								G									C	
1	G37	IIa	T	A					A																										C	T	C	
1	G39	*p*			A	A						T								C	A					C	G	G		G			G	C			
1	G41	IIa	T	A			T		A																			G							C	T	C	A
1	G43	IIa	T	A																											C				C	T	C	
1	G51	IId			A	A						T					C			T		T		A		C				G			T	G	C			
1	G54	IIa	T	A			T		A																						C					C		
1	G56	IIa		A																											C				C			

h= C. hominis; p= C. parvum, m= C. meleagridis, f = C. felis and x= not typed. Bovine samples are labelled with an alphanumeric code starting with a "G" (G1-G65B). Underlined results represent moderate quality of base calling. Heterozygous calls show both alternative alleles separated by a slash. Additional SNVs selected to reinforce the accuracy of base calling in a subset of 83 samples with uncertain genotypes are typed in bold. The number of samples showing identical results is indicated in the last column and the name of the sample is provided when a genotype appears just once.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3. Aetiology and epidemiology of human cryptosporidiosis cases in Galicia (NW Spain), 2000-2008

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Epidemiol. Infect. (2015), 143, 3022–3035. © Cambridge University Press 2015 doi:10.1017/S0950268815000163

Aetiology and epidemiology of human cryptosporidiosis cases in Galicia (NW Spain), 2000–2008

J. L. ABAL-FABEIRO[1,2]*, X. MASIDE[1,2,3], J. LLOVO[2,4] AND C. BARTOLOMÉ[1,2]

[1] *Grupo de Medicina Xenómica, CIMUS, Universidade de Santiago de Compostela, Santiago de Compostela, Galicia, Spain*
[2] *Xenómica Comparada de Parasitos Humanos, IDIS, Santiago de Compostela, Spain*
[3] *Departamento de Anatomía Patolóxica e Ciencias Forenses, Universidade de Santiago de Compostela, Galicia, Spain*
[4] *Servizo de Microbioloxía e Parasitoloxía, Complexo Hospitalario Universitario de Santiago de Compostela, Santiago de Compostela, Galicia, Spain*

Received 27 June 2014; Final revision 19 December 2014; Accepted 20 January 2015; first published online 20 February 2015

SUMMARY

Cryptosporidium infects millions of people worldwide causing acute gastroenteritis, but despite its remarkable epidemiological and economic impact, information on the epidemiological trends of human cryptosporidiosis is still scarce in most countries. Here we investigate a panel of 486 cases collected in Galicia (NW Iberian Peninsula) between 2000 and 2008, which sheds new light on the epidemiology in this region of the South Atlantic European façade. Incidence rates in Galicia are one order of magnitude higher than those reported in other regions of Spain, suggesting that this parasite remains largely underdiagnosed in this country, and are also larger than those typical of other European countries with available data. Two species dominate our dataset, *Cryptosporidium hominis* (65%) and *C. parvum* (34%). The sex ratio of patients infected by either species was 0·5, but *C. hominis* was significantly more common in younger males. *C. parvum* infections were more acute and required more specialized medical attention, which suggests a differential adaptation of each species to human hosts. The parasites display strong seasonal and geographical variation. *C. parvum* incidence peaked during summer and was mainly detected in rural areas while *C. hominis* infections were more frequent in autumn and exhibited a more even geographical distribution. Such differences probably reflect their distinct sources of infection – *C. parvum* is mainly zoonotic and *C. hominis* anthroponotic – and the effects of climatic variables, like temperature and rainfall.

Key words: *Cryptosporidium*, molecular epidemiology, seasonal variation, virulence.

INTRODUCTION

Cryptosporidium is a genus of protozoan parasites that infect many vertebrate species and cause diarrhoeal diseases [1]. They affect humans worldwide and the increasing number of cases reported in the last decades has prompted the recognition of cryptosporidiosis as an emerging disease by the WHO [1–4]. This pathogen has a faecal–oral transmission route, and propagates through the ingestion of oocysts present in contaminated water supplies or food [2, 5–7]. Most human infections are caused by *Cryptosporidium hominis* and *C. parvum*, while other species are found only rarely [3, 8]. *C. hominis* is almost

* Author for correspondence: Mr J. L. Abal-Fabeiro, CIMUS, Laboratorio P2L2, Avda Barcelona, s/n 15782, Santiago de Compostela, Galicia, Spain.
(Email: j.lfabeiro@hotmail.com)

exclusively anthroponotic, in contrast to *C. parvum*, which is commonly detected in domestic animals such as cows and sheep [3].

Cryptosporidiosis is a matter of public health concern in many countries and has inspired a growing number of epidemiological surveys aimed at understanding its demographic and clinical features [9–14]. However, there is little data on the worldwide incidence of this parasite in humans and only a few South European countries have reported studies on its incidence and prevalence [15, 16]. Spain, in common with most countries, lacks a specific cryptosporidiosis surveillance system (notification of cases to the health authorities was not mandatory until 2009 except for outbreaks), which means that the disease is likely to be underdiagnosed and underreported. Thus, although there are some surveys involving Spanish human samples, most of them include a small number of patients and are devoted to the analysis of particular cases or isolated outbreaks [17–19].

In order to improve our understanding of cryptosporidiosis in Europe, we present the results of a large-scale epidemiological study performed on a panel of samples corresponding to all patients with gastrointestinal illness in the Santiago de Compostela health area (Galicia, NW Iberian Peninsula), over a 9-year period. All faecal samples received at the Servizo de Microbioloxía of the Complexo Hospitalario Universitario de Santiago (CHUS) were routinely checked for cryptosporidiosis and subsequently characterized at the species and subtype levels [20]. In order to ascertain relevant epidemiological patterns of cryptosporidiosis in the study region, incidence rates were combined with genetic (species and subtype), environmental (temperature, rainfall, humidity) geographical (location; rural vs. urban area), demographic (sex and age of patients) and clinical (medical care required, oocyst counts and stool consistency) data, and time-series analyses were performed. Our results can be taken as a proxy for the epidemiology of cryptosporidiosis in the NW Iberian Peninsula, and represent the largest-scale study of the whole South-Western European façade.

MATERIALS AND METHODS

Sample genotyping

Stool samples were collected from all patients suffering from diarrhoeal disease between January 2000 and December 2008 in the health district of Santiago de Compostela (Galicia, NW Iberian Peninsula), an area with 515 700 inhabitants (2004). Diarrhoea was defined as the passage of three unformed stools in 8 h or of >3 loose stools in 24 h in addition to the presence of one or more symptoms of an enteric infection [21]. Samples were checked for enteric pathogens by routine microbiological methods at the Microbiology and Parasitology Laboratory of CHUS. The presence of viral antigens (adenovirus, astrovirus, rotavirus, norovirus) was assessed by applying a commercial immunochromatography test (CerTest Biotec faecal antigen rapid test; CerTest Biotec, Spain), bacteria (*Campylobacter*, *Escherichia*, *Salmonella*, *Shigella*, *Yersinia*) were identified using conventional coproculture techniques [22] and protozoan parasites were visualized by microscope examination after concentrating the samples with a modification of the Ritchie method [23]. Coccidian oocysts were detected by fluorescence microscopy (100–400x magnification) after staining stool smears with phenol-auramine [22, 24]. The identification was conducted on the basis of size and shape (for details see the microscopic identification module in http://water.epa.gov/lawsregs/rulesregs/sdwa/lt2/lab_home.cfm#training) by two experienced microscopists from the Microbiology and Parasitology Laboratory of CHUS.

A total of 822 cases of cryptosporidiosis were reported during the study period. Those with enough stool to perform DNA extractions (486 cases) were genotyped by means of iPLEX™ technology (Agena Bioscience, USA) to determine the *Cryptosporidium* species and subtype present in each sample [20]. This method is based on the use of single base extension of a panel of selected single nucleotide variants (SNVs) [25], used as species and subtype markers, followed by sequencing by mass spectrometry (MALDI-TOF) [26].

Demographic and clinical data

All relevant demographic (municipality, age and sex) and clinical (observed co-infections, associated symptoms and immunocompromised status) data were collected from each patient's record. Medical records were kept confidential pursuant to current legislation. Other variables, like specimen collection date, reported outbreaks, recent foreign travel and patient's close contacts affected by enteric problems, i.e. relatives and classmates, were also compiled.

The stage of the parasitization process and clinical profile of each case were assessed by means of three

parameters: (i) excretion rate, (ii) sought medical care and (iii) stool consistency. (i) The excretion rate, or parasitic load, was quantified by scoring the number of oocysts observed per microscope field in stained faecal smears using 100x magnification, and used to typify the stage of the illness [27]. Medical treatment is usually sought after the onset of the first symptoms, mainly within the first week of the infection. In this initial, or acute, stage there is an exponential growth of the parasite population, and oocysts are shed in large numbers (>50 oocysts/field). In the second stage of the infection, usually within the second and third weeks, the parasite population usually reaches a plateau and experiences a progressive reduction, along with the oocyst count (20–50 oocysts/field), and symptoms ameliorate. In the third stage, patients may still shed oocysts (5–20 oocysts/field) for a long period of time (up to 2 months after infection), prior to the final non-proliferative stage (<5 oocysts/field) (J. Llovo, unpublished data). These data were available for 482 samples (99% of the genotyped samples), which were divided into four groups (1–4) according to oocyst counts (<5, 5–20, 20–50, >50, respectively) [28]. (ii) Medical care, as defined according to the kind of medical assistance required by patients, was available for 480 samples (98% of the genotyped samples). It was classified as primary health care (PHC), emergency ward (EW) or hospital admission (HA) [29], following a criterion of increasing symptomatic seriousness. Patients treated at PHC are assumed to present less virulent infections than those who use the EW or are admitted into hospital, which obviously are the most severe cases. (iii) Stool consistency, categorized as formed, pasty, and liquid stools, was evaluated in 369 samples (76% of the genotyped samples) [30]. This parameter can be used as a proxy for the stage of the illness, since the production of loose faeces is more frequent in the acute phase of the infection [31], and it is also expected to be associated with the clinical profile, as watery diarrhoea has a stronger impact on patients (dehydration, weight loss, etc.) than the excretion of pasty or formed stools.

Environmental and climatic analyses

The health district of CHUS is distributed in 14 administrative regions called *comarcas* (analogous to shires; Fig. 1, Table 1), which include 58 lesser administrative units or parishes. A rural–urban index (RUI) was devised to represent the global environment of each *comarca* by combining four socioeconomic factors: (i) human density (population/km^2), (ii) livestock abundance (number of cattle), (iii) availability of public water supply and (iv) sewerage services (fraction of the population with access to these services). Data were obtained from the Instituto Galego de Estatística (IGE; http://www.ige.eu/). To categorize each *comarca*, average values for each variable were compared with their corresponding average value at Galician level. Variables above the Galician average were counted as 0 (otherwise counted as 1), except for livestock abundance, where values were assigned the other way around. Finally the index for each *comarca* was estimated as the sum of the four variables. Thus, this index ranges from 0, which defines an urban *comarca* (high population density, low presence of cattle and high percentage of population with access to public water services) to 4, a rural *comarca*.

Climatic data (i.e. monthly averages for humidity, rainfall and temperature [32]) were collected from the IGE and the Spanish National Institute of Statistics (INE; http://www.ine.es/) at two different weather stations located in Santiago de Compostela (Fig. 1, region 12), which were considered representative of all *comarcas*. The monthly data for each variable was calculated as the mean of the values from the two stations. For each variable, months were sorted according to the value of the variable and grouped into equal-sized bins (36 months in each bin), and the corresponding numbers of parasitized cases was obtained for each bin.

Statistical analyses

Time-series analyses were performed to characterize the seasonal component of the number of patients infected by *C. parvum* or *C. hominis*. The seasonal variation index (SVI), which quantifies the deviation of the number of cryptosporidiosis cases recorded each season relative to the overall mean, was estimated by applying the ratio-to-moving average method under a multiplicative time-series model. These calculations were performed with the aid of IBM SPSS software v. 20·0 (IBM Corp., USA).

Associations between *Cryptosporidium* species – and their corresponding subtypes – and demographical (age, sex, geographical distribution), clinical (excretion rates, medical care, stool consistency or symptoms) and environmental (seasonality, rainfall, humidity, temperature) parameters were evaluated by means of χ^2 tests of homogeneity [33]. These were

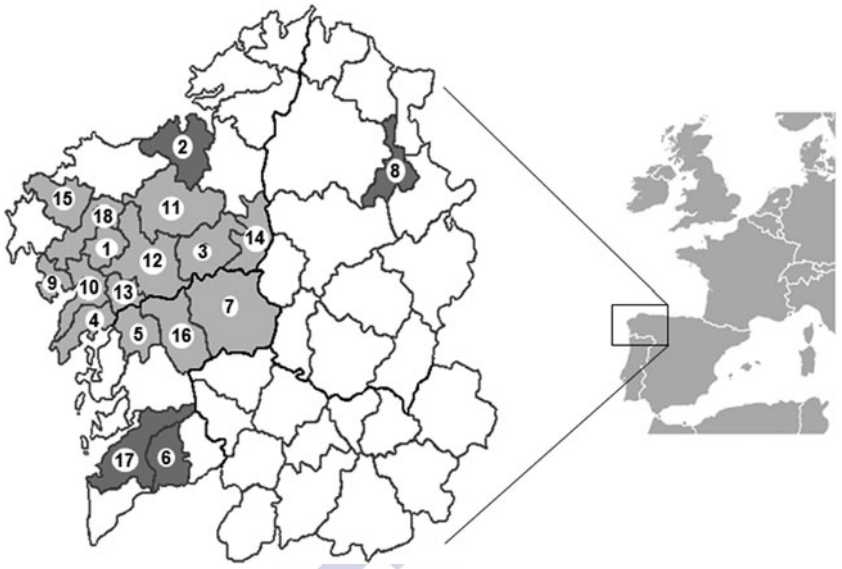


Fig. 1. Comarcas included in Santiago de Compostela health district. Several patients from *comarcas* 2, 6, 8 and 17, which are not part of this health area (depicted in dark grey), received medical assistance at CHUS and were included in the analyses. Comarcas are: 1, A Barcala; 2, A Coruña; 3, Arzúa; 4, Barbanza; 5, Caldas; 6, Condado; 7, Deza; 8, Meira; 9, Muros; 10, Noia; 11, Ordes; 12, Santiago; 13, Sar; 14, T. Melide; 15, T. Soneira; 16, Tabeirós; 17, Vigo; 18, Xallas.

also used to investigate associations between clinical parameters, such as excretion rate, medical care and stool consistency. The distribution of parasites' species in age groups was assessed by means of a Mann–Whitney *U* test [33]. To do this, patients were grouped into 10-year bins. Given the large number of children, these were further divided into two age groups (0–3 and 4–9 years, respectively [10]). Spearman's rho (ρ) correlation coefficient [33] was used to evaluate associations between *Cryptosporidium* species and the regional development index (RUI). All statistical analyses were performed using Microsoft Excel (Microsoft Corp., USA) and IBM SPSS software v. 20·0 (IBM Corp., USA). In all cases, *P* values $\leq$0·05 were considered significant.

RESULTS

The incidence rates of cryptosporidiosis in terms of number of cases per 100 000 population per year in the Santiago de Compostela health area (Galicia, NW Iberian Peninsula) were 9·44 (2000), 9·25 (2001), 17·57 (2002), 29·18 (2003), 13·96 (2004), 20·31 (2005), 27·64 (2006), 5·79 (2007) and 25·77 (2008), with an average (±S.E.) rate of 17·65±2·88, and 91·33 ± 7·44 cryptosporidiosis cases per year.

The epidemiological patterns were studied in a subset of these cases (*N* = 486), which were genotyped at the species and subtype levels [20]. Of these, 318 (65·43%) samples were confirmed as *C. hominis*, 164 (33·95%) as *C. parvum*, three (0·62%) as *C. meleagridis* and one (0·21%) as *C. felis*. The *C. hominis* samples corresponded to *gp60* subtypes Ib (*n* = 289), Id (*n* = 23), Ia and Ie (*n* = 3, each). *C. parvum* samples were assigned to subtypes IIa (*n* = 146), IIn (*n* = 14), IId (*n* = 3) and one undetermined subtype. Of the three *C. meleagridis* samples, two were of subtype IIIf and one IIIb. Only the main species (*C. hominis* and *C. parvum*) and subtypes (IIa, IIn, Ib, Id) were used in the analyses (Table 1).

Demographic data analysis

In our sample, *Cryptosporidium* mainly affects young children. The mean age of *C. hominis* patients (9·34 ± 17·58 years, range 1 month to 91 years, median 2 years, mode 1 year) was slightly higher than that of those infected by *C. parvum* (7·53±15·07 years, range 5 months to 80 years, median 2 years, mode 1 year), although the observed difference was not statistically significant by Mann–Whitney *U* test. No significant variation of the relative frequency of the two parasite species was observed across age bins (Table 1, Fig. 2).

The sex ratio of the patients was nearly 0·5 (239 females and 247 males, including three cases of parasitization with *C. meleagridis* and one with *C. felis*)

Table 1. *Demographic data*

		Cryptosporidium species			*C. hominis* subtypes			*C. parvum* subtypes		
Variable	Outcome	*C. hominis*	*C. parvum*	*P*	Ib	Id	*P*	IIa	IIn	*P*
*Comarca**	1. A Barcala (3)	2	7	*<10⁻⁴*	2	—	*0·83*	7	—	*0·51*
	2. A Coruña (0)	—	1		—	—		1	—	
	3. Arzúa (4)	3	10		1	1		8	2	
	4. Barbanza (1)	22	7		17	3		3	3	
	5. Caldas (0)	6	1		6	—		1	—	
	6. Condado (1)	1	—		1	—		—	—	
	7. Deza (3)	3	10		2	1		8	1	
	8. Meira (3)	—	1		—	—		1	—	
	9. Muros (1)	5	2		4	—		2	—	
	10. Noia (2)	11	5		10	1		5	—	
	11. Ordes (4)	8	24		7	1		20	3	
	12. Santiago (1)	213	62		199	13		57	4	
	13. Sar (2)	8	3		8	—		3	—	
	14. T. Melide (4)	2	7		2	—		7	—	
	15. T. Soneira (3)	1	—		1	—		—	—	
	16. Tabeirós (3)	29	5		26	2		4	1	
	17. Vigo (0)	1	—		1	—		—	—	
	18. Xallas (4)	2	17		2	—		17	—	
Age groups† (years)	1–3	201	107	*0·99*	184	14	*0·98*	95	10	*0·73*
	4–9	54	33		46	6		31	2	
	10–19	18	7		17	1		5	—	
	20–29	13	5		12	1		4	1	
	30–39	8	3		7	—		3	—	
	40–49	5	2		5	—		2	—	
	50–59	3	1		3	—		—	1	
	60–69	5	2		5	—		2	—	
	70–79	8	3		7	1		3	—	
	>80	2	1		2	—		1	—	
Sex	Male	164	82	0·74	149	13	0·65	72	8	0·58
	Female	154	82		140	10		74	6	

All statistical analyses were performed using the χ^2 test of homogeneity.
P values obtained with Yates' correction are in italics.
Values in parentheses are the rural–urban index for each *comarca*.
* Unknown in three cases.
† Unknown in one case.

and the distribution of parasite species across patients' sex did not depart from random expectations ($P = 0{\cdot}74$, χ^2 test; Table 1). But, if age groups are also considered, a differential incidence of *C. hominis* in males and females was detected (Fig. 2): there were more *C. hominis* cases in boys aged <3 years (115 cases) than in girls of the same age (84 cases), whereas this ratio was inverted in patients aged ≥4 years (49 in males vs. 69 in females, respectively; $P < 0{\cdot}01$, χ^2 test; Fig. 2). This effect was not detected in *C. parvum* patients, with 54 and 52 cases in boys and girls, and 28 and 30 cases in older males and females, respectively ($P = 0{\cdot}74$, χ^2 test). No deviation from randomness was observed in the distribution of the major subtypes (Ib, Id, IIa, IIn) across sexes, considering their relative abundances (Table 1).

Clinical analysis

Seriousness of the infection

To check if the pathological course of the infections was in any way associated with the parasite species, we searched for associations between the abundance of the two *Cryptosporidium* species and three parameters which were used as proxies for the stage of the infection and its symptomatic seriousness: the type of medical attention required, the consistency of the stools

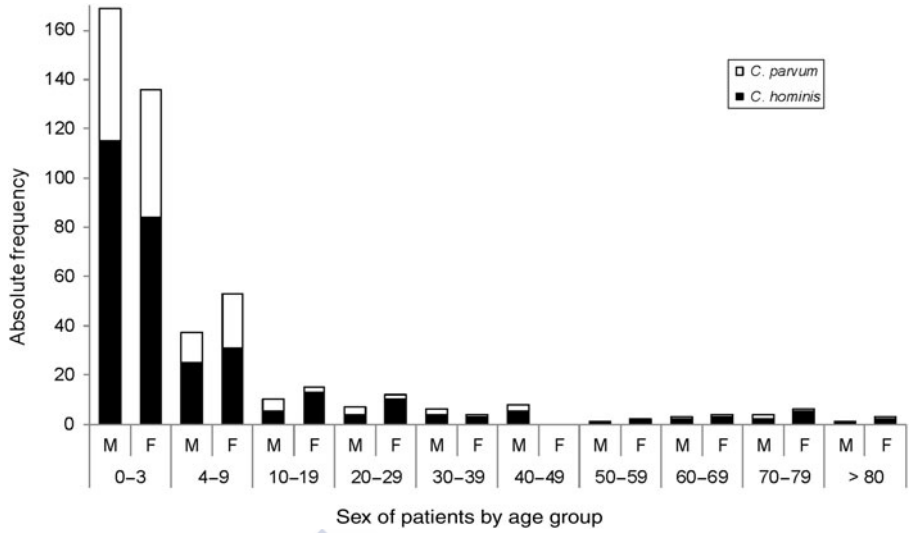


Fig. 2. Absolute frequencies of *Cryptosporidium* cases ranked by the sex and age group of patients.

and the parasitic load. Indeed, (i) *C. parvum* infections required HA in a larger proportion of cases than those caused by *C. hominis* (34·36% vs. 17·46%, respectively; $P < 0·0001$, χ^2 test; Table 2), which were more often dealt with at PHC (67·62% of the *C. hominis* cases). No significant differences in required treatment were observed in within-species subtypes. (ii) Although *Cryptosporidium* infections usually presented pasty faeces (more than 50% of cases; Table 2), *C. parvum* were more often associated with liquid stools than *C. hominis* (41·23% vs. 25·59%, respectively; $P < 0·01$, χ^2 test). This is interesting, because patients with formed or pasty stools were usually assisted at PHC, while those suffering from watery diarrhoea were mainly treated in the EW or were admitted to hospital ($P < 0·0001$, χ^2 test; Fig. 3a, Supplementary Table S1). This was also observed within *Cryptosporidium* species when they were individually assessed (*C. hominis*, $P < 0·01$ and *C. parvum*, $P < 0·05$, χ^2 test; Fig. 3a, Supplementary Table S1), which suggests that the association between stool consistency and medical care is independent of the infecting species. The same pattern was found for *C. hominis* subtype Ib ($P < 0·01$, χ^2 test, data not shown).

Patients loads (measured in terms of oocyst excretion rates) did not vary significantly across parasite species (Table 2). Patients with high loads required HA more frequently than those with lower excretion rates ($P < 0·01$, χ^2 test, Fig. 3b, Supplementary Table S2). Markedly, this effect seems to be restricted to *C. parvum* infections ($P < 0·0001$, χ^2 test), and it was not observed in *C. hominis* patients ($P = 0·23$, χ^2 test; Fig. 3b, Supplementary Table S2). The main *C. parvum* subtype (IIa) displayed a similar pattern ($P < 0·01$, χ^2 test, data not shown).

No significant association was found between oocyst excretion rates and stool consistency, neither for the whole dataset ($P = 0·24$, χ^2 test; Fig. 3c, Supplementary Table S3) nor within either species (*C. hominis*, $P = 0·32$ and *C. parvum*, $P = 0·49$, χ^2 test; Supplementary Table S3).

Associated co-infections

Enteropathogens other than *Cryptosporidium* were detected in 80 out of the 486 samples, including 11 dif- ferent organisms. Eight samples had more than one concurrent pathogen. There were three cases of co-infection with either *Endolimax nana*, *Cystoisospora belli* or *Pentatrichomonas hominis*, two with noroviruses, two with *Entamoeba histolytica*, seven with rotaviruses, eight with adenoviruses, nine with *Salmonella enterica*, 13 with *Campylobacter jejuni*, 19 with *Blastocystis hominis* and 26 with *Giardia lamblia*. Some of these pathogens had previously been described as co-infective with *Cryptosporidium* [2, 34, 35]. Co-infections were more often associated with *C. hominis* (77·5% of the co-infection cases, $P < 0·05$, χ test; Table 2). No differences in subtypes were found.

Clinical symptoms, outbreaks and other factors

The classical symptoms of cryptosporidiosis are nausea, fever, abdominal pain, vomiting and mucus in the stool [36]. The latter two were more often associated with *C. parvum* infections ($P < 0·05$ and

Table 2. *Clinical data*

Variable	Outcome		*Cryptosporidium* species			*C. hominis* subtypes			*C. parvum* subtypes		
			C. hominis	*C. parvum*	*P*	Ib	Id	*P*	IIa	IIn	*P*
Excretion rate*		1	70	44	0·34	63	5	0·93	40	3	*0·58*
		2	76	43		69	6		36	6	
		3	70	37		66	4		35	1	
		4	99	39		88	8		34	4	
Stool consistency†		Formed	46	13	<0·01	39	5	0·39	10	3	*0·32*
		Pasty	143	54		135	6		50	3	
		Liquid	65	47		59	5		41	3	
Medical care‡		PHC	213	62	$<10^{-4}$	196	12	0·14	52	9	*0·10*
		EW	47	45		44	3		39	4	
		HA	55	56		46	8		54	1	
Co-infections		Yes	62	18	<0·05	55	6	0·58	16	1	*1·00*
		No	256	146		234	17		130	13	
Symptoms	Abdominal pain	Yes	11	5	0·81	11	—	*0·71*	4	—	*0·79*
		No	307	159		278	23		142	14	
	Fever	Yes	14	9	0·60	11	2	*0·56*	8	—	*0·80*
		No	304	155		278	21		138	14	
	Vomiting	Yes	8	11	<0·05	8	—	*0·90*	11	—	*0·61*
		No	310	153		281	23		135	14	
	Nausea	Yes	2	—	*0·79*	1	1	*0·34*	—	—	—
		No	316	164		288	22		146	14	
	Mucoid stools	Yes	5	13	<0·01	4	2	0·09	12	1	*0·71*
		No	313	151		285	21		134	13	
	Other symptoms§	Yes	6	3	*0·76*	5	1	*0·93*	4	—	*0·79*
		No	312	161		284	22		142	14	
Immunocompromised		Yes	6	4	*0·94*	6	—	*0·93*	4	—	*0·79*
		No	312	160		283	23		142	14	
Outbreaks		Yes	16	—	<0·01	16	—	*0·50*	—	—	—
		No	302	164		273	23		146	14	
Travel history		Yes	6	1	*0·48*	2	4	$<10^{-4}$	1	—	*0·14*
		No	312	163		287	19		145	14	
Family and school cases		Yes	22	11	0·93	19	3	*0·45*	11	—	*0·61*
		No	296	153		270	20		135	14	

PHC, Primary health care; EW, emergency ward; HA, hospital admission.
All statistical analyses were performed by using the χ^2 test of homogeneity.
P values obtained with Yates' correction are in italics.
* Data available for 482 samples.
† Data available for 480 samples.
‡ Data available for 369 samples.
§ Weight loss, dehydration and food rejection.

$P < 0·01$, respectively, χ^2 test; Table 2). No differences were found in subtypes.

Immunocompromised patients, who required HA in 90% of the cases, did not show significant differences in the predominance of any *Cryptosporidium* species or subtype (Table 2).

Samples from a *Cryptosporidium* outbreak in the *comarca* of Tabeirós in autumn 2003, were genotyped as part of our survey. The outbreak was caused exclusively by *C. hominis* subtype Ib and affected hundreds of people, although only 16 cases were included in the study (Table 2).

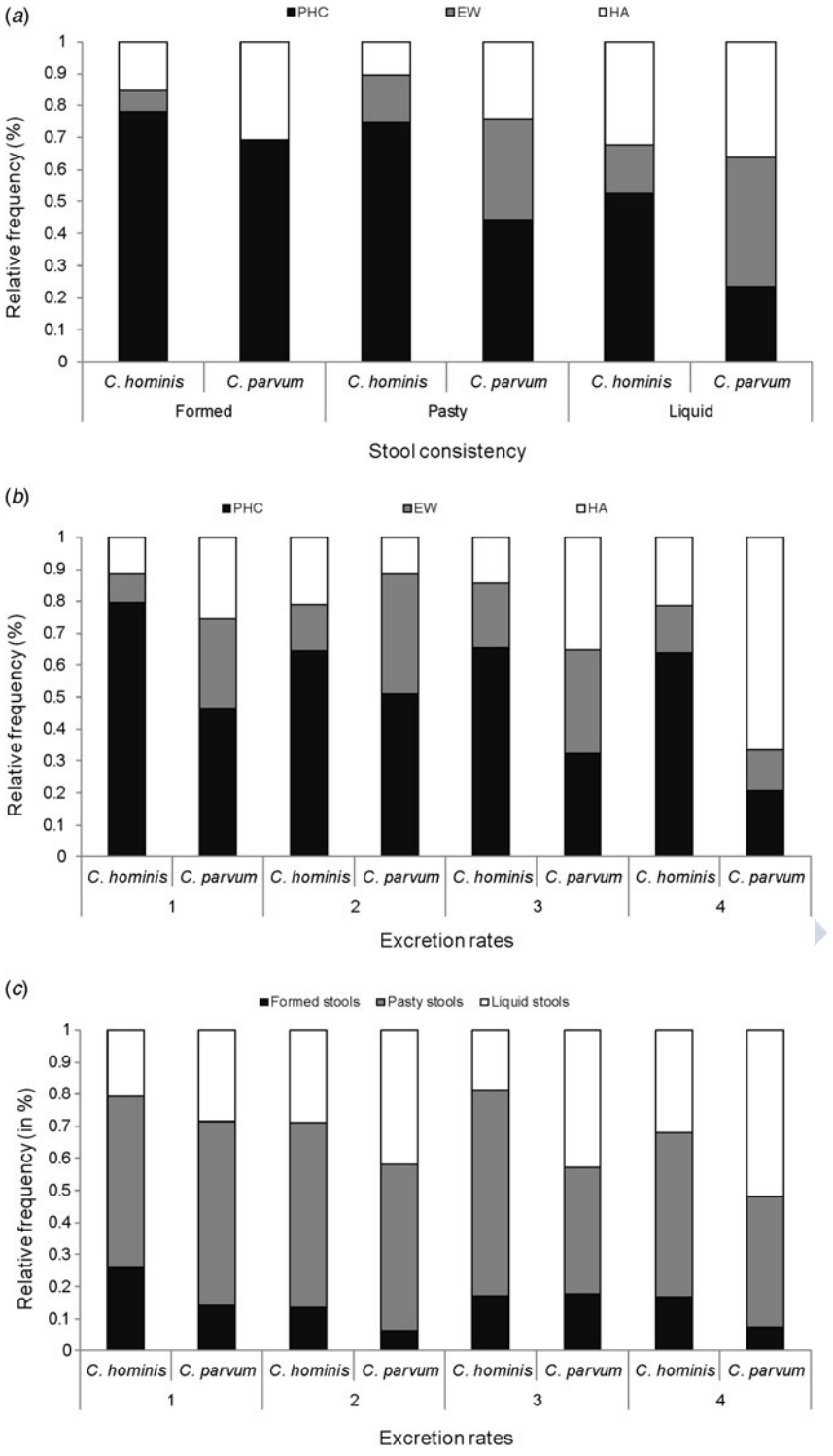


Fig. 3. Relationships in the three variables used to measure the virulence of each infection. (a) Stool consistency and type of medical assistance required by patients infected by *C. hominis* or *C. parvum*. (b) Excretion rates (parasitic load) and type of medical care required by patients infected by *C. hominis* or *C. parvum*. (c) Excretion rates (parasitic load) and stool consistency of patients infected by *C. hominis* or *C. parvum*.

The few cryptosporidiosis patients that reported a recent travel abroad or foreign origin were gathered and studied as a single group. These people came mainly from Africa (Ethiopia) and South America (Mexico, Bolivia, Ecuador). No significant inter-specific differences were reported, although subtype

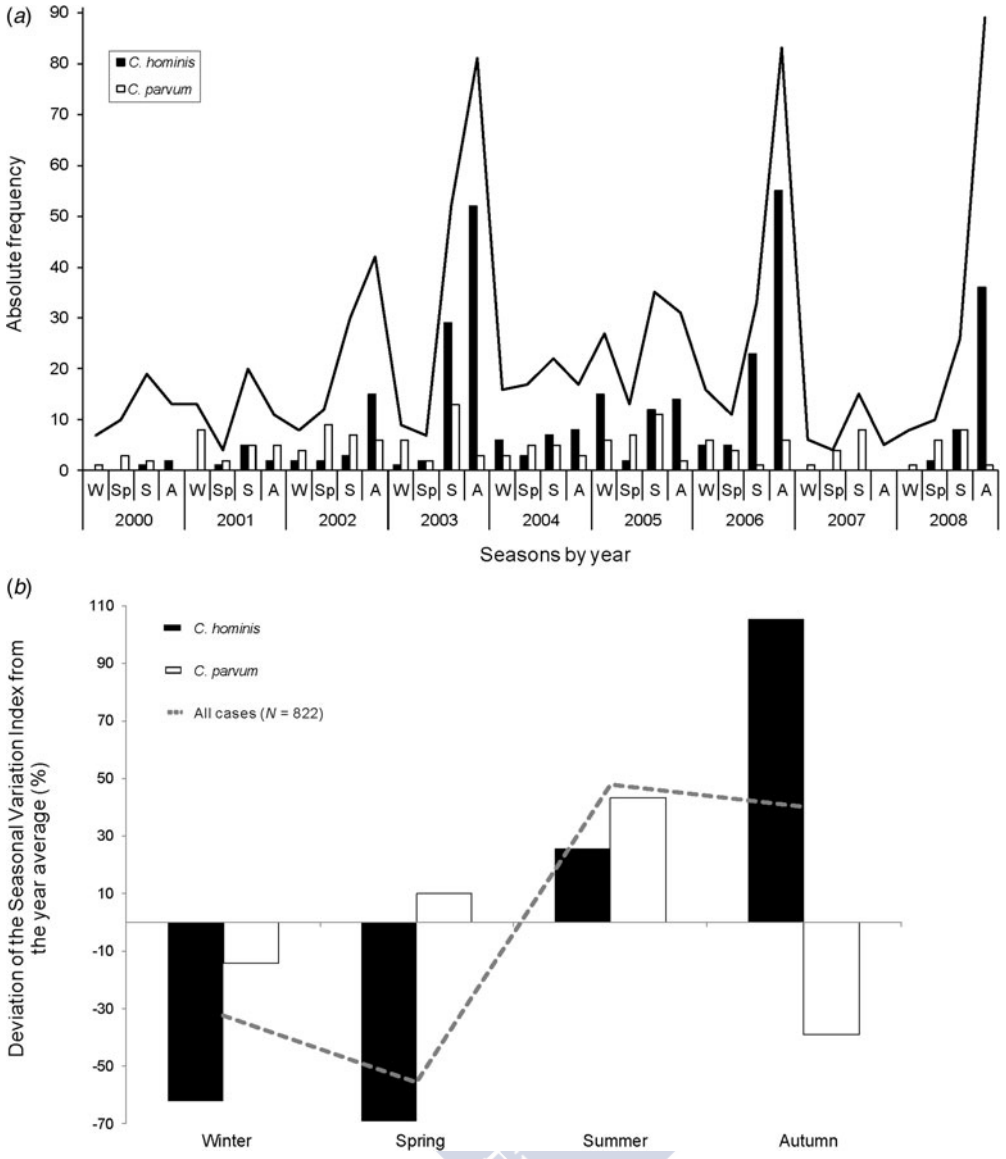


Fig. 4. Seasonality of *Cryptosporidium* incidence. (a) Absolute frequencies of cryptosporidiosis cases across the study period. The continuous line represents the total number of cryptosporidiosis cases. (b) Seasonal patterns of variation of incidence rates (W, winter; Sp, spring, S, summer, A, autumn).

Id was significantly more frequent in this group of patients ($P < 0{\cdot}0001$, χ^2 test; Table 2).

Finally, we observed that patients with *C. hominis* presented twice as many members of the same household or schoolmates with a gastroenteric problem than those suffering from *C. parvum* infections, although this difference was not statistically significant (Table 2).

Geographical distribution and environmental analysis

Spatial distribution of cases was different between species ($P < 0{\cdot}0001$, χ^2 test; Table 1). *C. parvum* incidence was positively associated with the RUI (Spearman's ρ correlation $\rho_{\text{RUI-}C.parvum} = 0{\cdot}552$, $P < 0{\cdot}05$), which indicates a significant association of this species with rural areas, while *C. hominis* infections were not associated with any particular social environment.

Seasonality and climatic analyses

Time-series analyses of the whole dataset revealed a strong seasonal pattern of cryptosporidiosis incidence, with higher numbers in summer and particularly in autumn, as opposed to winter and spring, when incidence rates regularly fell below the yearly average

Table 3. *Environmental data*

		Cryptosporidium species			*C. hominis* subtypes			*C. parvum* subtypes		
Variable*	Outcome	*C. hominis*	*C. parvum*	*P*	Ib	Id	*P*	IIa	IIn	*P*
Season	Winter	29	36	$<10^{-4}$	28	—	*0·07*	29	4	*0·87*
	Spring	17	42		25	2		37	5	
	Summer	88	60		64	11		55	4	
	Autumn	184	26		172	10		25	1	
Humidity (%)	<74	95	51	$<10^{-4}$	95	—	<0·01	43	8	*0·22*
(73·6 ± 12·9)	74–79·9	74	67		59	12		61	4	
	>80	149	46		135	11		42	2	
Rainfall (mm^3)	<60	61	71	$<10^{-4}$	47	11	<0·01	61	8	*0·57*
(136·5 ± 132·6)	60–140	102	49		98	3		45	2	
	>140	155	44		144	9		40	4	
Temperature (°C)	<10·4	60	35	0·39	57	1	0·13	29	3	*0·98*
(13·2 ± 4·1)	10·4–15·8	125	54		115	9		49	5	
	>15·8	133	75		117	13		68	6	
Max. temperature (°C)	<22	62	38	<0·01	59	1	<0·05	31	4	*0·92*
(25·6 ± 6·8)	22–29·4	165	57		154	10		52	5	
	>29·5	91	69		76	12		63	5	
Min. temperature (°C)	<1	87	45	0·90	82	3	0·13	40	3	*0·70*
(3·6 ± 4·2)	1–6	97	47		90	6		40	6	
	>6	134	72		117	14		66	5	

All statistical analyses were performed by using the χ^2 test of homogeneity.
P values obtained with Yates' correction are typed in italics.
* Average monthly values ± standard error for climatic each variable are given in parentheses.

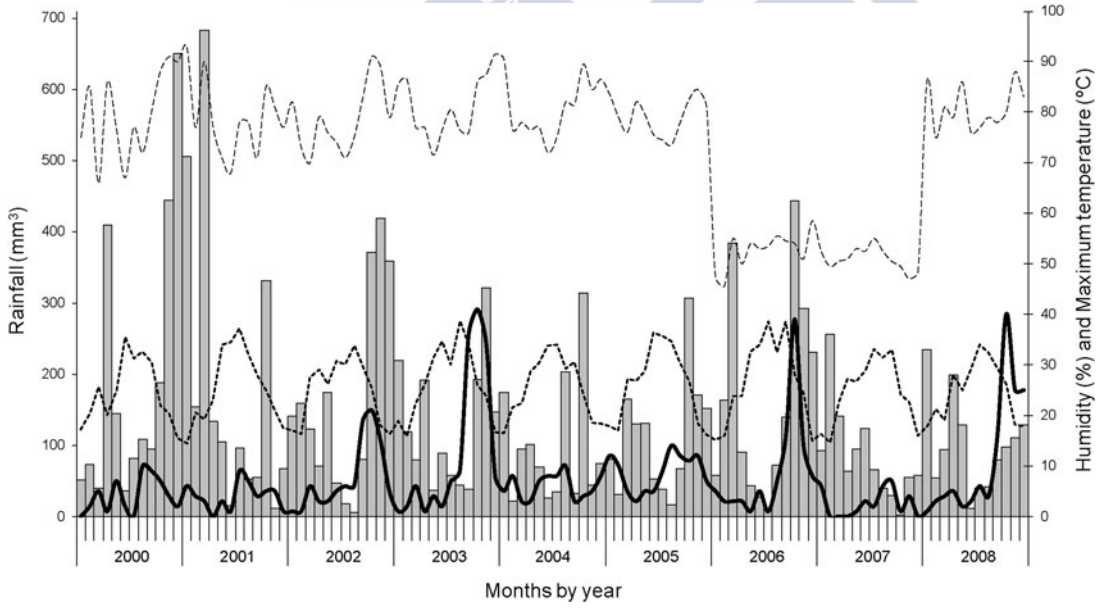


Fig. 5. Cryptosporidiosis cases reported (continuous line, $N = 822$) and monthly averages of rainfall (bars), maximum temperature (dotted line) and humidity (segmented line). All lines follow the right axis.

(Fig. 4a). The two species displayed different seasonal structuration: the number of *C. parvum* cases experienced moderate seasonal variation, with a large increase in summer (SVI = 43%, Fig. 4b) and a sharp reduction in autumn (SVI = −39%). By contrast, seasonal variation of *C. hominis* was much

greater, with a peak of cases in autumn (SVI = 105%), and below average incidences in winter and spring (−62% and −70%, respectively).

Potential associations of temporal patterns with climate parameters such as temperature, rainfall and humidity distributions were also investigated (Table 3). Monthly temperature was found not to be associated with the incidence of cryptosporidiosis at the species and subtype levels. However, since Galicia has a temperate climate, a role for monthly extreme temperatures (maximum and minimum) was also assessed [32]. Indeed, maximum monthly temperatures were associated with a higher incidence of both species ($P < 0{\cdot}01$, χ^2 test; Table 3). Humidity and rainfall also covaried with the incidence rates of both species, although they showed different patterns. *C. hominis* infections increased with rainfall and/or humidity (Figs 4a, 5), and the opposite pattern was observed for *C. parvum*, which was more common after dry periods ($P < 0{\cdot}0001$, χ^2 test; Table 3). In good agreement with these observations, only *C. parvum* cases were reported in 2007, which showed particularly low levels of rainfall in summer and autumn (seasons that usually display the highest number of *C. hominis* cases) and was one of the years with the lowest level of humidity reported (Figs 4a, 5). On the other hand, an increase in the number of infections caused by *C. hominis* was observed in 2003 and 2006, the warmest years of the temporal series (especially their summers and autumns) that also showed high rainfall rates (Figs 4a, 5).

DISCUSSION

This is one of the broadest epidemiological studies on cryptosporidiosis conducted in Southern Europe to date. The 822 cases reported in the 9-year period of analysis represent half of the cases reported elsewhere in Spain between 1995 and 2009 [17]. Considering that the population in the Santiago health area represents just about the 1·2% of the Spanish population, it seems clear that cryptosporidiosis cases were severely underdiagnosed in Spain during this period. In fact, the Microbiology and Parasitology Laboratory of CHUS is one of the few centres in Spain were all faecal samples from patients with enteric problems are routinely analysed for the presence of this and other pathogens. This high level of screening is reflected in the high incidence rates observed in our health area (17·65 ± 2·88 cases per 100000 population per year), that are far greater than those previously reported in most other large-scale surveys conducted in Europe [16, 17, 37, 38] or in the USA [12, 13]. In fact, only 2007 presented a lower incidence rate than those reported in Ireland (14·17), Great Britain (6·05) – countries that share climatic characteristics with Galicia – and some USA states [13, 38].

In the current dataset there was a strong predominance of *C. hominis* and *C. parvum* infections, involving the most common subtypes in humans (Ib and IIa, respectively) [3, 15, 39] and affecting mainly young children, as described worldwide [8, 10, 11]. As previously reported [11, 40], the number of cases decreased with age and no significant differences in the incidence of both species were observed.

In good agreement with data from other European countries, parasite species were equally distributed across sexes [10, 11]. However, in contrast to *C. parvum*, the fraction of *C. hominis* cases was usually larger in adult females than in males, suggesting human-to-human transmission that can probably be attributed to their greater involvement in childcare.

The analyses of various clinical variables showed differences in virulence between species; however, by contrast with previous studies [41–43], our results revealed that infections produced by *C. parvum* were more harmful than those caused by *C. hominis*, as they were significantly associated with more severe symptoms (vomiting or presence of mucous stools) that often required HA. These disparities in virulence suggest that humans could be better adapted to *C. hominis* infections, which seemed to have a milder effect on patient's physiology. Unlike previous studies [41, 42], the subtype families detected in our health area showed no significant differences in virulence.

Cryptosporidiosis caused by *C. hominis* exhibited clinical characteristics somewhat different to those caused by *C. parvum*. For instance, more than 75% of cases with concomitant enteropathogens were *C. hominis* infections. Moreover, the fact that *C. hominis*, and particularly its subtype Ib, is responsible for the majority of the outbreaks reported in Europe [3], as the one detected in Tabeirós in 2003, confirms the high infectivity of this species and its great capability of spreading both through rural or urban regions. On the contrary, subtype Id, which was found in a much smaller proportion in our panel of samples, was significantly more frequent in patients reporting recent foreign travel, reinforcing the assumption that it is not a typical European subtype [3, 15, 41, 42].

The results of the geographical and seasonal distribution of the Galician samples are concordant with

previous studies showing that *C. hominis* displays a widespread distribution with seasonal peaks in autumn [e.g. 6, 10, 11, 40], while *C. parvum* was significantly more prevalent in rural areas [3, 11], with increments in the number of cases that coincided with lambing and calving activity peaks (usually in spring and summer). This could be the reason for the increase in oocyst concentrations observed in drinking water obtained from the river Tambre – the main water source that flows through the study area – in spring and summer of 2007 [44]. In fact, *C. parvum* subtypes identified in human samples were commonly detected in livestock stools, which further supports their zoonotic origin [20, 45].

Finally, it has been proposed that climate variables such as those analysed here modulate the incidence rate of cryptosporidiosis and, thus, are likely to play a role in the seasonal distribution of the species [32, 46, 47]. Regarding temperature, the intervals of highest incidence occurred consistently after the warmest periods [47], as described in other mid-latitude and temperate regions [46]. On the other hand, variations of rainfall and/or humidity might also contribute to the different incidence rates observed between species. A negative association between rainfall and the risk of infection by *Cryptosporidium*, such as the one observed for *C. parvum*, has been attributed to a reduction of oocyst concentration in the soil and surface water after rainy periods [48, 49]. However, this hypothesis does not seem to apply to *C. hominis*. An explanation of this might reside in the mechanisms of transmission of the two species. *C. parvum* is mainly zoonotic and is commonly found in rural areas, thus the spread of its oocysts is probably associated with rain levels in contrast to person-to-person transmission of *C. hominis*, which is expected to be more affected by seasonal changes in human activity, such as use of recreational water and consumption of fresh vegetables in warmer seasons, or initiation of nursery-primary school attendance of children in late summer.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268815000163.

ACKNOWLEDGEMENTS

We thank Dr Manuel del Valle (Dirección Provincial de Saúde Pública, A Coruña), as well as the anonymous referees who reviewed this work, for helpful suggestions and constructive comments on the manuscript. This work was supported by grant PGIDIT06PXIB228073PR from Xunta de Galicia, Spain, to C.B. J.L.A-F. was supported by a student fellowship co-financed by Xunta de Galicia, Spain, and the European Social Fund; X.M. was supported by a Programa Ramón y Cajal contract (Ministerio de Ciencia e Innovación, Spain) and C.B. was supported by a Programa Isidro Parga Pondal contract (Xunta de Galicia, Spain).

DECLARATION OF INTEREST

None.

REFERENCES

1. **Mosier DA, Oberst RD**. Cryptosporidiosis. A global challenge. *Annals of the New York Academy of Sciences* 2000; 916: 102–111.
2. **Meinhardt PL, Casemore DP, Miller KB**. Epidemiologic aspects of human cryptosporidiosis and the role of waterborne transmission. *Epidemiologic Reviews* 1996; 18: 118–136.
3. **Xiao L**. Molecular epidemiology of cryptosporidiosis: an update. *Experimental Parasitology* 2010; 124: 80–89.
4. **World Health Organization**. Emerging issues in water and infectious disease. Geneva: WHO; 2003.
5. **Freire-Santos F, *et al***. Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. *Journal of Parasitology* 2000; 86: 853–854.
6. **Semenza JC, Nichols G**. Cryptosporidiosis surveillance and water-borne outbreaks in Europe. *Eurosurveillance* 2007; 12: E13–14.
7. **Craun GF, Calderón RL, Craun MF**. Outbreaks associated with recreational water in the United States. *International Journal of Environmental Health Research* 2005; 15: 243–262.
8. **Elwin K, *et al***. The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000–2008. *Epidemiology and Infection* 2011; 140: 673–683.
9. **Fournet N, *et al***. Simultaneous increase of *Cryptosporidium* infections in the Netherlands, the United Kingdom and Germany in late summer season, 2012. *Eurosurveillance* 2013; 18(2).
10. **Chalmers RM, *et al***. Long-term *Cryptosporidium* typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. *Eurosurveillance* 2009; 14(2).
11. **Chalmers RM, *et al***. Epidemiology of anthroponotic and zoonotic human cryptosporidiosis in England and Wales, 2004–2006. *Epidemiology and Infection* 2011; 139: 700–712.
12. **Yoder JS, Beach MJ**. Cryptosporidiosis surveillance – United States, 2003–2005. Morbidity and Mortality *Weekly Report Surveillance Summaries* 2007; 56: 1–10.

13. **Yoder JS, Harral C, Beach MJ**. Cryptosporidiosis surveillance - United States, 2006–2008. Morbidity and Mortality Weekly Report. *Surveillance Summaries* 2010; 59: 1–14.
14. **Kotloff KL**, ***et al***. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet* 2013; 382: 209–222.
15. **Alves M, *et al*.** Distribution of *Cryptosporidium* subtypes in humans and domestic and wild ruminants in Portugal. *Parasitology Research* 2006; 99: 287–292.
16. **The ANOFEL *Cryptosporidium* National Network**. Laboratory-based surveillance for *Cryptosporidium* in France, 2006–2009. *Eurosurveillance* 2010; 15: 19642.
17. **Navarro-i-Martínez L, del Águila C, Bornay-Llinares FJ.** *Cryptosporidium*: a genus in revision. The situation in Spain [in Spanish]. *Enfermedades Infecciosas y Microbiología Clínica* 2011; 29: 135–143.
18. **Artieda J, *et al*.** Outbreak of cryptosporidiosis in a child day-care centre in Gipuzkoa, Spain, October to December 2011. *Eurosurveillance* 2012; 17(5).
19. **Soler P**. Surveillance of cryptosporidiosis in Spain from 1995 to 2003. *Eurosurveillance* 2004; 8(10).
20. **Abal-Fabeiro JL, *et al***. High-throughput genotyping assay for the large-scale genetic characterization of *Cryptosporidium* parasites from human and bovine samples. *Parasitology* 2014; 141: 491–500.
21. **DuPont HL, *et al*.** The infectivity of *Cryptosporidium* parvum in healthy volunteers. *New England Journal of Medicine* 1995; 332: 855–859.
22. **Murray P, *et al***. *Manual of Clinical Microbiology*, 9th edn. Washington, DC: ASM Press, 2007.
23. **Allen AV, Ridley DS.** Further observations on the formol-ether concentration technique for faecal parasites. *Journal of Clinical Pathology* 1970; 23: 545–546.
24. **Nichols G, Thom B**. Screening for *Cryptosporidium* in stools. *Lancet* 1984; 1: 734–735.
25. **Gabriel S, Ziaugra L, Tabbaa D.** SNP genotyping using the Sequenom MassARRAY iPLEX platform. *Current Protocols in Human Genetics* 2009; Chapter 2: Unit 2 12.
26. **Sauer S, Gut IG**. Genotyping single-nucleotide polymorphisms by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. *Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences* 2002; 782: 73–87.
27. **Chappell CL, *et al*.** *Cryptosporidium parvum*: intensity of infection and oocyst excretion patterns in healthy volunteers. *Journal of Infectious Diseases* 1996; 173: 232–236.
28. **Garcia LS, Brewer TC, Bruckner DA.** Fluorescence detection of *Cryptosporidium* oocysts in human fecal specimens by using monoclonal antibodies. *Journal of Clinical Microbiology* 1987; 25: 119–121.
29. **Kendrick D, *et al*.** Relationships between child, family and neighbourhood characteristics and childhood injury: a cohort study. *Social Science & Medicine* 2005; 61: 1905–1915.
30. **Pereira SJ, *et al.*** Pathogenesis of human and bovine *Cryptosporidium parvum* in gnotobiotic pigs. *Journal of Infectious Diseases* 2002; 186: 715–718.
31. **Franzen C, Muller A**. Cryptosporidia and microsporidia – waterborne diseases in the immunocompromised host. *Diagnostic Microbiology & Infectious Disease* 1999; 34: 245–262.
32. **González-Moreno O, *et al*.** Prevalence and climatic associated factors of *Cryptosporidium* sp. infections in savanna chimpanzees from Ugalla, Western Tanzania. *Parasitology Research* 2013; 112: 393–399.
33. **Sokal RR, Rohlf FJ**. *Biometry: the Principles and Practice of Statistics in Biological Research*, 3rd edn. New York: W. H. Freeman, 1995, pp. 887.
34. **Jex AR, *et al*.** Detection of diarrhoeal pathogens in human faeces using an automated, robotic platform. *Molecular and Cellular Probes* 2012; 26: 11–15.
35. **Mengelle C, *et al*.** Simultaneous detection of gastrointestinal pathogens with a multiplex Luminex-based molecular assay in stool samples from diarrhoeic patients. *Clinical Microbiology and Infection* 2013; 19: E458–465.
36. **Chalmers RM, Davies AP**. Minireview: clinical cryptosporidiosis. *Experimental Parasitology* 2010; 124: 138–146.
37. **European Centre for Disease Prevention and Control**. Annual epidemiological report on communicable diseases in Europe. Report of the status of communicable diseases in the EU and EEA/EFTA countries. Stockholm: ECDC, 2007.
38. **European Centre for Disease Prevention and Control**. Annual epidemiological report 2012. Reporting on 2010 surveillance data and 2011 epidemic intelligence data. Stockholm: ECDC, 2013.
39. **Jex AR, Gasser RB**. Analysis of the genetic diversity within *Cryptosporidium hominis* and *Cryptosporidium parvum* from imported and autochtonous cases of human cryptosporidiosis by mutation scanning. *Electrophoresis* 2008; 29: 4119–4129.
40. **Garvey P, McKeown P**. Epidemiology of human cryptosporidiosis in Ireland, 2004–2006: analysis of national notification data. *Eurosurveillance* 2009; 14(8).
41. **Cama VA, *et al*.** Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. *Journal of Infectious Diseases* 2007; 196: 684–691.
42. **Cama VA, *et al*.** *Cryptosporidium* species and subtypes and clinical manifestations in children, Peru. *Emerging Infectious Diseases* 2008; 14: 1567–1574.
43. **Hunter PR, *et al*.** Health sequelae of human cryptosporidiosis in immunocompetent patients. *Clinical Infectious Diseases* 2004; 39: 504–510.
44. **Castro-Hermida JA, *et al*.** Presence of *Cryptosporidium* spp. and *Giardia duodenalis* through drinking water. *Science of the Total Environment* 2008; 405: 45–53.
45. **Quílez J, *et al***. *Cryptosporidium* species and subtype analysis from dairy calves in Spain. *Parasitology* 2008; 135: 1613–1620.

46. **Jagai JS, *et al.*** Seasonality of cryptosporidiosis: a meta-analysis approach. *Environmental Research* 2009; 109: 465-478.
47. **Naumova EN, *et al.*** Seasonality in six enterically transmitted diseases and arnbient temperature. *Epidemiology and Infection* 2007; 135: 281-292.
48. **Schijven J, *et al.*** A decision support tool to compare waterborne and foodbome infection and/or illness risks associated with clirnate change. *Risk Analysis* 2013; 33: 2154-2167.
49. **Tate KW, Pereira MD, Atwill ER.** Efficacy of vegetated buffer strips for retaining *Cryptosporidium parvum*. *Journal of Environmental Quality* 2004; 33: 2243-2251.

3.1 Material suplementario

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Supplementary Table S1. Stool consistency and type of medical assistance required by patients infected by *C. hominis* or *C. parvum* (absolute frequencies).

		Medical Assistance Required		
Species	Stool consistency	Primary Health Care	Emergency Wards	Hospital Admissions
C. hominis	Formed	36	3	7
	Pasty	106	21	15
	Liquid	34	10	21
C. parvum	Formed	9	0	4
	Pasty	24	17	13
	Liquid	11	19	17
All	Formed	45	3	11
	Pasty	130	38	28
	Liquid	45	29	38

Supplementary Table S2. Excretion rates (parasitic load) and type of medical care required by patients infected by *C. hominis* or *C. parvum* (absolute frequencies).

		Stool consitency		
Species	Excretion rate	Formed	Pasty	Liquid
C. hominis	1	15	31	12
	2	8	34	17
	3	10	38	11
	4	13	40	25
C. parvum	1	4	16	8
	2	2	16	13
	3	5	11	12
	4	2	11	14
All	1	19	47	20
	2	10	50	30
	3	15	49	23
	4	15	51	39

Supplementary Table S3. Excretion rates (parasitic load) and stool consistency of patients infected by *C. hominis* or *C. parvum* (absolute frequencies).

		Medical Assistance Required		
Species	Excretion rate	Primary Health Care	Emergency Wards	Hospital Admissions
C. hominis	1	55	6	8
	2	49	11	16
	3	45	14	10
	4	63	15	21
C. parvum	1	20	12	11
	2	22	16	5
	3	12	12	13
	4	8	5	26
All	1	75	18	19
	2	71	27	21
	3	57	26	23
	4	71	20	47

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DISCUSIÓN

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Análise da variabilidade xenética en dez *loci* codificadores de copia única

Neste traballo analizouse a variación xenética das principais especies de *Cryptosporidium* que parasitan a humanos –*C. parvum*, *C. hominis*, *C. meleagridis* e *C. felis*–. Para elo estudouse a diversidade nucleotídica de dous dos xenes máis amplamente empregados na caracterización a nivel de especie (*COWP1*) e de subtipo (*gp60*) dos criptosporidios (AMAR *et al.* 2004; RYAN *et al.* 2014), así como doutros oito *loci* (*ACoAs*, *dyn4*, *eIF4a*, *gtub*, *gtRNAl*, *ISWIr*, *KhRNAb* e *RNaseLi*), escollidos aleatoriamente ó longo do seu xenoma. Desbotouse a idea de empregala-la secuencia da *SSU rRNA* xa que nestes parasitos é codificado por un número variable de copias parálogas espalladas polo seu xenoma (LE BLANCQ *et al.* 1997; SPANO e CRISANTI 2000; ABRAHAMSEN *et al.* 2004; XU *et al.* 2004), circunstancia que podería ocasionar unha sobreestima dos niveis de variación no caso de existiren diferenzas entre elas.

Nove dos dez *loci* analizados amosaron uns niveis de diversidade neutra inferiores ó 1% e unhas estimas de diverxencia próximas ao 6% entre *C. hominis* e *C. parvum*, sendo estas cifras tres veces superiores cando se compararon as citadas especies con *C. meleagridis*. Estas observacións coinciden cos resultados descritos noutros traballos recentes levados a cabo con *loci* diferentes ós aquí estudados (BOUZID *et al.* 2010) e son semellantes ós obtidos na comparación dos xenomas completos de *C. hominis* e *C. parvum*; ditos estudos amosaron que a estrutura e organización dos xenomas destas especies estaban altamente conservadas, que existía unha grande similitude entre as súas secuencias e que presentaban uns niveis de diverxencia media arredor do 5% (XU *et al.* 2004; GE *et al.* 2008; MAZURIE *et al.* 2013).

Sen embargo, o *locus gp60* exhibiu uns padróns de diversidade moi diferentes, caracterizados por (i) elevada diversidade intraespecífica, (ii) exceso de polimorfismos compartidos entre especies e (iii) niveis moi superiores de diverxencia interespecífica (*e.g.* K_S = 31% entre *C. hominis* e *C. parvum*). En contra da interpretación comunmente aceptada de que *gp60* evoluciona baixo o efecto da selección positiva (WIDMER 2009; WIDMER e SULLIVAN 2012), nós reunimos evidencias suficientes que indican que a evolución de dito *locus* se axusta de forma máis precisa a un modelo de selección balanceadora, e máis concretamente a un réxime de selección dependente das frecuencias. Este modelo selectivo favorece o mantemento de múltiples alelos na poboación a frecuencias maiores ás esperadas por deriva ou mutación e durante longos períodos de tempo (CHARLESWORTH 2006; FIJARCZYK e BABIK 2015). Partindo da base de que *gp60* codifica dúas glicoproteinas (GP40

e GP15) e asumindo que xogan un papel importante nos procesos de recoñecemento e invasión dos enterocitos (CEVALLOS *et al.* 2000; STRONG *et al.* 2000; LENDNER e DAUGSCHIES 2014), resulta sinxelo postular un escenario de interacción hóspede-parásito no que a aparición de variantes de maior virulencia no segundo son sucesivamente contrarrestadas por alelos de resistencia no primeiro. Deste xeito, unha variante nova de *gp60* que confire maior virulencia ó parasito terá unha vantaxe selectiva e aumentará a súa frecuencia na poboación, e ó cabo do tempo será a forma predominante. Neste punto, as formas do hóspede resistentes ás outras formas do parasito carecerán de valor adaptativo e a súa frecuencia diminuirá de xeito progresivo na poboación nas sucesivas xeracións. Mais, no momento en que xurda unha nova forma de resistencia no hóspede eficaz contra a nova variante virulenta do parasito, esta terá un grande valor adaptativo e será seleccionada de forma positiva ata que a maior parte dos hóspedes sexan resistentes contra o alelo agora maioritario de *gp60*. Entón as formas máis comúns do parasito perderán eficacia na infección dos hóspedes e calquera variante pouco frecuente de *gp60* que persista na poboación de parasitos, para a cal o hóspede perdeu a capacidade de resistencia, resultará exitosa, iniciando un novo o ciclo selectivo (BROWN 2003; AGUILETA *et al.* 2009; BROWN e TELLIER 2011) (**Figura 1**). Escenarios semellantes a este foron propostos con anterioridade para explicar niveis elevados de variación en *loci* de resistencia noutros sistemas parasito–hóspede, como os detectados en *Neisseria*, *Campylobacter* ou *Plasmodium* (URWIN *et al.* 2004; DINGLE *et al.* 2005; AGUILETA *et al.* 2009; AMAMBUA-NGWA *et al.* 2012).

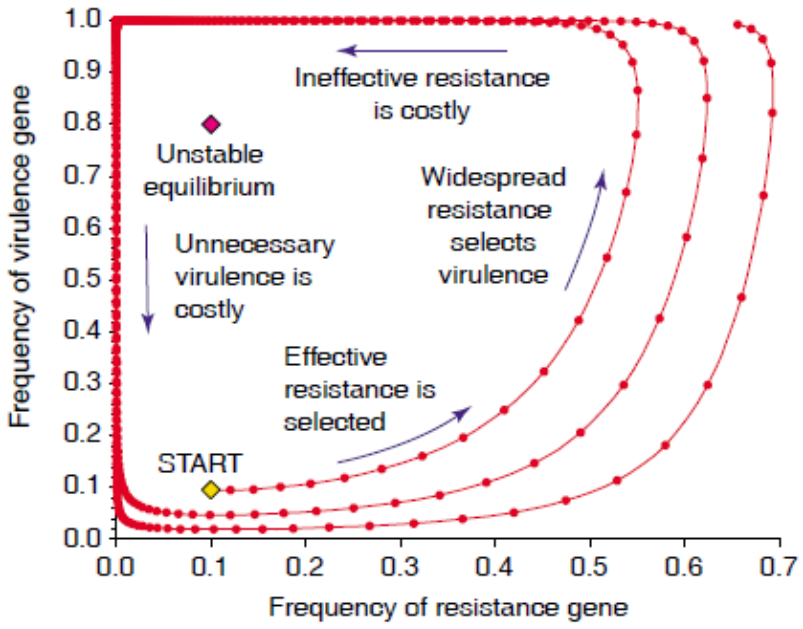


Figura 1. Dinámicas das frecuencias alélicas nun modelo simple de interacción xene-xene entre un parasito e o seu hóspede (BROWN 2003).

A busca de xenes potencialmente sometidos a selección balanceadora, como podería se-lo caso dos diversos receptores de membrana que participan no proceso de introdución do parasito na célula hóspede (LENDNER e DAUGSCHIES 2014), é extremadamente interesante dende o punto de vista terapéutico (MU *et al.* 2007) xa que pode axudar a identificar dianas para desenrolar medicamentos ou sustancias específicas que impidan o inicio do proceso patolóxico.

O estudo das secuencias dos dez *loci* analizados neste traballo permitiu tamén constatar que os eventos de recombinación nestes organismos son pouco frecuentes –ata o presente hai poucos traballos onde se describan procesos naturais ou experimentais de recombinación en criptosporidios (FENG *et al.* 2002; TANRIVERDI *et al.* 2007; TANRIVERDI *et al.* 2008) –. Do mesmo xeito, e en relación con esta baixa taxa de recombinación, observamos que os padróns de variación xenética noutros *loci* estaban estruturados de acordo ós distintos haplotipos (ou familias de subtipos) de *gp60*. Ese é o caso da variante fixada dentro do *locus ACoA* na súa posición 686 (Táboa 2 do artigo 2) a cal, xunto cun cambio dunha Guanina por unha Adenina na posición 278 da secuencia do xene de *gp60* serve para identifica-lo subtipo IIn de *C. parvum*; ou para os diversos haplotipos descritos para o *locus gtub*, os cales aparecen intimamente asociados a unha única variante alélica do xene *gp60* e non se comparten entre haplotipos distintos deste último *locus* (Táboa 4 do artigo 1). Este descubrimento suxire a posibilidade de diferenciación xenética a escala xenómica entre subtipos, o que xustificaría afondar na investigación da diversidade funcional e patoxénica que puidera existir entre eles e tería grande relevancia no eido terapéutico e da saúde pública.

En calquera caso é importante sinalar que aínda que *gp60* e *COWP1* están no mesmo grupo de ligamento (cdg6, Táboa S1 do artigo número 1), os seus padróns de diversidade son moi distintos: *gp60* amosa evidencia de selección balanceadora e *COWP1* non. Isto é síntoma inequívoco de que no longo prazo os dous *loci* evolucionaron de forma independente o que implica que a recombinación meiótica pode ter xogado un papel importante na distribución da variación xenética ó longo dese e posiblemente doutros cromosomas nalgún momento do pasado. Neste contexto, a ausencia de evidencias de recombinación nos datos de polimorfismo existentes suxire que esta debe ter lugar a taxas demasiado baixas para ser detectables, e que a diversidade observada na actualidade nas especies de *Cryptosporidium* ten unha orixe moi recente. Esta hipótese reconciliase cos baixos niveis de diversidade neutra media nas especies estudadas e suxire que a relación entre a especie humana e as variedades de *Cryptosporidium* que a parasitan na actualidade pode non ser moi antiga. Isto debuxa un

escenario dinámico entre os xenotipos de *Cryptosporidium* e as especies ás cales parasitan, o que coincide co seu probado carácter zoonótico.

Análises xenético-poboacionais como a aquí descrita permiten tamén a identificación de variantes moleculares (ou SNVs) específicos de especie e/ou subtipo de *Cryptosporidium* (BOUZID *et al.* 2010), as cales poden ser potencialmente empregadas como ferramentas diagnósticas para o xenotipado e identificación molecular dos mesmos, poñendo en valor as achegas que a ciencia básica pode ofrecer ó desenrolo da ciencia aplicada.

2. Desenrolo dun novo método diagnóstico da criptosporidiose de alto rendemento

A criptosporidiose é unha enfermidade emerxente e de declaración obrigatoria nos países occidentais (SEMENZA e NICHOLS 2007; YODER *et al.* 2012). Para realizar traballos epidemiolóxicos e filoxeográficos rigorosos sobre dita enfermidade faise necesario describi-la diversidade xenética das poboacións naturais destes parasitos, sendo os criterios moleculares os máis precisos para levar a cabo unha clasificación precisa da variación dos criptosporidios. Sen embargo, a pesares da importancia sanitaria global desta enfermidade e dos requirimentos moleculares marcados polo ICZN para a correcta asignación das especies de *Cryptosporidium*, a maioría dos traballos poboacionais e epidemiolóxicos realizados para aborda-lo estudo destes parasitos céntranse na análise dun número moi limitado de marcadores, xa sexan estes secuencias que permitan a identificación da especie presente nas mostras analizadas –como é o caso da SSU rRNA, *COWP1* ou a actina (SULAIMAN *et al.* 2002; TROTZ-WILLIAMS *et al.* 2006)– ou ben secuencias que permitan facer subclasificacións dentro das especies –como determinados microsatélites, minisatélites ou a secuencia do *locus gp60* (SPANO *et al.* 1998; STRONG *et al.* 2000; MALLON *et al.* 2003). Ademais destas limitacións, as achegas destes traballos vense tamén comprometidas polo escaso volume de mostras que son capaces de amplificar e procesar tanto por cuestións económicas como por dificultades técnicas dado que, ademais de ser un proceso laborioso, é complexo deseñar cebadores xenéricos que permitan amplifica-lo mesmo *locus* en especies diferentes (FAYER e XIAO 2008).

Ante estas limitacións, nos últimos anos na comunidade científica vénse propoñendo a necesidade de seleccionar un conxunto estruturado de marcadores suficientemente informativos que permitan a identificación de tódalas especies, xenotipos e subtipos de *Cryptosporidium* (FAYER e XIAO 2008). A finalidade última desta selección debería se-lo

desenrolo dun método de diagnóstico multilocus de uso universal que, empregando un número óptimo de marcadores moleculares (WIDMER e SULLIVAN 2012), fixese posible a análise rigorosa das poboacións naturais deste parasito empregando un método de tipado de alto rendemento preciso, rápido e económico. En concordancia con estes requirimentos, e utilizando información sobre os padróns de diversidade molecular descritos no apartado anterior, desenvolvemos un protocolo de xenotipado baseado na tecnoloxía MassARRAY (Agena Bioscience, antes Sequenom) que permite identifica-la especie e o subtipo presente en grandes coleccións de mostras.

Esta metodoloxía presenta multitude de avantaxes fronte ás técnicas empregadas tradicionalmente para o estudo da criptosporidiose. Entre elas destacan: (i) a posibilidade de empregar múltiples marcadores situados en distintos *loci*, en comparación coa análise dun ou dous xenes que se utiliza na maioría dos traballos (DÍAZ *et al.* 2010; WALDRON *et al.* 2011a; WALDRON *et al.* 2011b; RAMO *et al.* 2014); (ii) a capacidade de analizar un grande número de mostras de xeito simultáneo e automatizado, o que garante a súa rapidez con respecto a outros métodos; (iii) ser moito máis barata que a secuenciación Sanger (0,1 € por reacción de xenotipado fronte os 4 € que custa a reacción de secuenciación dun amplicón); (iv) o seu mellor rendemento con respecto á secuenciación Sanger no caso de mostras con baixas concentracións de ADN ou nas que este se atopa parcialmente degradado (MENDISCO *et al.* 2011); e (v) a plasticidade para adapta-la análise ás necesidades de cada experimento, xa que o panel de SNVs pode ser modificado e optimizado segundo as especies ou subtipos que se queiran identificar nas mostras. Ademais, métodos semellantes a este foron empregados recentemente para o xenotipado doutros organismos patóxenos como *Mycobacterium tuberculosis* (BOUAKAZE *et al.* 2011) e *Neisseria gonorrhoeae* (TREMBIZKI *et al.* 2014), o cal avala a posibilidade de implementa-lo mesmo procedemento con éxito noutro grupo de organismos con características semellantes.

No caso que nos ocupa, a utilización como control de 82 mostras previamente tipadas por secuenciación Sanger no Center for Disease Control and Prevention (CDC, Atlanta, USA) posibilitou a avaliación da *sensibilidade* (definida como a capacidade de identificar verdadeiros positivos) e da *especificidade* (definida como a capacidade de identificar verdadeiros negativos) do método desenvolvido. Ambos parámetros viñeron a certifica-la robustez da técnica ó acadar uns valores do 87,3 e do 98%, respectivamente, que poderían ser incluso maiores se unicamente se tiveran en conta os SNVs que amosaron un mellor rendemento (94,5 e 99,8%, respectivamente).

3. Epidemioloxía da criptosporidiose na área sanitaria de Santiago de Compostela

Procedeuse a analizar un panel de 671 mostras previamente identificadas como positivas para *Cryptosporidium* spp. mediante técnicas clásicas –tintura con auramina-fenol e inmunocromatografía–, das cales 608 procedían de feces de doentes da área sanitaria de Santiago de Compostela e as 63 restantes de gando vacún. A precisión e o bo rendemento amosados pola ferramenta diagnóstica anteriormente descrita fixo posible determina-la especie infectante de *Cryptosporidium* nun total de 610 destas mostras, e a familia de subtipos de *gp60* en 605, tendo así unha porcentaxe de éxito de máis do 90% en ambos casos. Nelas púidose identifica-la presenza de *C. hominis* (e da súa familia de subtipos Ia, Ib, Id e Ie) en 370 mostras, a de *C. parvum* (subtipos IIa, IId e IIn) en 176, a de *C. meleagridis* (subtipos IIIb e IIIf) en tres, e a de *C. felis* nunha única mostra.

A partires destes datos, e unha vez descartadas aquelas mostras que estaban duplicadas, foi posible levar a cabo a primeira análise epidemiolóxica a grande escala sobre a criptosporidiose nunha área sanitaria galega. O presente traballo é especialmente novidoso, xa que se analizaron unha ampla gama de variables demográficas, clínicas e medioambientais sobre un total de 486 mostras recollidas en doentes humanos durante case unha década. Isto constitúe o maior estudo realizado ata o presente sobre distintos aspectos epidemiolóxicos deste grupo de parasitos en doentes humanos en España (Navarro-i-Martínez *et al.* 2011; Artieda *et al.* 2012; de Lucio *et al.* 2015; Segura *et al.* 2015). As análises epidemiolóxicas efectuáronse considerando tanto a especie como o subtipo infectante, en contraste coa maioría dos estudos epidemiolóxicos publicados que se limitan a avaliar a presenza de ooquistes de *Cryptosporidium* nas feces dos doentes ou en mostras medioambientais (Castro-Hermida *et al.* 2006; Gallas-Lindemann *et al.* 2013; Kotloff *et al.* 2013; Gholami *et al.* 2014) ou, como moito, caracterizan os illados a nivel de especie (Chalmers *et al.* 2009; The Anofel *Cryptosporidium* National Network 2010; Elwin *et al.* 2012), sendo excepcionais aqueles onde se analiza a clínica que amosan os distintos subtipos (Cama *et al.* 2007; Cama *et al.* 2008).

A nivel demográfico, observouse que os casos de criptosporidiose detectados na área sanitaria de Santiago de Compostela teñen unha maior taxa de incidencia nas etapas infantís que na idade adulta, afectando de maneira semellante a ambos sexos en consonancia cos padróns descritos noutros traballos en Estados Unidos e Europa (Semenza e Nichols 2007; Chalmers *et al.* 2009; Chalmers *et al.* 2011; Yoder *et al.* 2012). O resultado máis

salientable desta análise demográfica foi observa-la distinta distribución de *C. hominis* nos doentes cando se tiñan en conta de xeito simultáneo a idade e o sexo dos doentes, xa que se ben dita especie parasita de maneira significativa a máis nenos que nenas durante a infancia, esta relación invertese conforme aumentan as franxas de idade. Dita observación xa fora descrita con anterioridade (CHALMERS *et al.* 2009; CHALMERS *et al.* 2011) e suxire que as mulleres poderían presentar unha maior susceptibilidade a padecer criptosporidiose antroponótica debido á súa maior implicación no coidado dos cativos.

No eido clínico, o estudo epidemiolóxico revelou grandes diferenzas na sintomatoloxía dos doentes segundo a especie infectante. Deste xeito, os infectados por *C. parvum* sufriron de síntomas máis severos e presentaban maiores taxas de ingreso hospitalario que os afectados por *C. hominis*. Sen embargo, cabe destacar que a sintomatoloxía diferencial observada para ambas especies no noso estudo contrasta co descrito na escasa literatura dispoñible sobre o tema e na que son os doentes infectados por *C. hominis* os que tenden a amosar unha clínica máis grave (CAMA *et al.* 2008; XIAO 2010). Nestes traballos a infección de *C. hominis* se atopa significativamente vinculada con procesos diarreicos que levan aparellados episodios de náusea, vómito e dor abdominal na poboación xeral. Nembargante, noutros estudos observouse que as infeccións causadas por *C. parvum* parecen cursar dun xeito máis virulento nos doentes inmunocomprometidos, pudendo causarlles diarreas crónicas potencialmente letais (CAMA *et al.* 2007). Esta falla de concordancia na bibliografía sobre a patoxenicidade das dúas principais especies de criptosporidios pon de manifesto a necesidade de realizar análises epidemiolóxicas máis profundas nas que non se analicen e avalíen unicamente a gravidade daqueles síntomas clásicos que mostran os doentes afectados pola criptosporidiose, senón que semella recomendable incluír ademais outras variables clínicas que permitan unha avaliación integral do cadro sintomático de dita doenza, como serían o tipo de atención médica que necesitaron os doentes, a consistencia das súas feces ou as súas taxas de excreción de ooquistes.

No referente á súa distribución, *C. hominis* amosou ser unha especie máis ubicua – apareceu tanto en doentes que vivían en ambientes rurais como urbanos aínda que, como se ven de constatar noutras zonas de España, parece claro que dita especie se atopa con maior frecuencia en ambientes máis urbanizados (DE LUCIO *et al.* 2015; SEGURA *et al.* 2015)– con picos de incidencia nos outonos, mentres que as infeccións por *C. parvum* foron máis frecuentes nos ambientes rurais e acadaron maior incidencia durante as primaveras e os veráns. Ditos padróns estacionais e de distribución xeográfica están en sintonía cos descritos anteriormente noutras rexións de Europa (SEMENZA e NICHOLS 2007; CHALMERS *et al.* 2009).

A análise medioambiental permitiu describi-lo papel que xogan algunhas variables climáticas na incidencia de *Cryptosporidium*. Así, por exemplo, o aumento da temperatura está asociado positivamente ás taxas de infección, independentemente da especie –característica xa descrita con anterioridade noutros traballos (NAUMOVA *et al.* 2007)–, mentres que os niveis de humidade e de precipitación influíron de xeito diferente segundo se tratase de *C. hominis* ou *C. parvum*: a primeira especie foi máis común durante os períodos húmidos mentres que a segunda o foi durante os períodos máis secos. A menor abundancia de casos debidos a *C. parvum* durante os períodos con maiores taxas de precipitacións pode deberse a que un grande número de ooquistes viables no medio ambiente son arrastrados coas chuvias e as augas de escorrentía, producíndose un lavado das terras que contiñan ditas formas de resistencia do parasito (TATE *et al*. 2004; SCHIJVEN *et al*. 2013).

Outras achegas salientables deste traballo foron constata-la irrelevancia clínica dos subtipos e descubri-las altas taxas de incidencia da criptosporidiose na área sanitaria estudada. No primeiro caso, aínda que hai evidencias previas da especial virulencia que amosa a familia de subtipos Ib de *C. hominis* –e particularmente o seu sub-subtipo IbA10G2 (XIAO 2010; LI *et al.* 2013)–, no presente estudo non se detectaron variacións significativas de severidade en ningún dos catro subtipos analizados. Isto pódese deber a que a virulencia dos distintos subtipos de *Cryptosporidium* varíe segundo a rexión do planeta onde nos atopemos –debido a unha maior patoxenicidade do parasito ou a unha maior susceptibilidade do hóspede humano, ligada polo xeral ó seu nivel socieconómico (XIAO e RYAN 2004; CAMA *et al.* 2007)– ou a que no noso estudo contabamos con poucos representantes das familias de subtipos non maioritarios (23 mostras da familia de subtipos Id e 14 da familia IIn, fronte ás 289 mostras da familia de subtipos Ib e ás 146 da familia IIa), o que podería ter nesgado os resultados. Deste xeito, e ó igual que o indicado anteriormente para o caso das especies, faise preciso elaborar novos estudos epidemiolóxicos que permitan determinar con maior precisión se existen diferenzas na virulencia das distintas familias de subtipos de *Cryptosporidium*.

No segundo caso, constatouse que a incidencia media da criptosporidiose na área sanitaria de Santiago de Compostela para o período 2000-2008 era de 17,65 casos por 100.000 habitantes e ano. Este dato é moi superior aos datos rexistrados no Centro Nacional de Epidemioloxía para o conxunto de España (incidencia media duns 0,30 casos por 100.000 habitantes e ano para o mesmo período) e no Centro Europeo para o Control e Prevención de Enfermidades (ECDC, nas siglas en inglés) para o conxunto de Europa (incidencia media que non chega a superar os 3 casos por 100.000 habitantes e ano) (CENTRO NACIONAL DE EPIDEMIOLOGÍA 2003; EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2007;

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2013). Esta enorme disparidade entre os valores obtidos no noso estudo e os ofrecidos polos organismos oficiais de Saúde Pública, xunto coa constatación de que os datos de incidencia observados na área sanitaria de Santiago son similares ós descritos en rexións que contan con programas específicos para o diagnóstico da criptosporidiose e que levan xa varios lustros estudándoa, como é o caso de Irlanda e o Reino Unido (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL 2007), indican que a criptosporidiose en España é unha enfermidade altamente infradiagnosticada. Afortunadamente, a recente inclusión deste parasito na lista de patóxenos de declaración obrigatoria, tanto a nivel estatal (RED NACIONAL DE VIGILANCIA EPIDEMIOLÓGICA 2013) como autonómico (CONSELLARÍA DE SANIDADE 2013), permitirá obter a partir de agora unha panorámica máis realista sobre a situación deste parasito, o cal probablemente dará orixe a novos estudos que permitan mellora-lo control epidemiolóxico das infeccións causadas por *Cryptosporidium* no noso entorno.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CONCLUSIÓNS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Os padróns de variación intra e interespecíficos son semellantes en nove dos dez *loci* estudados en diferentes especies do xénero *Cryptosporidium* (*ACoAs, COWP1, dyn4, eIF4a, gtRNAl, gtub, ISWIr, KhRNAb* e *RNaseLi*), polo que semella razoable utilizalos como referencia para comprende-los procesos moleculares que modelan a composición do xenoma destes parasitos. Estes *loci* presentan unha diversidade neutra promedio moderada (inferior ó 1%) e evolucionan baixo o efecto predominante da selección purificadora.

2. Os padróns de variación intra e interespecíficos descritos para o *locus gp60* son significativamente superiores ós observados no resto de *loci* estudados e suxiren que evoluciona baixo o efecto de selección balanceadora. Este réxime selectivo pode ser indicativo de que a función das glicoproteínas codificadas para o recoñecemento e a ancoraxe do parasito ás células hóspedes teñen un enorme valor funcional, o que as sinala como potenciais candidatas para seren empregadas como dianas terapéuticas.

3. A división artificial das diversas especies de *Cryptosporidium* en familias de subtipos segundo a secuencia do *locus gp60* reflicte unha realidade biolóxica, xa que as variantes haplotípicas atopadas noutros xenes tamén se estruturan de acordo a este patrón.

4. A estrutura haplotípica das secuencias obtidas non aporta evidencias de recombinación. Sen embargo, dados os particulares padróns de diversidade que se observan para o *locus gp60* pero non noutros *loci* –algún dos cales se atopa no mesmo grupo de ligamento que o do xene *gp60*– suxire a existencia de episodios de recombinación na historia evolutiva do parasito.

5. O método para o xenotipado masivo de illados de *Cryptosporidium* baseado na tecnoloxía MassARRAY demostrou excelentes propiedades funcionais –medidos en termos de sensibilidade (87,3%) e especificidade (98%)– para a determinación da especie e familia de subtipos para os que foi deseñado.

6. A criptosporidiose é unha enfermidade infradiagnosticada. A taxa de incidencia media obtida só para a área sanitaria de Santiago durante a pasada década (17,65 ± 2,88 casos por 100.000 habitantes) superou en dúas orde de magnitude a ofrecida polas

autoridades sanitarias españolas durante o mesmo período para o conxunto do Estado. As especies con maior presenza nesta área foron *C. hominis* e *C. parvum*.

7. A criptosporidiose afecta principalmente á poboación infantil, independentemente do seu sexo e da especie infectante. *Cryptosporidium hominis* parasita máis frecuentemente a nenos durante as etapas infantís mentres que tende a afectar dun xeito máis significativo ás mulleres durante as etapas adultas, o cal suxire que as mulleres poderían presentar unha maior susceptibilidade a padecer criptosporidiose antroponótica debido á súa maior implicación no coidado dos cativos.

8. *Cryptosporidium hominis* mostra unha distribución ubicua –aparece tanto en ambientes rurais como urbanos– e *C. parvum* é máis común en zonas rurais.

9. Os doentes afectados por *C. parvum* amosaron unha clínica máis severa, requirindo seren ingresados en maior medida e presentando unha sintomatoloxía más grave (xa que a infección soe vir acompañada en maior medida de vómitos e de moco nas deposicións) que aqueles afectados por *C. hominis*. Non se detectaron diferenzas significativas entre as diferentes familias de subtipos estudados no referente ó seu nivel de virulencia.

10. A incidencia da criptosporidiose presenta unha grande estacionalidade, con maior incidencia durante os meses do verán e do outono. Esta variación reflíctese tamén nas incidencias das dúas especies, aínda que de xeito diferente. *Cryptosporidium parvum* amosa unha variación estacional moderada, cun máximo no verán e un mínimo no outono. A estacionalidade de *C. hominis* é máis acentuada, presentando o seu máximo nos outonos.

11. As variables climáticas xogan un importante papel na incidencia desta enfermidade. Así, as temperaturas elevadas parecen aumenta-los casos de criptosporidiose causados por ambas especies. Do mesmo xeito, durante os períodos húmidos (alta humidade ambiental e alta taxa de precipitación) danse máis casos de criptosporidiose provocados pola infección de *C. hominis*, mentres que nos períodos secos aumentan os casos debidos a *C. parvum*.

BIBLIOGRAFÍA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Abe, N., K. Takami, I. Kimata and M. Iseki, 2004 Molecular characterization of a *Cryptosporidium* isolate from a banded mongoose *Mungos mungo*. J Parasitol 90**:** 167-171.

Abeywardena, H., A. R. Jex, A. V. Koehler, R. P. Rajapakse, K. Udayawarna *et al.*, 2014 First molecular characterization of *Cryptosporidium* and *Giardia* from bovines (*Bos taurus* and *Bubalus bubalis*) in Sri Lanka: unexpected absence of *C. parvum* from pre-weaned calves. Parasit Vectors 7**:** 75.

Abrahamsen, M. S., T. J. Templeton, S. Enomoto, J. E. Abrahante, G. Zhu *et al.*, 2004 Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304**:** 441-445.

Adl, S. M., A. G. Simpson, M. A. Farmer, R. A. Andersen, O. R. Anderson *et al.*, 2005 The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol 52**:** 399-451.

Aguileta, G., G. Refregier, R. Yockteng, E. Fournier and T. Giraud, 2009 Rapidly evolving genes in pathogens: methods for detecting positive selection and examples among fungi, bacteria, viruses and protists. Infect Genet Evol 9**:** 656-670.

Aiello, A. E., L. Xiao, J. R. Limor, C. Liu, M. S. Abrahamsen *et al.*, 1999 Microsatellite analysis of the human and bovine genotypes of *Cryptosporidium parvum*. J Eukaryot Microbiol 46**:** 46S-47S.

Ajioka, J. W. S., D.;, 2007 *Toxoplasma: Molecular and Cellular Biology*.

Akiyoshi, D. E., J. K. Tumwine, S. Bakeera-Kitaka and S. Tzipori, 2006 Subtype analysis of *Cryptosporidium* isolates from children in Uganda. J Parasitol 92**:** 1097-1100.

Alcock, F., C. T. Webb, P. Dolezal, V. Hewitt, M. Shingu-Vasquez *et al.*, 2012 A small Tim homohexamer in the relict mitochondrion of *Cryptosporidium*. Mol Biol Evol 29**:** 113-122.

Aldeyarbi, H. M., and P. Karanis, 2015 The Ultra-Structural Similarities between *Cryptosporidium parvum* and the Gregarines. J Eukaryot Microbiol.

Altzibar, J. M., C. Zigorraga, R. Rodriguez, N. Leturia, A. Garmendia *et al.*, 2015 Outbreak of acute gastroenteritis caused by contamination of drinking water in a factory, the Basque Country. J Water Health 13**:** 168-173.

Álvarez-Pellitero, P., and A. Sitja-Bobadilla, 2002 *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata* L. and *Dicentrarchus labrax* L. Int J Parasitol 32**:** 1007-1021.

Alves, M., O. Matos and F. Antunes, 2003a Microsatellite analysis of *Cryptosporidium hominis* and *C. parvum* in Portugal: a preliminary study. J Eukaryot Microbiol 50 Suppl**:** 529-530.

Alves, M., L. Xiao, F. Antunes and O. Matos, 2006 Distribution of *Cryptosporidium* subtypes in humans and domestic and wild ruminants in Portugal. Parasitol Res 99**:** 287-292.

Alves, M., L. Xiao, I. Sulaiman, A. A. Lal, O. Matos *et al.*, 2003b Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J Clin Microbiol 41**:** 2744-2747.

Amadi, B., M. Mwiya, J. Musuku, A. Watuka, S. Sianongo *et al.*, 2002 Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: a randomised controlled trial. Lancet 360**:** 1375-1380.

Amadi, B., M. Mwiya, S. Sianongo, L. Payne, A. Watuka *et al.*, 2009 High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive Zambian children: a randomised controlled trial. BMC Infect Dis 9**:** 195.

Amambua-Ngwa, A., K. K. Tetteh, M. Manske, N. Gomez-Escobar, L. B. Stewart *et al.*, 2012 Population genomic scan for candidate signatures of balancing selection to guide antigen characterization in malaria parasites. PLoS Genet 8**:** e1002992.

Amar, C. F., P. H. Dear and J. McLauchlin, 2004 Detection and identification by real time PCR/RFLP analyses of *Cryptosporidium* species from human faeces. Lett Appl Microbiol 38**:** 217-222.

Anderson, B. C., and M. S. Bulgin, 1981 Enteritis caused by *Cryptosporidium* in calves. Vet Med Small Anim Clin 76**:** 865-868.

Anon., 1982 Cryptosporidiosis: assessment of chemotherapy of males with acquired immunodificiency syndrome (AIDS). Morbid. Mortal. 31**:** 599.

Ares-Mazás, M. E., B. Fernandez-da Ponte, C. A. Vergara-Castiblanco, F. Freire-Santos, J. Quilez-Cinca *et al.*, 1999 Oocysts, IgG levels and immunoblot patterns determined for *Cryptosporidium parvum* in bovine examined during a visit to a farm (northeastern Spain). Vet Parasitol 81**:** 185-193.

Armson, A., R. C. Thompson and J. A. Reynoldson, 2003 A review of chemotherapeutic approaches to the treatment of cryptosporidiosis. Expert Rev Anti Infect Ther 1**:** 297-305.

Arrowood, M. J., and C. R. Sterling, 1987 Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J Parasitol 73**:** 314-319.

Arrowood, M. J., C. R. Sterling and M. C. Healey, 1991 Immunofluorescent microscopical visualization of trails left by gliding *Cryptosporidium parvum* sporozoites. J Parasitol 77**:** 315-317.

Artieda, J., M. Basterrechea, L. Arriola, M. Yague, E. Albisua *et al.*, 2012 Outbreak of cryptosporidiosis in a child day-care centre in Gipuzkoa, Spain, October to December 2011. Euro Surveill 17.

Ashbolt, R. H., D. J. Coleman, A. Misrachi, J. M. Conti and M. D. Kirk, 2003 An outbreak of cryptosporidiosis associated with an animal nursery at a regional fair. Commun Dis Intell Q Rep 27**:** 244-249.

Aspock, H., and A. Hassl, 1990 Parasitic infections in HIV patients in Austria: first results of a long-term study. Zentralbl Bakteriol 272**:** 540-546.

Atuma, C., V. Strugala, A. Allen and L. Holm, 2001 The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol 280**:** G922-929.

Augustin-Bichl, G., J. Boch and G. Henkel, 1984 [Cryptosporidial infections in dogs and cats]. Berl Munch Tierarztl Wochenschr 97**:** 179-181.

Ausina Ruíz, V., and S. Moreno Guillén, 2006 *Tratado SEIMC de Enfermedades Infecciosas y Microbiología Clínica*.

Austin, Z., R. E. Alcock, R. M. Christley, P. M. Haygarth, A. L. Heathwaite *et al.*, 2012 Policy, practice and decision making for zoonotic disease management: water and *Cryptosporidium*. Environ Int 40**:** 70-78.

Awad-El-Kariem, F. M., H. A. Robinson, F. Petry, V. McDonald, D. Evans *et al.*, 1998 Differentiation between human and animal isolates of *Cryptosporidium parvum* using molecular and biological markers. Parasitol Res 84**:** 297-301.

Awad-el-Kariem, F. M., D. C. Warhurst and V. McDonald, 1994 Detection and species identification of *Cryptosporidium* oocysts using a system based on PCR and endonuclease restriction. Parasitology 109 (Pt 1)**:** 19-22.

Bajer, A., B. Toczylowska, M. Bednarska and E. Sinski, 2012 Effectiveness of water treatment for the removal of *Cryptosporidium* and *Giardia* spp. Epidemiol Infect 140**:** 2014-2022.

Bakker, E. G., C. Toomajian, M. Kreitman and J. Bergelson, 2006 A genome-wide survey of *R* gene polymorphisms in *Arabidopsis*. Plant Cell 18**:** 1803-1818.

Balatbat, A. B., G. W. Jordan, Y. J. Tang and J. Silva, Jr., 1996 Detection of *Cryptosporidium parvum* DNA in human feces by nested PCR. J Clin Microbiol 34**:** 1769-1772.

Baldursson, S., and P. Karanis, 2011 Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010. Water Res 45**:** 6603-6614.

Bankier, A. T., H. F. Spriggs, B. Fartmann, B. A. Konfortov, M. Madera *et al.*, 2003 Integrated mapping, chromosomal sequencing and sequence analysis of *Cryptosporidium parvum*. Genome Res 13**:** 1787-1799.

Barbosa, J. M., S. Costa-de-Oliveira, A. G. Rodrigues, T. Hanscheid, H. Shapiro *et al.*, 2008 A flow cytometric protocol for detection of *Cryptosporidium* spp. Cytometry A 73**:** 44-47.

Barker, I. K., and P. L. Carbonell, 1974 *Cryptosporidium agni* sp.n. from lambs, and *Cryptosporidium bovis* sp.n. from a calf, with observations on the oocyst. Z Parasitenkd 44**:** 289-298.

Barta, J. R., M. C. Jenkins and H. D. Danforth, 1991 Evolutionary relationships of avian *Eimeria* species among other Apicomplexan protozoa: monophyly of the apicomplexa is supported. Mol Biol Evol 8**:** 345-355.

Barta, J. R., and R. C. Thompson, 2006 What is *Cryptosporidium*? Reappraising its biology and phylogenetic affinities. Trends Parasitol 22**:** 463-468.

Bartosova-Sojkova, P., R. D. Oppenheim, D. Soldati-Favre and J. Lukes, 2015 Epicellular Apicomplexans: Parasites "On the Way In". PLoS Pathog 11**:** e1005080.

Baxby, D., N. Blundell and C. A. Hart, 1984 The development and performance of a simple, sensitive method for the detection of *Cryptosporidium* oocysts in faeces. J Hyg (Lond) 93**:** 317-323.

Bhat, N., A. Joe, M. PereiraPerrin and H. D. Ward, 2007 *Cryptosporidium* p30, a galactose/N-acetylgalactosamine-specific lectin, mediates infection in vitro. J Biol Chem 282**:** 34877-34887.

Blackburn, B. G., J. M. Mazurek, M. Hlavsa, J. Park, M. Tillapaw *et al.*, 2006 Cryptosporidiosis associated with ozonated apple cider. Emerg Infect Dis 12**:** 684-686.

Blackman, M. J., and V. B. Carruthers, 2013 Recent insights into apicomplexan parasite egress provide new views to a kill. Curr Opin Microbiol 16**:** 459-464.

Boletín Epidemiolóxico de Galicia, 2014 Modificación do Sistema Básico da Rede Galega de Vixilancia en Saúde Pública. Boletín Epidemiolóxico de Galicia XXVI**:** 1-21.

Borowski, H., P. L. Clode and R. C. Thompson, 2008 Active invasion and/or encapsulation? A reappraisal of host-cell parasitism by *Cryptosporidium*. Trends Parasitol 24**:** 509-516.

Bosch, S. S., T. Kronenberger, K. A. Meissner, F. M. Zimbres, D. Stegehake *et al.*, 2015 Oxidative stress control by apicomplexan parasites. Biomed Res Int 2015**:** 351289.

Bouakaze, C., C. Keyser, A. Gonzalez, W. Sougakoff, N. Veziris *et al.*, 2011 Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based single nucleotide polymorphism genotyping assay using iPLEX gold technology for identification of *Mycobacterium tuberculosis* complex species and lineages. J Clin Microbiol 49**:** 3292-3299.

Bouzid, M., P. R. Hunter, R. M. Chalmers and K. M. Tyler, 2013 *Cryptosporidium* pathogenicity and virulence. Clin Microbiol Rev 26**:** 115-134.

Bouzid, M., K. M. Tyler, R. Christen, R. M. Chalmers, K. Elwin *et al.*, 2010 Multi-locus analysis of human infective *Cryptosporidium* species and subtypes using ten novel genetic loci. BMC Microbiol 10**:** 213.

Brown, J. K., 2003 A cost of disease resistance: paradigm or peculiarity? Trends Genet 19: 667-671.

Brown, J. K., and A. Tellier, 2011 Plant-parasite coevolution: bridging the gap between genetics and ecology. Annu Rev Phytopathol 49**:** 345-367.

Budu-Amoako, E., S. J. Greenwood, B. R. Dixon, H. W. Barkema and J. T. McClure, 2012 Occurrence of *Cryptosporidium* and *Giardia* on beef farms and water sources within the vicinity of the farms on Prince Edward Island, Canada. Vet Parasitol 184**:** 1-9.

Bushen, O. Y., A. Kohli, R. C. Pinkerton, K. Dupnik, R. D. Newman *et al.*, 2007 Heavy cryptosporidial infections in children in northeast Brazil: comparison of *Cryptosporidium hominis* and *Cryptosporidium parvum*. Trans R Soc Trop Med Hyg 101**:** 378-384.

Butaeva, F., G. Paskerova and R. Entzeroth, 2006 *Ditrypanocystis* sp. (Apicomplexa, Gregarinia, Selenidiidae): the mode of survival in the gut of *Enchytraeus albidus* (Annelida, Oligochaeta, Enchytraeidae) is close to that of the coccidian genus *Cryptosporidium*. Tsitologiia 48**:** 695-704.

Cabada, M. M., and A. C. White, Jr., 2010 Treatment of cryptosporidiosis: do we know what we think we know? Curr Opin Infect Dis 23**:** 494-499.

Cacciò, S., W. Homan, R. Camilli, G. Traldi, T. Kortbeek *et al.*, 2000 A microsatellite marker reveals population heterogeneity within human and animal genotypes of *Cryptosporidium parvum*. Parasitology 120 (Pt 3)**:** 237-244.

Cacciò, S., W. Homan, K. van Dijk and E. Pozio, 1999 Genetic polymorphism at the beta-tubulin locus among human and animal isolates of *Cryptosporidium parvum*. FEMS Microbiol Lett 170**:** 173-179.

Cacciò, S., F. Spano and E. Pozio, 2001 Large sequence variation at two microsatellite loci among zoonotic (genotype C) isolates of *Cryptosporidium parvum*. Int J Parasitol 31**:** 1082-1086.

Cacciò, S. M., V. de Waele and G. Widmer, 2015 Geographical segregation of *Cryptosporidium parvum* multilocus genotypes in Europe. Infect Genet Evol 31**:** 245-249.

Cacciò, S. M., R. C. Thompson, J. McLauchlin and H. V. Smith, 2005 Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol 21**:** 430-437.

Calvo, M., M. Carazo, M. L. Arias, C. Chaves, R. Monge *et al.*, 2004 [Prevalence of *Cyclospora* sp., *Cryptosporidium* sp, microsporidia and fecal coliform determination in fresh fruit and vegetables consumed in Costa Rica]. Arch Latinoam Nutr 54**:** 428-432.

Cama, V. A., C. Bern, J. Roberts, L. Cabrera, C. R. Sterling *et al.*, 2008 *Cryptosporidium* species and subtypes and clinical manifestations in children, Peru. Emerg Infect Dis 14**:** 1567-1574.

Cama, V. A., C. Bern, I. M. Sulaiman, R. H. Gilman, E. Ticona *et al.*, 2003 *Cryptosporidium* species and genotypes in HIV-positive patients in Lima, Peru. J Eukaryot Microbiol 50 Suppl**:** 531-533.

Cama, V. A., J. M. Ross, S. Crawford, V. Kawai, R. Chavez-Valdez *et al.*, 2007 Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. J Infect Dis 196**:** 684-691.

Carmena, D., X. Aguinagalde, C. Zigorraga, J. C. Fernandez-Crespo and J. A. Ocio, 2007 Presence of *Giardia* cysts and *Cryptosporidium* oocysts in drinking water supplies in northern Spain. J Appl Microbiol 102**:** 619-629.

Carreno, R. A., D. S. Martin and J. R. Barta, 1999 *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitol Res 85**:** 899-904.

Casemore, D. P., 1991 ACP Broadsheet 128: June 1991. Laboratory methods for diagnosing cryptosporidiosis. J Clin Pathol 44**:** 445-451.

Casemore, D. P., M. Armstrong and R. L. Sands, 1985a Laboratory diagnosis of cryptosporidiosis. J Clin Pathol 38**:** 1337-1341.

Casemore, D. P., R. L. Sands and A. Curry, 1985b *Cryptosporidium* species a "new" human pathogen. J Clin Pathol 38**:** 1321-1336.

Castro-Hermida, J. A., A. Almeida, M. Gonzalez-Warleta, J. M. Correia da Costa, C. Rumbo-Lorenzo *et al.*, 2007 Occurrence of *Cryptosporidium parvum* and *Giardia duodenalis* in healthy adult domestic ruminants. Parasitol Res 101**:** 1443-1448.

Castro-Hermida, J. A., A. Almeida, M. Gonzalez-Warleta, J. M. Da Costa and M. Mezo, 2006a Prevalence and preliminary genetic characterization of *Cryptosporidium* spp. isolated from asymptomatic heifers in Galicia (NW, Spain). J Eukaryot Microbiol 53 Suppl 1**:** S22-23.

Castro-Hermida, J. A., C. Carro-Corral, M. Gonzalez-Warleta and M. Mezo, 2006b Prevalence and intensity of infection of *Cryptosporidium* spp. and *Giardia duodenalis* in dairy cattle in Galicia (NW Spain). J Vet Med B Infect Dis Vet Public Health 53**:** 244-246.

Castro-Hermida, J. A., A. Delafosse, I. Pors, E. Ares-Mazas and C. Chartier, 2005 *Giardia duodenalis* and *Cryptosporidium parvum* infections in adult goats and their implications for neonatal kids. Vet Rec 157**:** 623-627.

Castro-Hermida, J. A., I. Garcia-Presedo, A. Almeida, M. Gonzalez-Warleta, J. M. Correia Da Costa *et al.*, 2008a Contribution of treated wastewater to the contamination of recreational river areas with *Cryptosporidium* spp. and *Giardia duodenalis*. Water Res 42**:** 3528-3538.

Castro-Hermida, J. A., I. Garcia-Presedo, A. Almeida, M. Gonzalez-Warleta, J. M. Correia Da Costa *et al.*, 2008b Presence of *Cryptosporidium* spp. and *Giardia duodenalis* through drinking water. Sci Total Environ 405**:** 45-53.

Castro-Hermida, J. A., I. Garcia-Presedo, A. Almeida, M. Gonzalez-Warleta, J. M. Correia Da Costa *et al.*, 2011a *Cryptosporidium* spp. and *Giardia duodenalis* in two areas of Galicia (NW Spain). Sci Total Environ 409**:** 2451-2459.

Castro-Hermida, J. A., I. Garcia-Presedo, A. Almeida, M. Gonzalez-Warleta, J. M. Da Costa *et al.*, 2009 Detection of *Cryptosporidium* spp. and *Giardia duodenalis* in surface water: a health risk for humans and animals. Water Res 43**:** 4133-4142.

Castro-Hermida, J. A., I. Garcia-Presedo, M. Gonzalez-Warleta and M. Mezo, 2010 *Cryptosporidium* and *Giardia* detection in water bodies of Galicia, Spain. Water Res 44**:** 5887-5896.

Castro-Hermida, J. A., I. Garcia-Presedo, M. Gonzalez-Warleta and M. Mezo, 2011b Prevalence of *Cryptosporidium* and *Giardia* in roe deer (*Capreolus capreolus*) and wild boars (*Sus scrofa*) in Galicia (NW, Spain). Vet Parasitol 179**:** 216-219.

Castro-Hermida, J. A., M. Gonzalez-Warleta and M. Mezo, 2015 *Cryptosporidium* spp. and *Giardia duodenalis* as pathogenic contaminants of water in Galicia, Spain: the need for safe drinking water. Int J Hyg Environ Health 218**:** 132-138.

Causer, L. M., T. Handzel, P. Welch, M. Carr, D. Culp *et al.*, 2006 An outbreak of *Cryptosporidium hominis* infection at an Illinois recreational waterpark. Epidemiol Infect 134**:** 147-156.

Cavalier-Smith, T., 2014 Gregarine site-heterogeneous 18S rDNA trees, revision of gregarine higher classification, and the evolutionary diversification of Sporozoa. Eur J Protistol 50**:** 472-495.

Centro Nacional de Epidemiología, 2003 Boletín Epidemiológico Semanal. Semana 45, pp. 277-284. Instituto de Salud Carlos III. Ministerio de Sanidad y Consumo.

Cevallos, A. M., N. Bhat, R. Verdon, D. H. Hamer, B. Stein *et al.*, 2000a Mediation of *Cryptosporidium parvum* infection in vitro by mucin-like glycoproteins defined by a neutralizing monoclonal antibody. Infect Immun 68**:** 5167-5175.

Cevallos, A. M., X. Zhang, M. K. Waldor, S. Jaison, X. Zhou *et al.*, 2000b Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15. Infect Immun 68**:** 4108-4116.

Chacín-Bonilla, L., M. C. Bonilla, L. Soto-Torres, Y. Rios-Candida, M. Sardina *et al.*, 1997 *Cryptosporidium parvum* in children with diarrhea in Zulia State, Venezuela. Am J Trop Med Hyg 56**:** 365-369.

Chalmers, R. M., 2012 Waterborne outbreaks of cryptosporidiosis. Ann Ist Super Sanita 48**:** 429-446.

Chalmers, R. M., and A. P. Davies, 2010 Minireview: clinical cryptosporidiosis. Exp Parasitol 124**:** 138-146.

Chalmers, R. M., K. Elwin, A. L. Thomas, E. C. Guy and B. Mason, 2009 Long-term *Cryptosporidium* typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. Euro Surveill 14.

Chalmers, R. M., C. Ferguson, S. Caccio, R. B. Gasser, E. L. O. Y. G. Abs *et al.*, 2005 Direct comparison of selected methods for genetic categorisation of *Cryptosporidium parvum* and *Cryptosporidium hominis* species. Int J Parasitol 35**:** 397-410.

Chalmers, R. M., S. J. Hadfield, C. J. Jackson, K. Elwin, L. Xiao *et al.*, 2008 Geographic linkage and variation in *Cryptosporidium hominis*. Emerg Infect Dis 14**:** 496-498.

Chalmers, R. M., R. Smith, K. Elwin, F. A. Clifton-Hadley and M. Giles, 2011 Epidemiology of anthroponotic and zoonotic human cryptosporidiosis in England and Wales, 2004-2006. Epidemiol Infect 139**:** 700-712.

Chapman, P. A., B. A. Rush and J. McLauchlin, 1990 An enzyme immunoassay for detecting *Cryptosporidium* in faecal and environmental samples. J Med Microbiol 32**:** 233-237.

Chappell, C. L., and P. C. Okhuysen, 2002 Cryptosporidiosis. Curr Opin Infect Dis 15**:** 523-527.

Chappell, C. L., P. C. Okhuysen, R. Langer-Curry, G. Widmer, D. E. Akiyoshi *et al.*, 2006 *Cryptosporidium hominis*: experimental challenge of healthy adults. Am J Trop Med Hyg 75**:** 851-857.

Chappell, C. L., P. C. Okhuysen, C. R. Sterling and H. L. DuPont, 1996 *Cryptosporidium parvum*: intensity of infection and oocyst excretion patterns in healthy volunteers. J Infect Dis 173**:** 232-236.

Charlesworth, D., 2006 Balancing selection and its effects on sequences in nearby genome regions. PLoS Genet 2**:** e64.

Checkley, W., A. C. White, Jr., D. Jaganath, M. J. Arrowood, R. M. Chalmers *et al.*, 2015 A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. Lancet Infect Dis 15**:** 85-94.

Chen, X. M., and N. F. LaRusso, 2000 Mechanisms of attachment and internalization of *Cryptosporidium parvum* to biliary and intestinal epithelial cells. Gastroenterology 118**:** 368-379.

Chen, X. M., S. P. O'Hara, B. Q. Huang, J. B. Nelson, J. J. Lin *et al.*, 2004a Apical organelle discharge by *Cryptosporidium parvum* is temperature, cytoskeleton, and intracellular calcium dependent and required for host cell invasion. Infect Immun 72**:** 6806-6816.

Chen, X. M., P. L. Splinter, P. S. Tietz, B. Q. Huang, D. D. Billadeau *et al.*, 2004b Phosphatidylinositol 3-kinase and frabin mediate *Cryptosporidium parvum* cellular invasion via activation of Cdc42. J Biol Chem 279**:** 31671-31678.

Clark, D. P., and C. L. Sears, 1996 The pathogenesis of cryptosporidiosis. Parasitol Today 12**:** 221-225.

Clode, P. L., W. H. Koh and R. C. Thompson, 2015 Life without a Host Cell: What is *Cryptosporidium*? Trends Parasitol.

Cohen, S., F. Dalle, A. Gallay, M. Di Palma, A. Bonnin *et al.*, 2006 Identification of Cpgp40/15 Type Ib as the predominant allele in isolates of *Cryptosporidium* spp. from a waterborne outbreak of gastroenteritis in South Burgundy, France. J Clin Microbiol 44**:** 589-591.

Consellaría de Sanidade, 2013 Orde do 11 de decembro pola que se regula o sistema básico da Rede galega de vixilancia en saúde pública, pp., edited by X. d. Galicia, Santiago de Compostela.

Corso, P. S., M. H. Kramer, K. A. Blair, D. G. Addiss, J. P. Davis *et al.*, 2003 Cost of illness in the 1993 waterborne *Cryptosporidium* outbreak, Milwaukee, Wisconsin. Emerg Infect Dis 9**:** 426-431.

Coupe, S., C. Sarfati, S. Hamane and F. Derouin, 2005 Detection of *Cryptosporidium* and identification to the species level by nested PCR and restriction fragment length polymorphism. J Clin Microbiol 43**:** 1017-1023.

Current, W. L., 1985 Cryptosporidiosis. J Am Vet Med Assoc 187**:** 1334-1338.

Current, W. L., and L. S. Garcia, 1991 Cryptosporidiosis. Clin Microbiol Rev 4**:** 325-358.

Current, W. L., and T. B. Haynes, 1984 Complete development of *Cryptosporidium* in cell culture. Science 224**:** 603-605.

Current, W. L., and P. L. Long, 1983 Development of human and calf *Cryptosporidium* in chicken embryos. J Infect Dis 148**:** 1108-1113.

Current, W. L., and N. C. Reese, 1986 A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. J Protozool 33**:** 98-108.

Current, W. L., Reese, N. C., Ernst, J. V., Bailey, W. S., Heyman, M. B., and Weinstein, W. M., 1983 Human cryptosporidiosis in immunocompetent and immunodeficient persons. Studies of an outbreak and experimental transmission. N. Engl. J. Med. 308**:** 1252.

Current, W. L., S. J. Upton and T. B. Haynes, 1986 The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. J Protozool 33**:** 289-296.

D'Antonio, R. G., R. E. Winn, J. P. Taylor, T. L. Gustafson, W. L. Current *et al.*, 1985 A waterborne outbreak of cryptosporidiosis in normal hosts. Ann Intern Med 103**:** 886-888.

Dawson, D., 2005 Foodborne protozoan parasites. Int J Food Microbiol 103**:** 207-227.

de Graaf, D. C., E. Vanopdenbosch, L. M. Ortega-Mora, H. Abbassi and J. E. Peeters, 1999 A review of the importance of cryptosporidiosis in farm animals. Int J Parasitol 29**:** 1269-1287.

de Lucio, A., F. J. Merino, R. Martinez-Ruiz, B. Bailo, M. Aguilera *et al.*, 2015 Molecular genotyping and sub-genotyping of *Cryptosporidium* spp. isolates from symptomatic individuals attending two major public hospitals in Madrid, Spain. Infect Genet Evol.

De Waele, V., F. Van den Broeck, T. Huyse, G. McGrath, I. Higgins *et al.*, 2013 Panmictic structure of the *Cryptosporidium parvum* population in Irish calves: influence of prevalence and host movement. Appl Environ Microbiol 79**:** 2534-2541.

Denton, H., S. M. Brown, C. W. Roberts, J. Alexander, V. McDonald *et al.*, 1996 Comparison of the phosphofructokinase and pyruvate kinase activities of *Cryptosporidium parvum*, *Eimeria tenella* and *Toxoplasma gondii*. Mol Biochem Parasitol 76**:** 23-29.

Díaz, P., J. Quilez, R. M. Chalmers, R. Panadero, C. Lopez *et al.*, 2010a Genotype and subtype analysis of *Cryptosporidium* isolates from calves and lambs in Galicia (NW Spain). Parasitology 137**:** 1187-1193.

Díaz, P., J. Quilez, G. Robinson, R. M. Chalmers, P. Diez-Banos *et al.*, 2010b Identification of *Cryptosporidium xiaoi* in diarrhoeic goat kids (*Capra hircus*) in Spain. Vet Parasitol 172**:** 132-134.

Dingle, K. E., F. M. Colles, D. Falush and M. C. Maiden, 2005 Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. J Clin Microbiol 43**:** 340-347.

Dreelin, E. A., R. L. Ives, S. Molloy and J. B. Rose, 2014 *Cryptosporidium* and *Giardia* in surface water: a case study from Michigan, USA to inform management of rural water systems. Int J Environ Res Public Health 11**:** 10480-10503.

Drumo, R., G. Widmer, L. J. Morrison, A. Tait, V. Grelloni *et al.*, 2012 Evidence of host-associated populations of *Cryptosporidium parvum* in Italy. Appl Environ Microbiol 78**:** 3523-3529.

Dubey, J. P., J. E. Markovits and K. A. Killary, 2002 *Cryptosporidium muris*-like infection in stomach of cynomolgus monkeys (*Macaca fascicularis*). Vet Pathol 39**:** 363-371.

DuPont, H. L., C. L. Chappell, C. R. Sterling, P. C. Okhuysen, J. B. Rose *et al.*, 1995 The infectivity of *Cryptosporidium parvum* in healthy volunteers. N Engl J Med 332**:** 855-859.

Egyed, Z., T. Sreter, Z. Szell and I. Varga, 2003 Characterization of *Cryptosporidium* spp.--recent developments and future needs. Vet Parasitol 111**:** 103-114.

el-Sibaei, M. M., M. M. Rifaat, D. M. Hameed and H. M. el-Din, 2003 Nosocomial sources of cryptosporidial infection in newly admitted patients in Ain Shams University Pediatric Hospital. J Egypt Soc Parasitol 33**:** 177-188.

Ellegren, H., 2004 Microsatellites: simple sequences with complex evolution. Nat Rev Genet 5**:** 435-445.

Elliott, D. A., D. J. Coleman, M. A. Lane, R. C. May, L. M. Machesky *et al.*, 2001 *Cryptosporidium parvum* infection requires host cell actin polymerization. Infect Immun 69**:** 5940-5942.

Elwin, K., S. J. Hadfield, G. Robinson and R. M. Chalmers, 2012a The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000-2008. Epidemiol Infect 140**:** 673-683.

Elwin, K., S. J. Hadfield, G. Robinson, N. D. Crouch and R. M. Chalmers, 2012b *Cryptosporidium viatorum* n. sp. (Apicomplexa: Cryptosporidiidae) among travellers returning to Great Britain from the Indian subcontinent, 2007-2011. Int J Parasitol 42**:** 675-682.

Enemark, H. L., P. Ahrens, V. Bille-Hansen, P. M. Heegaard, H. Vigre *et al.*, 2003 *Cryptosporidium parvum*: infectivity and pathogenicity of the 'porcine' genotype. Parasitology 126**:** 407-416.

Enemark, H. L., P. Ahrens, C. D. Juel, E. Petersen, R. F. Petersen *et al.*, 2002 Molecular characterization of Danish *Cryptosporidium parvum* isolates. Parasitology 125**:** 331-341.

Enríquez, C., N. Nwachuku and C. P. Gerba, 2001 Direct exposure to animal enteric pathogens. Rev Environ Health 16**:** 117-131.

Esteban, J. G., C. Aguirre, A. Flores, W. Strauss, R. Angles *et al.*, 1998 High *Cryptosporidium* prevalences in healthy Aymara children from the northern Bolivian Altiplano. Am J Trop Med Hyg 58**:** 50-55.

European Centre for Disease Prevention and Control, 2007 Annual epidemiological report on communicable diseases in Europe. Report of the status of communicable diseases in the EU and EEA/EFTA countries., pp., edited by ECDC. ECDC, Stockholm.

European Centre for Disease Prevention and Control, 2013 Annual epidemiological report 2012. Reporting on 2010 surveillance data and 2011 epidemic intelligence data., pp. ECDC, Stockholm:.

Fall, A., R. C. Thompson, R. P. Hobbs and U. Morgan-Ryan, 2003 Morphology is not a reliable tool for delineating species within *Cryptosporidium*. J Parasitol 89**:** 399-402.

Farizawati, S., Y. A. Lim, R. A. Ahmad, C. T. Fatimah and Y. Siti-Nor, 2005 Contribution of cattle farms towards river contamination with *Giardia* cysts and *Cryptosporidium* oocysts in Sungai Langat Basin. Trop Biomed 22**:** 89-98.

Fayer, R., 2004 *Cryptosporidium*: a water-borne zoonotic parasite. Vet Parasitol 126**:** 37-56.

Fayer, R., 2010 Taxonomy and species delimitation in *Cryptosporidium*. Exp Parasitol 124**:** 90-97.

Fayer, R., U. Morgan and S. J. Upton, 2000 Epidemiology of *Cryptosporidium*: transmission, detection and identification. Int J Parasitol 30**:** 1305-1322.

Fayer, R., and M. Santin, 2009 *Cryptosporidium xiaoi* n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (*Ovis aries*). Vet Parasitol 164**:** 192-200.

Fayer, R., M. Santin and D. Macarisin, 2010 *Cryptosporidium ubiquitum* n. sp. in animals and humans. Vet Parasitol 172**:** 23-32.

Fayer, R., M. Santin and J. M. Trout, 2008 *Cryptosporidium ryanae* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). Vet Parasitol 156**:** 191-198.

Fayer, R., M. Santin and L. Xiao, 2005 *Cryptosporidium bovis* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). J Parasitol 91**:** 624-629.

Fayer, R., J. M. Trout, L. Xiao, U. M. Morgan, A. A. Lai *et al.*, 2001 *Cryptosporidium canis* n. sp. from domestic dogs. J Parasitol 87**:** 1415-1422.

Fayer, R., and B. L. Ungar, 1986 *Cryptosporidium* spp. and cryptosporidiosis. Microbiol Rev 50**:** 458-483.

Fayer, R., and L. Xiao, 2008 *Cryptosporidium and cryptosporidiosis*. CRC Press, Boca Raton.

Feltus, D. C., C. W. Giddings, B. L. Schneck, T. Monson, D. Warshauer *et al.*, 2006 Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin. J Clin Microbiol 44**:** 4303-4308.

Feng, X., S. M. Rich, D. Akiyoshi, J. K. Tumwine, A. Kekitiinwa *et al.*, 2000 Extensive polymorphism in *Cryptosporidium parvum* identified by multilocus microsatellite analysis. Appl Environ Microbiol 66**:** 3344-3349.

Feng, X., S. M. Rich, S. Tzipori and G. Widmer, 2002 Experimental evidence for genetic recombination in the opportunistic pathogen *Cryptosporidium parvum*. Mol Biochem Parasitol 119**:** 55-62.

Feng, Y., A. A. Lal, N. Li and L. Xiao, 2011a Subtypes of *Cryptosporidium* spp. in mice and other small mammals. Exp Parasitol 127**:** 238-242.

Feng, Y., Y. Ortega, G. He, P. Das, M. Xu *et al.*, 2007 Wide geographic distribution of *Cryptosporidium bovis* and the deer-like genotype in bovines. Vet Parasitol 144**:** 1-9.

Feng, Y., N. Tiao, N. Li, M. Hlavsa and L. Xiao, 2014 Multilocus sequence typing of an emerging *Cryptosporidium hominis* subtype in the United States. J Clin Microbiol 52**:** 524-530.

Feng, Y., E. Torres, N. Li, L. Wang, D. Bowman *et al.*, 2013 Population genetic characterisation of dominant *Cryptosporidium parvum* subtype IIaA15G2R1. Int J Parasitol 43**:** 1141-1147.

Feng, Y., W. Yang, U. Ryan, L. Zhang, M. Kvac *et al.*, 2011b Development of a multilocus sequence tool for typing *Cryptosporidium* muris and *Cryptosporidium andersoni*. J Clin Microbiol 49**:** 34-41.

Fijarczyk, A., and W. Babik, 2015 Detecting balancing selection in genomes: limits and prospects. Mol Ecol 24**:** 3529-3545.

Fischer, P., H. Taraschewski, R. Ringelmann and B. Eing, 1998 Detection of *Cryptosporidium parvum* in human feces by PCR. Tokai J Exp Clin Med 23**:** 309-311.

Forney, J. R., D. B. DeWald, S. Yang, C. A. Speer and M. C. Healey, 1999 A role for host phosphoinositide 3-kinase and cytoskeletal remodeling during *Cryptosporidium parvum* infection. Infect Immun 67**:** 844-852.

Forney, J. R., D. K. Vaughan, S. Yang and M. C. Healey, 1998 Actin-dependent motility in *Cryptosporidium parvum* sporozoites. J Parasitol 84**:** 908-913.

Fournet, N., M. P. Deege, A. T. Urbanus, G. Nichols, B. M. Rosner *et al.*, 2013 Simultaneous increase of *Cryptosporidium* infections in the Netherlands, the United Kingdom and Germany in late summer season, 2012. Euro Surveill 18.

Freire-Santos, F., H. Gomez-Couso, M. R. Ortega-Inarrea, J. A. Castro-Hermida, A. M. Oteiza-Lopez *et al.*, 2002 Survival of *Cryptosporidium parvum* oocysts recovered from experimentally contaminated oysters (*Ostrea edulis*) and clams (*Tapes decussatus*). Parasitol Res 88**:** 130-133.

Freire-Santos, F., A. M. Oteiza-Lopez, J. A. Castro-Hermida, O. Garcia-Martin and M. E. Ares-Mazas, 2001 Viability and infectivity of oocysts recovered from clams, *Ruditapes philippinarum*, experimentally contaminated with *Cryptosporidium parvum*. Parasitol Res 87**:** 428-430.

Freire-Santos, F., A. M. Oteiza-Lopez, C. A. Vergara-Castiblanco, E. Ares-Mazas, E. Alvarez-Suarez *et al.*, 2000 Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. J Parasitol 86**:** 853-854.

Gabriel, S., L. Ziaugra and D. Tabbaa, 2009 SNP genotyping using the Sequenom MassARRAY iPLEX platform. Curr Protoc Hum Genet Chapter 2**:** Unit 2 12.

Gajria, B., A. Bahl, J. Brestelli, J. Dommer, S. Fischer *et al.*, 2008 ToxoDB: an integrated *Toxoplasma gondii* database resource. Nucleic Acids Res 36**:** D553-556.

Gallas-Lindemann, C., I. Sotiriadou, J. Plutzer and P. Karanis, 2013 Prevalence and distribution of *Cryptosporidium* and *Giardia* in wastewater and the surface, drinking and ground waters in the Lower Rhine, Germany. Epidemiol Infect 141**:** 9-21.

García-Presedo, I., S. Pedraza-Diaz, M. Gonzalez-Warleta, M. Mezo, M. Gomez-Bautista *et al.*, 2013a The first report of *Cryptosporidium bovis*, *C. ryanae* and *Giardia*

duodenalis sub-assemblage A-II in roe deer (*Capreolus capreolus*) in Spain. Vet Parasitol 197**:** 658-664.

García-Presedo, I., S. Pedraza-Diaz, M. Gonzalez-Warleta, M. Mezo, M. Gomez-Bautista *et al.*, 2013b Presence of *Cryptosporidium scrofarum*, *C. suis* and *C. parvum* subtypes IIaA16G2R1 and IIaA13G1R1 in Eurasian wild boars (*Sus scrofa*). Vet Parasitol 196**:** 497-502.

García, L. S., R. Y. Shimizu, S. Novak, M. Carroll and F. Chan, 2003 Commercial assay for detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. J Clin Microbiol 41**:** 209-212.

Gardner, M. J., R. Bishop, T. Shah, E. P. de Villiers, J. M. Carlton *et al.*, 2005 Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. Science 309**:** 134-137.

Gardner, M. J., N. Hall, E. Fung, O. White, M. Berriman *et al.*, 2002 Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419**:** 498-511.

Garvey, P., and P. McKeown, 2009 Epidemiology of human cryptosporidiosis in Ireland, 2004-2006: analysis of national notification data. Euro Surveill 14.

Gasser, R. B., E. L. O. Y. G. Abs, S. Prepens and R. M. Chalmers, 2004 An improved 'cold SSCP' method for the genotypic and subgenotypic characterization of *Cryptosporidium*. Mol Cell Probes 18**:** 329-332.

Gasser, R. B., Y. G. El-Osta and R. M. Chalmers, 2003 Electrophoretic analysis of genetic variability within *Cryptosporidium parvum* from imported and autochthonous cases of human cryptosporidiosis in the United Kingdom. Appl Environ Microbiol 69**:** 2719-2730.

Gasser, R. B., X. Zhu, S. Caccio, R. Chalmers, G. Widmer *et al.*, 2001 Genotyping *Cryptosporidium parvum* by single-strand conformation polymorphism analysis of ribosomal and heat shock gene regions. Electrophoresis 22**:** 433-437.

Gatei, W., P. Das, P. Dutta, A. Sen, V. Cama *et al.*, 2007 Multilocus sequence typing and genetic structure of *Cryptosporidium hominis* from children in Kolkata, India. Infect Genet Evol 7**:** 197-205.

Gatei, W., C. A. Hart, R. H. Gilman, P. Das, V. Cama *et al.*, 2006 Development of a multilocus sequence typing tool for *Cryptosporidium hominis*. J Eukaryot Microbiol 53 Suppl 1**:** S43-48.

Gelletlie, R., J. Stuart, N. Soltanpoor, R. Armstrong and G. Nichols, 1997 Cryptosporidiosis associated with school milk. Lancet 350**:** 1005-1006.

Gherasim, A., M. Lebbad, M. Insulander, V. Decraene, A. Kling *et al.*, 2012 Two geographically separated food-borne outbreaks in Sweden linked by an unusual *Cryptosporidium parvum* subtype, October 2010. Euro Surveill 17.

Gholami, S., M. Khanmohammadi, E. Ahmadpour, A. S. Pagheh, S. Khadem Nakhjiri *et al.*, 2014 *Cryptosporidium* infection in patients with gastroenteritis in sari, Iran. Iran J Parasitol 9**:** 226-232.

Giangaspero, A., R. Cirillo, V. Lacasella, A. Lonigro, M. Marangi *et al.*, 2009 *Giardia* and *Cryptosporidium* in inflowing water and harvested shellfish in a lagoon in Southern Italy. Parasitol Int 58**:** 12-17.

Gibbons-Matthews, C., and A. M. Prescott, 2003 Intra-isolate variation of *Cryptosporidium parvum* small subunit ribosomal RNA genes from human hosts in England. Parasitol Res 90**:** 439-444.

Glaberman, S., J. E. Moore, C. J. Lowery, R. M. Chalmers, I. Sulaiman *et al.*, 2002 Three drinking-water-associated cryptosporidiosis outbreaks, Northern Ireland. Emerg Infect Dis 8**:** 631-633.

Glaberman, S., I. M. Sulaiman, C. Bern, J. Limor, M. M. Peng *et al.*, 2001 A multilocus genotypic analysis of *Cryptosporidium meleagridis*. J Eukaryot Microbiol Suppl**:** 19S-22S.

Gobet, P., and S. Toze, 2001 Sensitive genotyping of *Cryptosporidium parvum* by PCR-RFLP analysis of the 70-kilodalton heat shock protein (HSP70) gene. FEMS Microbiol Lett 200**:** 37-41.

Goldstein, D. B., and D. D. Pollock, 1997 Launching microsatellites: a review of mutation processes and methods of phylogenetic interference. J Hered 88**:** 335-342.

Goldstein, S. T., D. D. Juranek, O. Ravenholt, A. W. Hightower, D. G. Martin *et al.*, 1996 Cryptosporidiosis: an outbreak associated with drinking water despite state-of-the-art water treatment. Ann Intern Med 124**:** 459-468.

Gómez-Bautista, M., L. M. Ortega-Mora, E. Tabares, V. Lopez-Rodas and E. Costas, 2000 Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). Appl Environ Microbiol 66**:** 1866-1870.

Gómez-Couso, H., C. F. Amar, J. McLauchlin and E. Ares-Mazas, 2005 Characterisation of a *Cryptosporidium* isolate from water buffalo (*Bubalus bubalis*) by sequencing of a fragment of the *Cryptosporidium* oocyst wall protein gene (COWP). Vet Parasitol 131**:** 139-144.

Gómez-Couso, H., F. Freire-Santos, C. F. Amar, K. A. Grant, K. Williamson *et al.*, 2004a Detection of *Cryptosporidium* and *Giardia* in molluscan shellfish by multiplexed nested-PCR. Int J Food Microbiol 91**:** 279-288.

Gómez-Couso, H., F. Freire-Santos, M. R. Ortega-Inarrea, J. A. Castro-Hermida and M. E. Ares-Mazas, 2003 Environmental dispersal of *Cryptosporidium parvum* oocysts and cross transmission in cultured bivalve molluscs. Parasitol Res 90**:** 140-142.

Gómez-Couso, H., F. Mendez-Hermida and E. Ares-Mazas, 2007 First report of *Cryptosporidium parvum* 'ferret' genotype in American mink (*Mustela vison* Shreber 1777). Parasitol Res 100**:** 877-879.

Gómez-Couso, H., F. Mendez-Hermida, J. A. Castro-Hermida and E. Ares-Mazas, 2006a Cooking mussels (*Mytilus galloprovincialis*) by steam does not destroy the infectivity of *Cryptosporidium parvum*. J Food Prot 69**:** 948-950.

Gómez-Couso, H., F. Mendez-Hermida, J. A. Castro-Hermida and E. Ares-Mazas, 2006b *Cryptosporidium* contamination in harvesting areas of bivalve molluscs. J Food Prot 69**:** 185-190.

Gómez-Couso, H., F. Méndez-Hermida, J. A. Castro-Hermida, A. Pereira-Lorenzo and E. Ares-Mazás, 2004b Report about an outbreak of cryptosporidiosis in a nursery centre of Santiago de Compostela

(NW-Spain). pp. 486 in *IX European Multicolloquium of Parasitology*, Valencia, España.

Gómez Morales, M. A., E. Pozio and G. P. Croppo, 1992 Serodiagnosis of cryptosporidiosis in Italian HIV-positive patients by means of an oocyst soluble antigen in an ELISA. J Infect 25**:** 229-236.

Goncalves, E. M., A. J. da Silva, M. B. Eduardo, I. H. Uemura, I. N. Moura *et al.*, 2006 Multilocus genotyping of *Cryptosporidium hominis* associated with diarrhea outbreak in a day care unit in Sao Paulo. Clinics (Sao Paulo) 61**:** 119-126.

Goodgame, R. W., R. M. Genta, A. C. White and C. L. Chappell, 1993 Intensity of infection in AIDS-associated cryptosporidiosis. J Infect Dis 167**:** 704-709.

Graczyk, T. K., D. B. Conn, D. J. Marcogliese, H. Graczyk and Y. De Lafontaine, 2003 Accumulation of human waterborne parasites by zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*). Parasitol Res 89**:** 107-112.

Graczyk, T. K., R. Fayer and M. R. Cranfield, 1997 Zoonotic transmission of *Cryptosporidium parvum*: Implications for water-borne cryptosporidiosis. Parasitol Today 13**:** 348-351.

Gubbels, M. J., and M. T. Duraisingh, 2012 Evolution of apicomplexan secretory organelles. Int J Parasitol 42**:** 1071-1081.

Guerrant, R. L., 1997 Cryptosporidiosis: an emerging, highly infectious threat. Emerg Infect Dis 3**:** 51-57.

Guo, Y., K. Tang, L. A. Rowe, N. Li, D. M. Roellig *et al.*, 2015 Comparative genomic analysis reveals occurrence of genetic recombination in virulent *Cryptosporidium hominis* subtypes and telomeric gene duplications in *Cryptosporidium parvum*. BMC Genomics 16**:** 320.

Gut, J., and R. G. Nelson, 1994 *Cryptosporidium parvum* sporozoites deposit trails of 11A5 antigen during gliding locomotion and shed 11A5 antigen during invasion of MDCK cells in vitro. J Eukaryot Microbiol 41**:** 42S-43S.

Hadfield, S. J., G. Robinson, K. Elwin and R. M. Chalmers, 2011 Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of real-time PCR. J Clin Microbiol 49**:** 918-924.

Harris, J. R., M. Adrian and F. Petry, 2003 Structure of the *Cryptosporidium parvum* microneme: a metabolically and osmotically labile apicomplexan organelle. Micron 34**:** 65-78.

Hashim, A., G. Mulcahy, B. Bourke and M. Clyne, 2006 Interaction of *Cryptosporidium hominis* and *Cryptosporidium parvum* with primary human and bovine intestinal cells. Infect Immun 74**:** 99-107.

Heiges, M., H. Wang, E. Robinson, C. Aurrecoechea, X. Gao *et al.*, 2006 CryptoDB: a *Cryptosporidium* bioinformatics resource update. Nucleic Acids Res 34**:** D419-422.

Heine, J., J. F. Pohlenz, H. W. Moon and G. N. Woode, 1984 Enteric lesions and diarrhea in gnotobiotic calves monoinfected with *Cryptosporidium* species. J Infect Dis 150**:** 768-775.

Heinz, E., and T. Lithgow, 2013 Back to basics: a revealing secondary reduction of the mitochondrial protein import pathway in diverse intracellular parasites. Biochim Biophys Acta 1833**:** 295-303.

Hellard, M., J. Hocking, J. Willis, G. Dore and C. Fairley, 2003 Risk factors leading to *Cryptosporidium* infection in men who have sex with men. Sex Transm Infect 79**:** 412-414.

Henriksen, S. A., and J. F. Pohlenz, 1981 Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acta Vet Scand 22**:** 594-596.

Herges, G. R., G. Widmer, M. E. Clark, E. Khan, C. W. Giddings *et al.*, 2012 Evidence that *Cryptosporidium parvum* populations are panmictic and unstructured in the Upper Midwest of the United States. Appl Environ Microbiol 78**:** 8096-8101.

Hijjawi, N. S., B. P. Meloni, M. Ng'anzo, U. M. Ryan, M. E. Olson *et al.*, 2004 Complete development of *Cryptosporidium parvum* in host cell-free culture. Int J Parasitol 34**:** 769-777.

Hijjawi, N. S., B. P. Meloni, U. M. Ryan, M. E. Olson and R. C. Thompson, 2002 Successful in vitro cultivation of *Cryptosporidium andersoni*: evidence for the existence of novel

extracellular stages in the life cycle and implications for the classification of *Cryptosporidium*. Int J Parasitol 32**:** 1719-1726.

Homan, W., T. van Gorkom, Y. Y. Kan and J. Hepener, 1999 Characterization of *Cryptosporidium parvum* in human and animal feces by single-tube nested polymerase chain reaction and restriction analysis. Parasitol Res 85**:** 707-712.

Horger, A. C., M. Ilyas, W. Stephan, A. Tellier, R. A. van der Hoorn *et al.*, 2012 Balancing selection at the tomato *RCR3* Guardee gene family maintains variation in strength of pathogen defense. PLoS Genet 8**:** e1002813.

Huang, B. Q., X. M. Chen and N. F. LaRusso, 2004 *Cryptosporidium parvum* attachment to and internalization by human biliary epithelia in vitro: a morphologic study. J Parasitol 90**:** 212-221.

Huang, D. B., and A. C. White, 2006 An updated review on *Cryptosporidium* and *Giardia*. Gastroenterol Clin North Am 35**:** 291-314, viii.

Hudson, R. R., M. Kreitman and M. Aguade, 1987 A test of neutral molecular evolution based on nucleotide data. Genetics 116**:** 153-159.

Hunter, P. R., S. Hughes, S. Woodhouse, N. Raj, Q. Syed *et al.*, 2004a Health sequelae of human cryptosporidiosis in immunocompetent patients. Clin Infect Dis 39**:** 504-510.

Hunter, P. R., S. Hughes, S. Woodhouse, Q. Syed, N. Q. Verlander *et al.*, 2004b Sporadic cryptosporidiosis case-control study with genotyping. Emerg Infect Dis 10**:** 1241-1249.

Hunter, P. R., and G. Nichols, 2002 Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. Clin Microbiol Rev 15**:** 145-154.

Inman, L. R., and A. Takeuchi, 1979 Spontaneous cryptosporidiosis in an adult female rabbit. Vet Pathol 16**:** 89-95.

Insulander, M., M. Lebbad, T. A. Stenstrom and B. Svenungsson, 2005 An outbreak of cryptosporidiosis associated with exposure to swimming pool water. Scand J Infect Dis 37**:** 354-360.

Isaacs, D., G. H. Hunt, A. D. Phillips, E. H. Price, F. Raafat *et al.*, 1985 Cryptosporidiosis in immunocompetent children. J Clin Pathol 38**:** 76-81.

Iseki, M., 1979 *Cryptosporidium felis* sp. n. (Protozoa: Eimeriorina) from the domestic cat. Jap J Parasitol 28**:** 285-307.

Jakobi, V., and F. Petry, 2006 Differential expression of *Cryptosporidium parvum* genes encoding sporozoite surface antigens in infected HCT-8 host cells. Microbes Infect 8**:** 2186-2194.

Jex, A. R., and R. B. Gasser, 2010 Genetic richness and diversity in *Cryptosporidium hominis* and *C. parvum* reveals major knowledge gaps and a need for the application of "next generation" technologies--research review. Biotechnol Adv 28**:** 17-26.

Jex, A. R., A. Pangasa, B. E. Campbell, M. Whipp, G. Hogg *et al.*, 2008 Classification of *Cryptosporidium* species from patients with sporadic cryptosporidiosis by use of sequence-based multilocus analysis following mutation scanning. J Clin Microbiol 46**:** 2252-2262.

Jex, A. R., U. M. Ryan, J. Ng, B. E. Campbell, L. Xiao *et al.*, 2007 Specific and genotypic identification of *Cryptosporidium* from a broad range of host species by nonisotopic SSCP analysis of nuclear ribosomal DNA. Electrophoresis 28**:** 2818-2825.

Jiang, J., and L. Xiao, 2003 An evaluation of molecular diagnostic tools for the detection and differentiation of human-pathogenic *Cryptosporidium* spp. J Eukaryot Microbiol 50 Suppl**:** 542-547.

Jirku, M., A. Valigurova, B. Koudela, J. Krizek, D. Modry *et al.*, 2008 New species of *Cryptosporidium* Tyzzer, 1907 (Apicomplexa) from amphibian host: morphology, biology and phylogeny. Folia Parasitol (Praha) 55**:** 81-94.

Johnson, A. M., R. Fielke, R. Lumb and P. R. Baverstock, 1990 Phylogenetic relationships of *Cryptosporidium* determined by ribosomal RNA sequence comparison. Int J Parasitol 20**:** 141-147.

Johnson, D. W., N. J. Pieniazek, D. W. Griffin, L. Misener and J. B. Rose, 1995 Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl Environ Microbiol 61**:** 3849-3855.

Johnson, D. W., N. J. Pieniazek and J. B. Rose, 1993 DNA probe hybridization and PCR detection of *Cryptosporidium* compared to immunofluorescence assay. 27**:** 77-84.

Jokipii, L., and A. M. Jokipii, 1986 Timing of symptoms and oocyst excretion in human cryptosporidiosis. N Engl J Med 315**:** 1643-1647.

Jothikumar, N., A. J. da Silva, I. Moura, Y. Qvarnstrom and V. R. Hill, 2008 Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays. J Med Microbiol 57**:** 1099-1105.

Juranek, D. D., 1995 Cryptosporidiosis: sources of infection and guidelines for prevention. Clin Infect Dis 21 Suppl 1**:** S57-61.

Kaneta, Y., and Y. Nakai, 1998 Survey of *Cryptosporidium* oocysts from adult cattle in a slaughter house. J Vet Med Sci 60**:** 585-588.

Karasov, T. L., J. M. Kniskern, L. Gao, B. J. DeYoung, J. Ding *et al.*, 2014 The long-term maintenance of a resistance polymorphism through diffuse interactions. Nature 512**:** 436-440.

Katanik, M. T., S. K. Schneider, J. E. Rosenblatt, G. S. Hall and G. W. Procop, 2001 Evaluation of ColorPAC *Giardia*/*Cryptosporidium* rapid assay and ProSpecT *Giardia*/*Cryptosporidium* microplate assay for detection of *Giardia* and *Cryptosporidium* in fecal specimens. J Clin Microbiol 39**:** 4523-4525.

Keeling, P. J., 2004 Reduction and compaction in the genome of the apicomplexan parasite *Cryptosporidium parvum*. Dev Cell 6**:** 614-616.

Keithly, J. S., S. G. Langreth, K. F. Buttle and C. A. Mannella, 2005 Electron tomographic and ultrastructural analysis of the *Cryptosporidium parvum* relict mitochondrion, its associated membranes, and organelles. J Eukaryot Microbiol 52**:** 132-140.

Keithly, J. S., G. Zhu, S. J. Upton, K. M. Woods, M. P. Martinez *et al.*, 1997 Polyamine biosynthesis in *Cryptosporidium parvum* and its implications for chemotherapy. Mol Biochem Parasitol 88**:** 35-42.

Keusch, G. T., D. Hamer, A. Joe, M. Kelley, J. Griffiths *et al.*, 1995 Cryptosporidia--who is at risk? Schweiz Med Wochenschr 125**:** 899-908.

Khan, W. A., K. A. Rogers, M. M. Karim, S. Ahmed, P. L. Hibberd *et al.*, 2004 Cryptosporidiosis among Bangladeshi children with diarrhea: a prospective, matched, case-control study of clinical features, epidemiology and systemic antibody responses. Am J Trop Med Hyg 71**:** 412-419.

Khramtsov, N. V., M. Tilley, D. S. Blunt, B. A. Montelone and S. J. Upton, 1995 Cloning and analysis of a *Cryptosporidium parvum* gene encoding a protein with homology to cytoplasmic form Hsp70. J Eukaryot Microbiol 42**:** 416-422.

Kimbell, L. M., 3rd, D. L. Miller, W. Chavez and N. Altman, 1999 Molecular analysis of the 18S rRNA gene of *Cryptosporidium serpentis* in a wild-caught corn snake (*Elaphe guttata guttata*) and a five-species restriction fragment length polymorphism- based assay that can additionally discern *C. parvum* from *C. wrairi*. Appl Environ Microbiol 65**:** 5345-5349.

Kniel, K. E., and M. C. Jenkins, 2005 Detection of *Cryptosporidium parvum* oocysts on fresh vegetables and 1 herbs using antibodies specific for a *Cryptosporidium parvum* viral antigen. J Food Prot 68**:** 1093-1096.

Koch, K. L., D. J. Phillips, R. C. Aber and W. L. Current, 1985 Cryptosporidiosis in hospital personnel. Evidence for person-to-person transmission. Ann Intern Med 102**:** 593-596.

Koehler, A. V., R. S. Bradbury, M. A. Stevens, S. R. Haydon, A. R. Jex *et al.*, 2013 Genetic characterization of selected parasites from people with histories of gastrointestinal disorders using a mutation scanning-coupled approach. Electrophoresis 34**:** 1720-1728.

Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair and C. R. Sterling, 1990 Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl Environ Microbiol 56**:** 1423-1428.

Kotloff, K. L., J. P. Nataro, W. C. Blackwelder, D. Nasrin, T. H. Farag *et al.*, 2013 Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382**:** 209-222.

Koyama, Y., M. Satoh, K. Maekawa, K. Hikosaka and Y. Nakai, 2005 Isolation of *Cryptosporidium andersoni* Kawatabi type in a slaughterhouse in the northern island of Japan. Vet Parasitol 130**:** 323-326.

Kvac, M., L. Hofmannova, L. Hlaskova, D. Kvetonova, J. Vitovec *et al.*, 2014 *Cryptosporidium erinacei* n. sp. (Apicomplexa: Cryptosporidiidae) in hedgehogs. Vet Parasitol 201**:** 9-17.

Kvac, M., M. Kestranova, M. Pinkova, D. Kvetonova, J. Kalinova *et al.*, 2013 *Cryptosporidium scrofarum* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (*Sus scrofa*). Vet Parasitol 191**:** 218-227.

Lalonde, L. F., J. Reyes and A. A. Gajadhar, 2013 Application of a qPCR assay with melting curve analysis for detection and differentiation of protozoan oocysts in human fecal samples from Dominican Republic. Am J Trop Med Hyg 89**:** 892-898.

Lane, C. E., and J. M. Archibald, 2008 The eukaryotic tree of life: endosymbiosis takes its TOL. Trends Ecol Evol 23**:** 268-275.

Laxer, M. A., B. K. Timblin and R. J. Patel, 1991 DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. Am J Trop Med Hyg 45**:** 688-694.

Le Blancq, S. M., N. V. Khramtsov, F. Zamani, S. J. Upton and T. W. Wu, 1997 Ribosomal RNA gene organization in *Cryptosporidium parvum*. Mol Biochem Parasitol 90**:** 463-478.

Leander, B. S., R. E. Clopton and P. J. Keeling, 2003a Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and beta-tubulin. Int J Syst Evol Microbiol 53**:** 345-354.

Leander, B. S., J. T. Harper and P. J. Keeling, 2003b Molecular phylogeny and surface morphology of marine aseptate gregarines (Apicomplexa): *Selenidium* spp. and *Lecudina* spp. J Parasitol 89**:** 1191-1205.

Leav, B. A., M. R. Mackay, A. Anyanwu, O. C. RM, A. M. Cevallos *et al.*, 2002 Analysis of sequence diversity at the highly polymorphic Cpgp40/15 locus among *Cryptosporidium* isolates from human immunodeficiency virus-infected children in South Africa. Infect Immun 70**:** 3881-3890.

Léger, L., 1911 *Caryospora simplex*, coccidie monosporée et la classification des coccidies. Arch. Protistenkd 22**:** 71-88.

Lendner, M., and A. Daugschies, 2014 *Cryptosporidium* infections: molecular advances. Parasitology 141**:** 1511-1532.

Leng, X., D. A. Mosier and R. D. Oberst, 1996a Differentiation of *Cryptosporidium parvum*, *C. muris*, and *C. baileyi* by PCR-RFLP analysis of the 18S rRNA gene. Vet Parasitol 62**:** 1-7.

Leng, X., D. A. Mosier and R. D. Oberst, 1996b Simplified method for recovery and PCR detection of *Cryptosporidium* DNA from bovine feces. Appl Environ Microbiol 62**:** 643-647.

Levine, M. M., K. L. Kotloff, R. F. Breiman and A. K. Zaidi, 2013 Diarrheal disease constitutes one of the top two causes of mortality among young children in developing countries. Preface. Am J Trop Med Hyg 89**:** 1-2.

Levine, N. D., 1980 Some corrections of coccidian (Apicomplexa: Protozoa) nomenclature. J Parasitol 66**:** 830-834.

Levine, N. D., 1988 *The Protozoan Phylum Apicomplexa, vols. 1 and 2*. CRC, Boca Raton, FL.

Levine, N. D., J. O. Corliss, F. E. Cox, G. Deroux, J. Grain *et al.*, 1980 A newly revised classification of the protozoa. J Protozool 27**:** 37-58.

Levine, W. C., W. T. Stephenson and G. F. Craun, 1990 Waterborne disease outbreaks, 1986-1988. MMWR CDC Surveill Summ 39**:** 1-13.

Li, N., L. Xiao, K. Alderisio, K. Elwin, E. Cebelinski *et al.*, 2014 Subtyping *Cryptosporidium ubiquitum*,a zoonotic pathogen emerging in humans. Emerg Infect Dis 20**:** 217-224.

Li, N., L. Xiao, V. A. Cama, Y. Ortega, R. H. Gilman *et al.*, 2013 Genetic recombination and *Cryptosporidium hominis* virulent subtype IbA10G2. Emerg Infect Dis 19**:** 1573-1582.

Lim, Y. A., A. Iqbal, J. Surin, B. L. Sim, A. R. Jex *et al.*, 2011 First genetic classification of *Cryptosporidium* and *Giardia* from HIV/AIDS patients in Malaysia. Infect Genet Evol 11**:** 968-974.

Lindsay, D. S., S. J. Upton, D. S. Owens, U. M. Morgan, J. R. Mead *et al.*, 2000 *Cryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporiidae) from cattle, *Bos taurus*. J Eukaryot Microbiol 47**:** 91-95.

Llorente, M. T., A. Clavel, M. P. Goni, M. Varea, C. Seral *et al.*, 2007 Genetic characterization of *Cryptosporidium* species from humans in Spain. Parasitol Int 56**:** 201-205.

Llovo Taboada, J., 1990 *Cryptosporidium*-Cryptosporidiosis. Diagnóstico, incidencia, epidemiología, clínica y respuesta inmunológica, pp. Universidade de Santiago de Compostela, Santiago de Compostela.

Lorenzo, M. J., B. Ben, F. Mendez, I. Villacorta and M. E. Ares-Mazas, 1995 *Cryptosporidium parvum* oocyst antigens recognized by sera from infected asymptomatic adult cattle. Vet Parasitol 60**:** 17-25.

Lowery, C. J., J. E. Moore, B. C. Millar, D. P. Burke, K. A. McCorry *et al.*, 2000 Detection and speciation of *Cryptosporidium* spp. in environmental water samples by immunomagnetic separation, PCR and endonuclease restriction. J Med Microbiol 49**:** 779-785.

Lumb, R., K. Smith, P. J. O'Donoghue and J. A. Lanser, 1988 Ultrastructure of the attachment of *Cryptosporidium* sporozoites to tissue culture cells. Parasitol Res 74**:** 531-536.

Lv, C., L. Zhang, R. Wang, F. Jian, S. Zhang *et al.*, 2009 *Cryptosporidium* spp. in wild, laboratory, and pet rodents in china: prevalence and molecular characterization. Appl Environ Microbiol 75**:** 7692-7699.

Ma, P., and R. Soave, 1983 Three-step stool examination for cryptosporidiosis in 10 homosexual men with protracted watery diarrhea. J Infect Dis 147**:** 824-828.

MacKenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair *et al.*, 1994 A Massive Outbreak in Milwaukee of *Cryptosporidium* Infection Transmitted through the Public Water Supply. N Engl J Med 331**:** 161-167.

MacKenzie, W. R., W. L. Schell, K. A. Blair, D. G. Addiss, D. E. Peterson *et al.*, 1995 Massive outbreak of waterborne cryptosporidium infection in Milwaukee, Wisconsin: recurrence of illness and risk of secondary transmission. Clin Infect Dis 21**:** 57-62.

Mallon, M., A. MacLeod, J. Wastling, H. Smith, B. Reilly *et al.*, 2003a Population structures and the role of genetic exchange in the zoonotic pathogen *Cryptosporidium parvum*. J Mol Evol 56**:** 407-417.

Mallon, M. E., A. MacLeod, J. M. Wastling, H. Smith and A. Tait, 2003b Multilocus genotyping of *Cryptosporidium parvum* Type 2: population genetics and sub-structuring. Infect Genet Evol 3**:** 207-218.

Manabe, Y. C., D. P. Clark, R. D. Moore, J. A. Lumadue, H. R. Dahlman *et al.*, 1998 Cryptosporidiosis in patients with AIDS: correlates of disease and survival. Clin Infect Dis 27**:** 536-542.

Marcial, M. A., and J. L. Madara, 1986 *Cryptosporidium*: cellular localization, structural analysis of absorptive cell-parasite membrane-membrane interactions in guinea pigs, and suggestion of protozoan transport by M cells. Gastroenterology 90**:** 583-594.

Mary, C., E. Chapey, E. Dutoit, K. Guyot, L. Hasseine *et al.*, 2013 Multicentric evaluation of a new real-time PCR assay for quantification of *Cryptosporidium* spp. and identification of *Cryptosporidium parvum* and *Cryptosporidium hominis*. J Clin Microbiol 51**:** 2556-2563.

Mason, B. W., R. M. Chalmers, D. Carnicer-Pont and D. P. Casemore, 2010 A *Cryptosporidium hominis* outbreak in north-west Wales associated with low oocyst counts in treated drinking water. J Water Health 8**:** 299-310.

Matos, O., A. Tomas, P. Aguiar, D. Casemore and F. Antunes, 1998 Prevalence of cryptosporidiosis in AIDS patients with diarrhoea in Santa Maria Hospital, Lisbon. Folia Parasitol (Praha) 45**:** 163-166.

Matsui, T., T. Fujino and M. Tsuji, 1999 Infectivity to hosts of the endogenous stages of chicken and murine *Cryptosporidium*. J Vet Med Sci 61**:** 471-474.

Matukaitis, J. M., 1997 The emerging recognition of *Cryptosporidium* as a health hazard. J Community Health Nurs 14**:** 135-140.

Mazurie, A. J., J. M. Alves, L. S. Ozaki, S. Zhou, D. C. Schwartz *et al.*, 2013 Comparative genomics of *Cryptosporidium*. Int J Genomics 2013**:** 832756.

McCuin, R. M., and J. L. Clancy, 2005 Methods for the recovery, isolation and detection of *Cryptosporidium* oocysts in wastewaters. J Microbiol Methods 63**:** 73-88.

McLauchlin, J., C. F. Amar, S. Pedraza-Diaz, G. Mieli-Vergani, N. Hadzic *et al.*, 2003 Polymerase chain reaction-based diagnosis of infection with *Cryptosporidium* in children with primary immunodeficiencies. Pediatr Infect Dis J 22**:** 329-335.

McLauchlin, J., S. Pedraza-Diaz, C. Amar-Hoetzeneder and G. L. Nichols, 1999 Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. J Clin Microbiol 37**:** 3153-3158.

McNabb, S. J., D. M. Hensel, D. F. Welch, H. Heijbel, G. L. McKee *et al.*, 1985 Comparison of sedimentation and flotation techniques for identification of *Cryptosporidium* sp. oocysts in a large outbreak of human diarrhea. J Clin Microbiol 22**:** 587-589.

Mead, J. R., R. C. Humphreys, D. W. Sammons and C. R. Sterling, 1990 Identification of isolate-specific sporozoite proteins of *Cryptosporidium parvum* by two-dimensional gel electrophoresis. Infect Immun 58**:** 2071-2075.

Medema, G. J., and J. F. Schijven, 2001 Modelling the sewage discharge and dispersion of *Cryptosporidium* and *Giardia* in surface water. Water Res 35**:** 4307-4316.

Meisel, J. L., D. R. Perera, C. Meligro and C. E. Rubin, 1976 Overwhelming watery diarrhea associated with a *Cryptosporidium* in an immunosuppressed patient. Gastroenterology 70**:** 1156-1160.

Mendisco, F., C. Keyser, C. Hollard, V. Seldes, A. E. Nielsen *et al.*, 2011 Application of the iPLEX Gold SNP genotyping method for the analysis of Amerindian ancient DNA samples: benefits for ancient population studies. Electrophoresis 32**:** 386-393.

Meutin, D. J., H. J. Van Kruiningen and D. H. Lein, 1974 Cryptosporidiosis in a calf. J Am Vet Med Assoc 165**:** 914-917.

Millard, P. S., K. F. Gensheimer, D. G. Addiss, D. M. Sosin, G. A. Beckett *et al.*, 1994 An outbreak of cryptosporidiosis from fresh-pressed apple cider. JAMA 272**:** 1592-1596.

Millership, J. J., X. Cai and G. Zhu, 2004 Functional characterization of replication protein A2 (RPA2) from *Cryptosporidium parvum*. Microbiology 150**:** 1197-1205.

Millership, J. J., and G. Zhu, 2002 Heterogeneous expression and functional analysis of two distinct replication protein A large subunits from *Cryptosporidium parvum*. Int J Parasitol 32**:** 1477-1485.

Miron, D., J. Kenes and R. Dagan, 1991 Calves as a source of an outbreak of cryptosporidiosis among young children in an agricultural closed community. Pediatr Infect Dis J 10**:** 438-441.

Mogi, T., and K. Kita, 2010 Diversity in mitochondrial metabolic pathways in parasitic protists *Plasmodium* and *Cryptosporidium*. Parasitol Int 59**:** 305-312.

Moon, H. W., and W. J. Bemrick, 1981 Fecal transmission of calf cryptosporidia between calves and pigs. Vet Pathol 18**:** 248-255.

Moore, A. C., B. L. Herwaldt, G. F. Craun, R. L. Calderon, A. K. Highsmith *et al.*, 1993 Surveillance for waterborne disease outbreaks--United States, 1991-1992. MMWR CDC Surveill Summ 42**:** 1-22.

Morgan-Ryan, U. M., A. Fall, L. A. Ward, N. Hijjawi, I. Sulaiman *et al.*, 2002 *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. J Eukaryot Microbiol 49**:** 433-440.

Morgan, U., R. Weber, L. Xiao, I. Sulaiman, R. C. Thompson *et al.*, 2000 Molecular characterization of Cryptosporidium isolates obtained from human immunodeficiency virus-infected individuals living in Switzerland, Kenya, and the United States. J Clin Microbiol 38**:** 1180-1183.

Morgan, U., L. Xiao, I. Sulaiman, R. Weber, A. A. Lal *et al.*, 1999a Which genotypes/species of *Cryptosporidium* are humans susceptible to? J Eukaryot Microbiol 46**:** 42S-43S.

Morgan, U. M., C. C. Constantine, P. O'Donoghue, B. P. Meloni, P. A. O'Brien *et al.*, 1995 Molecular characterization of *Cryptosporidium* isolates from humans and other animals using random amplified polymorphic DNA analysis. Am J Trop Med Hyg 52**:** 559-564.

Morgan, U. M., P. Deplazes, D. A. Forbes, F. Spano, H. Hertzberg *et al.*, 1999b Sequence and PCR-RFLP analysis of the internal transcribed spacers of the rDNA repeat unit in isolates of *Cryptosporidium* from different hosts. Parasitology 118 (Pt 1)**:** 49-58.

Morgan, U. M., P. T. Monis, R. Fayer, P. Deplazes and R. C. Thompson, 1999c Phylogenetic relationships among isolates of *Cryptosporidium*: evidence for several new species. J Parasitol 85**:** 1126-1133.

Morgan, U. M., P. A. O'Brien and R. C. Thompson, 1996 The development of diagnostic PCR primers for *Cryptosporidium* using RAPD-PCR. Mol Biochem Parasitol 77**:** 103-108.

Morgan, U. M., L. Xiao, R. Fayer, A. A. Lal and R. C. Thompson, 1999d Variation in *Cryptosporidium*: towards a taxonomic revision of the genus. Int J Parasitol 29**:** 1733-1751.

Moriarty, E. M., J. M. McEvoy, C. J. Lowery, H. P. Thompson, M. Finn *et al.*, 2005 Prevalence and characterisation of *Cryptosporidium* species in cattle faeces and on beef carcases at slaughter. Vet Rec 156**:** 165-168.

Morrison, D. A., and J. T. Ellis, 1997 Effects of nucleotide sequence alignment on phylogeny estimation: a case study of 18S rDNAs of apicomplexa. Mol Biol Evol 14**:** 428-441.

Mosier, D. A., and R. D. Oberst, 2000 Cryptosporidiosis. A global challenge. Ann N Y Acad Sci 916**:** 102-111.

Mu, J., P. Awadalla, J. Duan, K. M. McGee, J. Keebler *et al.*, 2007 Genome-wide variation and identification of vaccine targets in the *Plasmodium falciparum* genome. Nat Genet 39**:** 126-130.

Muthusamy, D., S. S. Rao, S. Ramani, B. Monica, I. Banerjee *et al.*, 2006 Multilocus genotyping of *Cryptosporidium* sp. isolates from human immunodeficiency virus-infected individuals in South India. J Clin Microbiol 44**:** 632-634.

Naumova, E. N., J. S. Jagai, B. Matyas, A. DeMaria, Jr., I. B. MacNeill *et al.*, 2007 Seasonality in six enterically transmitted diseases and ambient temperature. Epidemiol Infect 135**:** 281-292.

Navarro-i-Martínez, L., F. J. Bornay-Llinares, C. Rueda, C. del Aguila, A. J. da Silva *et al.*, 2003 Molecular characterization of *Cryptosporidium* sp. from animals in Spain. J Eukaryot Microbiol 50 Suppl**:** 553-554.

Navarro-i-Martínez, L., C. del Aguila and F. J. Bornay-Llinares, 2011 [*Cryptosporidium*: a genus in revision. The situation in Spain]. Enferm Infecc Microbiol Clin 29**:** 135-143.

Nchito, M., P. Kelly, S. Sianongo, N. P. Luo, R. Feldman *et al.*, 1998 Cryptosporidiosis in urban Zambian children: an analysis of risk factors. Am J Trop Med Hyg 59**:** 435-437.

Neto, R. C., L. U. Santos and R. M. Franco, 2006 Evaluation of activated sludge treatment and the efficiency of the disinfection of *Giardia* species cysts and *Cryptosporidium* oocysts by UV at a sludge treatment plant in Campinas, south-east Brazil. Water Sci Technol 54**:** 89-94.

Ngouanesavanh, T., K. Guyot, G. Certad, Y. Le Fichoux, C. Chartier *et al.*, 2006 *Cryptosporidium* population genetics: evidence of clonality in isolates from France and Haiti. J Eukaryot Microbiol 53 Suppl 1**:** S33-36.

Nguyen, S. T., Y. Fukuda, C. Tada, R. Sato, B. Duong *et al.*, 2012 Molecular characterization of *Cryptosporidium* in native beef calves in central Vietnam. Parasitol Res 111**:** 1817-1820.

Nichols, G., and B. T. Thom, 1984 Screening for *Cryptosporidium* in stools. Lancet 1**:** 734-735.

Nichols, R. A., B. M. Campbell and H. V. Smith, 2003 Identification of *Cryptosporidium* spp. oocysts in United Kingdom noncarbonated natural mineral waters and drinking waters by using a modified nested PCR-restriction fragment length polymorphism assay. Appl Environ Microbiol 69**:** 4183-4189.

Nime, F. A., J. D. Burek, D. L. Page, M. A. Holscher and J. H. Yardley, 1976 Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. Gastroenterology 70**:** 592-598.

Nissapatorn, V., and N. Sawangjaroen, 2011 Parasitic infections in HIV infected individuals: diagnostic and therapeutic challenges. Idian J Med Res 134: 878-897

Nydam, D. V., S. E. Wade, S. L. Schaaf and H. O. Mohammed, 2001 Number of *Cryptosporidium* parvum oocysts or *Giardia* spp cysts shed by dairy calves after natural infection. Am J Vet Res 62**:** 1612-1615.

O'Connor, R. M., J. W. Wanyiri, A. M. Cevallos, J. W. Priest and H. D. Ward, 2007 *Cryptosporidium parvum* glycoprotein gp40 localizes to the sporozoite surface by association with gp15. Mol Biochem Parasitol 156**:** 80-83.

O'Donoghue, P. J., 1995 *Cryptosporidium* and cryptosporidiosis in man and animals. Int J Parasitol 25**:** 139-195.

O'Handley, R. M., and M. E. Olson, 2006 Giardiasis and cryptosporidiosis in ruminants. Vet Clin North Am Food Anim Pract 22**:** 623-643.

O'Hara, S. P., B. Q. Huang, X. M. Chen, J. Nelson and N. F. LaRusso, 2005 Distribution of *Cryptosporidium parvum* sporozoite apical organelles during attachment to and internalization by cultured biliary epithelial cells. J Parasitol 91**:** 995-999.

Ogunkolade, B. W., H. A. Robinson, V. McDonald, K. Webster and D. A. Evans, 1993 Isoenzyme variation within the genus *Cryptosporidium*. Parasitol Res 79**:** 385-388.

Ortega-Mora, L. M., and S. E. Wright, 1994 Age-related resistance in ovine cryptosporidiosis: patterns of infection and humoral immune response. Infect Immun 62**:** 5003-5009.

Ortega-Pierres, G., 2009 *Giardia and Cryptosporidium : from molecules to disease*. CABI, Wallingford, UK ; Cambridge, MA.

Ortega, Y. R., C. R. Roxas, R. H. Gilman, N. J. Miller, L. Cabrera *et al.*, 1997 Isolation of *Cryptosporidium parvum* and *Cyclospora cayetanensis* from vegetables collected in markets of an endemic region in Peru. Am J Trop Med Hyg 57**:** 683-686.

Paabo, S., D. M. Irwin and A. C. Wilson, 1990 DNA damage promotes jumping between templates during enzymatic amplification. J Biol Chem 265**:** 4718-4721.

Pain, A., L. Crossman and J. Parkhill, 2005 Comparative apicomplexan genomics. Nat Rev Microbiol 3**:** 454-455.

Palmer, S. R., and A. H. Biffin, 1990 Cryptosporidiosis in England and Wales: prevalence and clinical and epidemiological features. British Medical Journal 300**:** 774-777.

Panciera, R. J., Thomassen, R.W., Garner, F. M., 1971 Cryptosporidial infection in a calf. Vet. Pathol. 8**:** 479.

Paraud, C., I. Pors, A. Rieux and S. Brunet, 2014 High excretion of *Cryptosporidium ubiquitum* by peri-parturient goats in one flock in western France. Vet Parasitol 202**:** 301-304.

Patel, S., S. Pedraza-Diaz, J. McLauchlin and D. P. Casemore, 1998 Molecular characterisation of *Cryptosporidium parvum* from two large suspected waterborne outbreaks. Outbreak Control Team South and West Devon 1995, Incident Management Team and Further Epidemiological and Microbiological Studies Subgroup North Thames 1997. Commun Dis Public Health 1**:** 231-233.

Pavlasek, I., 1999 Cryptosporidia: Biology, diagnosis, host spectrum, specificity, and the environment. . Remedia—Klinicka Mikrobiologie 3**:** 290-301.

Pavlasek, I., and U. Ryan, 2008 *Cryptosporidium varanii* takes precedence over *C. saurophilum*. Exp Parasitol 118**:** 434-437.

Pedraza-Díaz, S., C. Amar, G. L. Nichols and J. McLauchlin, 2001 Nested polymerase chain reaction for amplification of the *Cryptosporidium* oocyst wall protein gene. Emerg Infect Dis 7**:** 49-56.

Peng, M. M., O. Matos, W. Gatei, P. Das, M. Stantic-Pavlinic *et al.*, 2001 A comparison of *Cryptosporidium* subgenotypes from several geographic regions. J Eukaryot Microbiol Suppl**:** 28S-31S.

Peng, M. M., S. R. Meshnick, N. A. Cunliffe, B. D. Thindwa, C. A. Hart *et al.*, 2003a Molecular epidemiology of cryptosporidiosis in children in Malawi. J Eukaryot Microbiol 50 Suppl**:** 557-559.

Peng, M. M., M. L. Wilson, R. E. Holland, S. R. Meshnick, A. A. Lal *et al.*, 2003b Genetic diversity of *Cryptosporidium* spp. in cattle in Michigan: implications for understanding the transmission dynamics. Parasitol Res 90**:** 175-180.

Petersen, C., 1992 Cryptosporidiosis in patients infected with the human immunodeficiency virus. Clin Infect Dis 15**:** 903-909.

Petry, F., 2004 Structural analysis of *Cryptosporidium parvum*. Microsc Microanal 10**:** 586-601.

Pitlik, S. D., V. Fainstein, D. Garza, L. Guarda, R. Bolivar *et al.*, 1983a Human cryptosporidiosis: spectrum of disease. Report of six cases and review of the literature. Arch Intern Med 143**:** 2269-2275.

Pitlik, S. D., V. Fainstein, A. Rios, L. Guarda, P. W. Mansell *et al.*, 1983b Cryptosporidial cholecystitis. N Engl J Med 308**:** 967.

Plutzer, J., and P. Karanis, 2009 Genetic polymorphism in *Cryptosporidium* species: an update. Vet Parasitol 165**:** 187-199.

Pohlenz, J., H. W. Moon, N. F. Cheville and W. J. Bemrick, 1978 Cryptosporidiosis as a probable factor in neonatal diarrhea of calves. J Am Vet Med Assoc 172**:** 452-457.

Portman, N., and J. Slapeta, 2014 The flagellar contribution to the apical complex: a new tool for the eukaryotic Swiss Army knife? Trends Parasitol 30**:** 58-64.

Power, M. L., C. Cheung-Kwok-Sang, M. Slade and S. Williamson, 2009 *Cryptosporidium fayeri*: diversity within the GP60 locus of isolates from different marsupial hosts. Exp Parasitol 121**:** 219-223.

Power, M. L., M. Holley, U. M. Ryan, P. Worden and M. R. Gillings, 2011 Identification and differentiation of *Cryptosporidium* species by capillary electrophoresis single-strand conformation polymorphism. FEMS Microbiol Lett 314**:** 34-41.

Power, M. L., and U. M. Ryan, 2008 A new species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) from eastern grey kangaroos (*Macropus giganteus*). J Parasitol 94**:** 1114-1117.

Pozio, E., and M. A. Morales, 2005 The impact of HIV-protease inhibitors on opportunistic parasites. Trends Parasitol 21**:** 58-63.

Pozio, E., G. Rezza, A. Boschini, P. Pezzotti, A. Tamburrini *et al.*, 1997 Clinical cryptosporidiosis and human immunodeficiency virus (HIV)-induced immunosuppression: findings from a longitudinal study of HIV-positive and HIV-negative former injection drug users. J Infect Dis 176**:** 969-975.

Priest, J. W., J. P. Kwon, M. J. Arrowood and P. J. Lammie, 2000 Cloning of the immunodominant 17-kDa antigen from *Cryptosporidium parvum*. Mol Biochem Parasitol 106**:** 261-271.

Puiu, D., S. Enomoto, G. A. Buck, M. S. Abrahamsen and J. C. Kissinger, 2004 CryptoDB: the *Cryptosporidium* genome resource. Nucleic Acids Res 32**:** D329-331.

Putignani, L., 2005 The unusual architecture and predicted function of the mitochondrion organelle in *Cryptosporidium parvum* and *hominis* species: the strong paradigm of the structure-function relationship. Parassitologia 47**:** 217-225.

Putignani, L., A. Tait, H. V. Smith, D. Horner, J. Tovar *et al.*, 2004 Characterization of a mitochondrion-like organelle in *Cryptosporidium parvum*. Parasitology 129**:** 1-18.

Quílez, J., C. Vergara-Castiblanco, L. Monteagudo, E. del Cacho and C. Sanchez-Acedo, 2013 Host association of *Cryptosporidium parvum* populations infecting domestic ruminants in Spain. Appl Environ Microbiol 79**:** 5363-5371.

Quiroz, E. S., C. Bern, J. R. MacArthur, L. Xiao, M. Fletcher *et al.*, 2000 An outbreak of cryptosporidiosis linked to a foodhandler. J Infect Dis 181**:** 695-700.

Raja, K., Z. Abbas, S. M. Hassan, N. H. Luck, T. Aziz *et al.*, 2014 Prevalence of cryptosporidiosis in renal transplant recipients presenting with acute diarrhea at a single center in Pakistan. J Nephropathol 3**:** 127-131.

Ramírez, N. E., L. A. Ward and S. Sreevatsan, 2004 A review of the biology and epidemiology of cryptosporidiosis in humans and animals. Microbes Infect 6**:** 773-785.

Ramo, A., J. Quilez, E. Del Cacho and C. Sanchez-Acedo, 2014 Optimization of a fragment size analysis tool for identification of *Cryptosporidium* species and Gp60 alleles infecting domestic ruminants. Vet Parasitol 205**:** 466-471.

Ramo, A., J. Quilez, C. Vergara-Castiblanco, L. Monteagudo, E. Del Cacho *et al.*, 2015 Multilocus typing and population structure of *Cryptosporidium* from children in Zaragoza, Spain. Infect Genet Evol 31**:** 190-197.

Reboredo-Fernández, A., E. Ares-Mazas, S. M. Caccio and H. Gomez-Couso, 2015a Occurrence of *Giardia* and *Cryptosporidium* in wild birds in Galicia (Northwest Spain). Parasitology 142**:** 917-925.

Reboredo-Fernández, A., E. Ares-Mazas, J. A. Martinez-Cedeira, R. Romero-Suances, S. M. Caccio *et al.*, 2015b *Giardia* and *Cryptosporidium* in cetaceans on the European Atlantic coast. Parasitol Res 114**:** 693-698.

Red Nacional de Vigilancia Epidemiológica, 2013 Protocolos de las enfermedades de declaración obligatoria, pp. 721, edited by ISCIII, Madrid.

Ren, X., J. Zhao, L. Zhang, C. Ning, F. Jian *et al.*, 2012 *Cryptosporidium tyzzeri* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic mice (*Mus musculus*). Exp Parasitol 130**:** 274-281.

Rider, S. D., Jr., X. Cai, W. J. Sullivan, Jr., A. T. Smith, J. Radke *et al.*, 2005 The protozoan parasite *Cryptosporidium parvum* possesses two functionally and evolutionarily divergent replication protein A large subunits. J Biol Chem 280**:** 31460-31469.

Rider, S. D., Jr., and G. Zhu, 2008 Differential expression of the two distinct replication protein A subunits from *Cryptosporidium parvum*. J Cell Biochem 104**:** 2207-2216.

Rider, S. D., Jr., and G. Zhu, 2010 *Cryptosporidium*: genomic and biochemical features. Exp Parasitol 124**:** 2-9.

Riordan, C. E., J. G. Ault, S. G. Langreth and J. S. Keithly, 2003 *Cryptosporidium parvum* Cpn60 targets a relict organelle. Curr Genet 44**:** 138-147.

Robertson, L. J., A. T. Campbell and H. V. Smith, 1992 Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl Environ Microbiol 58**:** 3494-3500.

Robertson, L. J., and B. Gjerde, 2001 Factors affecting recovery efficiency in isolation of *Cryptosporidium* oocysts and *Giardia* cysts from vegetables for standard method development. J Food Prot 64**:** 1799-1805.

Robinson, G., S. Wright, K. Elwin, S. J. Hadfield, F. Katzer *et al.*, 2010 Re-description of *Cryptosporidium cuniculus* Inman and Takeuchi, 1979 (Apicomplexa: Cryptosporidiidae): morphology, biology and phylogeny. Int J Parasitol 40**:** 1539-1548.

Rochelle, P. A., E. M. Jutras, E. R. Atwill, R. De Leon and M. H. Stewart, 1999 Polymorphisms in the beta-tubulin gene of *Cryptosporidium parvum* differentiate

between isolates based on animal host but not geographic origin. J Parasitol 85**:** 986-989.

Rosales, M. J., G. P. Cordon, M. S. Moreno and C. M. Sanchez, 2005 Extracellular like-gregarine stages of *Cryptosporidium parvum*. Acta Trop 95**:** 74-78.

Rosenblatt, J. E., and L. M. Sloan, 1993 Evaluation of an enzyme-linked immunosorbent assay for detection of *Cryptosporidium* spp. in stool specimens. J Clin Microbiol 31**:** 1468-1471.

Rosenthal, M., R. Pedersen, S. Leibsle, V. Hill, K. Carter *et al.*, 2015 Notes from the field: cryptosporidiosis associated with consumption of unpasteurized goat milk - Idaho, 2014. MMWR Morb Mortal Wkly Rep 64**:** 194-195.

Roy, S. W., and D. Penny, 2007 Widespread intron loss suggests retrotransposon activity in ancient apicomplexans. Mol Biol Evol 24**:** 1926-1933.

Ruecker, N. J., R. M. Hoffman, R. M. Chalmers and N. F. Neumann, 2011 Detection and resolution of *Cryptosporidium* species and species mixtures by genus-specific nested PCR-restriction fragment length polymorphism analysis, direct sequencing, and cloning. Appl Environ Microbiol 77**:** 3998-4007.

Ryan, U., R. Fayer and L. Xiao, 2014 *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitology 141**:** 1667-1685.

Ryan, U., and N. Hijjawi, 2015 New developments in *Cryptosporidium* research. Int J Parasitol 45**:** 367-373.

Ryan, U., L. Xiao, C. Read, L. Zhou, A. A. Lal *et al.*, 2003a Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl Environ Microbiol 69**:** 4302-4307.

Ryan, U. M., P. Monis, H. L. Enemark, I. Sulaiman, B. Samarasinghe *et al.*, 2004 *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). J Parasitol 90**:** 769-773.

Ryan, U. M., M. Power and L. Xiao, 2008 *Cryptosporidium fayeri* n. sp. (Apicomplexa: Cryptosporidiidae) from the Red Kangaroo (*Macropus rufus*). J Eukaryot Microbiol 55**:** 22-26.

Ryan, U. M., L. Xiao, C. Read, I. M. Sulaiman, P. Monis *et al.*, 2003b A redescription of *Cryptosporidium galli* Pavlasek, 1999 (Apicomplexa: Cryptosporidiidae) from birds. J Parasitol 89**:** 809-813.

Sam-Yellowe, T. Y., 1996 Rhoptry organelles of the apicomplexa: Their role in host cell invasion and intracellular survival. Parasitol Today 12**:** 308-316.

Santín, M., and D. S. Zarlenga, 2009 A multiplex polymerase chain reaction assay to simultaneously distinguish *Cryptosporidium* species of veterinary and public health concern in cattle. Vet Parasitol 166**:** 32-37.

Savioli, L., H. Smith and A. Thompson, 2006 *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. Trends Parasitol 22**:** 203-208.

Schijven, J., Bouwknegt, M., de Roda Husman, A. M., Rutjes S., B. Sudre *et al.*, 2013 A decision support tool to compare waterborne and foodborne infection and/or illness risks associated with climate change. Risk Anal 33: 2154-2167.

Schindler, A. R., Y. G. Abs El-Osta, M. Stevens, M. I. Sinclair and R. B. Gasser, 2005 Capillary electrophoretic analysis of fragment length polymorphism in ribosomal markers of *Cryptosporidium* from humans. Mol Cell Probes 19**:** 394-399.

Segura, R., N. Prim, M. Montemayor, M. E. Valls and C. Munoz, 2015 Predominant virulent IbA10G2 subtype of *Cryptosporidium hominis* in human isolates in Barcelona: a five-year study. PLoS One 10**:** e0121753.

Semenza, J. C., and G. Nichols, 2007 Cryptosporidiosis surveillance and water-borne outbreaks in Europe. Euro Surveill 12**:** E13-14.

Sestak, K., L. A. Ward, A. Sheoran, X. Feng, D. E. Akiyoshi *et al.*, 2002 Variability among *Cryptosporidium parvum* genotype 1 and 2 immunodominant surface glycoproteins. Parasite Immunol 24**:** 213-219.

Shirley, D. A., S. N. Moonah and K. L. Kotloff, 2012 Burden of disease from cryptosporidiosis. Curr Opin Infect Dis 25**:** 555-563.

Shore García, L., 2007 *Diagnostic Medical Parasitology*, Washington D.C.

Singh, A., I. Bairy and P. G. Shivananda, 2003 Spectrum of opportunistic infections in AIDS cases. Indian J Med Sci 57**:** 16-21.

Slapeta, J., 2012 The name *Cryptosporidium tyzzeri* Ren, Zhao, Zhang, Ning, Jian, Wang, Lv, Wang, Arrowood and Xiao, 2012 is permanently invalid. Exp Parasitol 130**:** 306-307.

Slavin, D., 1955 *Cryptosporidium meleagridis* (sp. nov.). J Comp Pathol 65**:** 262-266.

Smith, H. V., S. M. Caccio, A. Tait, J. McLauchlin and R. C. Thompson, 2006 Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections in humans. Trends Parasitol 22**:** 160-167.

Smith, H. V., R. A. Nichols and A. M. Grimason, 2005 *Cryptosporidium* excystation and invasion: getting to the guts of the matter. Trends Parasitol 21**:** 133-142.

Smith, H. V., and J. B. Rose, 1998 Waterborne cryptosporidiosis: current status. Parasitol Today 14**:** 14-22.

Soave, R., J. Ruiz, V. Garcia-Saucedo, C. Garrocho and B. H. Kean, 1989 Cryptosporidiosis in a rural community in central Mexico. J Infect Dis 159**:** 1160-1162.

Soler, P., 2004 Surveillance of cryptosporidiosis in Spain from 1995 to 2003. Euro Surveill 8**:** 2402.

Spanakos, G., A. Biba, A. Mavridou and P. Karanis, 2015 Occurrence of *Cryptosporidium* and *Giardia* in recycled waters used for irrigation and first description of *Cryptosporidium* parvum and *C. muris* in Greece. Parasitol Res 114**:** 1803-1810.

Spano, F., and A. Crisanti, 2000 *Cryptosporidium parvum*: the many secrets of a small genome. Int J Parasitol 30**:** 553-565.

Spano, F., L. Putignani, S. Guida and A. Crisanti, 1998 *Cryptosporidium parvum*: PCR-RFLP analysis of the TRAP-C1 (thrombospondin-related adhesive protein of *Cryptosporidium*-1) gene discriminates between two alleles differentially associated with parasite isolates of animal and human origin. Exp Parasitol 90**:** 195-198.

Spano, F., L. Putignani, J. McLauchlin, D. P. Casemore and A. Crisanti, 1997 PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. FEMS Microbiol Lett 150**:** 209-217.

Sreedharan, A., R. S. Jayshree and H. Sridhar, 1996 Cryptosporidiosis among cancer patients: an observation. J Diarrhoeal Dis Res 14**:** 211-213.

Staggs, S. E., E. M. Beckman, S. P. Keely, R. Mackwan, M. W. Ware *et al.*, 2013 The Applicability of TaqMan-Based Quantitative Real-Time PCR Assays for Detecting and Enumerating *Cryptosporidium* spp. Oocysts in the Environment. PLoS One 8**:** e66562.

Stantic-Pavlinic, M., L. Xiao, S. Glaberman, A. A. Lal, T. Orazen *et al.*, 2003 Cryptosporidiosis associated with animal contacts. Wien Klin Wochenschr 115**:** 125-127.

Striepen, B., 2013 Parasitic infections: Time to tackle cryptosporidiosis. Nature 503**:** 189-191.

Strong, W. B., J. Gut and R. G. Nelson, 2000 Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infect Immun 68**:** 4117-4134.

Strong, W. B., and R. G. Nelson, 2000 Preliminary profile of the *Cryptosporidium parvum* genome: an expressed sequence tag and genome survey sequence analysis. Mol Biochem Parasitol 107**:** 1-32.

Sturbaum, G. D., C. Reed, P. J. Hoover, B. H. Jost, M. M. Marshall *et al.*, 2001 Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. Appl Environ Microbiol 67**:** 2665-2668.

Suárez-Luengas, L., A. Clavel, J. Quilez, M. P. Goni-Cepero, E. Torres *et al.*, 2007 Molecular characterization of *Cryptosporidium* isolates from pigs in Zaragoza (northeastern Spain). Vet Parasitol 148**:** 231-235.

Sulaiman, I. M., P. R. Hira, L. Zhou, F. M. Al-Ali, F. A. Al-Shelahi *et al.*, 2005 Unique endemicity of cryptosporidiosis in children in Kuwait. J Clin Microbiol 43**:** 2805-2809.

Sulaiman, I. M., A. A. Lal, M. J. Arrowood and L. Xiao, 1999 Biallelic polymorphism in the intron region of beta-tubulin gene of *Cryptosporidium* parasites. J Parasitol 85**:** 154-157.

Sulaiman, I. M., A. A. Lal and L. Xiao, 2001 A population genetic study of the *Cryptosporidium parvum* human genotype parasites. J Eukaryot Microbiol Suppl**:** 24S-27S.

Sulaiman, I. M., A. A. Lal and L. Xiao, 2002 Molecular phylogeny and evolutionary relationships of *Cryptosporidium* parasites at the actin locus. J Parasitol 88**:** 388-394.

Sulaiman, I. M., U. M. Morgan, R. C. Thompson, A. A. Lal and L. Xiao, 2000 Phylogenetic relationships of *Cryptosporidium* parasites based on the 70-kilodalton heat shock protein (HSP70) gene. Appl Environ Microbiol 66**:** 2385-2391.

Tamburrini, A., and E. Pozio, 1999 Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). Int J Parasitol 29**:** 711-715.

Tanriverdi, S., J. C. Blain, B. Deng, M. T. Ferdig and G. Widmer, 2007 Genetic crosses in the apicomplexan parasite *Cryptosporidium parvum* define recombination parameters. Mol Microbiol 63**:** 1432-1439.

Tanriverdi, S., A. Grinberg, R. M. Chalmers, P. R. Hunter, Z. Petrovic *et al.*, 2008 Inferences about the global population structures of *Cryptosporidium parvum* and *Cryptosporidium hominis*. Appl Environ Microbiol 74**:** 7227-7234.

Tanriverdi, S., A. Markovics, M. O. Arslan, A. Itik, V. Shkap *et al.*, 2006 Emergence of distinct genotypes of *Cryptosporidium parvum* in structured host populations. Appl Environ Microbiol 72**:** 2507-2513.

Tanriverdi, S., and G. Widmer, 2006 Differential evolution of repetitive sequences in *Cryptosporidium parvum* and *Cryptosporidium hominis*. Infect Genet Evol 6**:** 113-122.

Tanyuksel, M., H. Gun and L. Doganci, 1995 Prevalence of *Cryptosporidium* sp. in patients with neoplasia and diarrhea. Scand J Infect Dis 27**:** 69-70.

Tate, K. W., Pereira, M. D. and E. R. Atwill 2004 Efficacy of vegetated buffer strips for retaining *Cryptosporidium parvum*. J Environ Qual 33: 2243-2251

Templeton, T. J., S. Enomoto, W. J. Chen, C. G. Huang, C. A. Lancto *et al.*, 2010 A genome-sequence survey for *Ascogregarina taiwanensis* supports evolutionary affiliation but

metabolic diversity between a *Gregarine* and *Cryptosporidium*. Mol Biol Evol 27**:** 235-248.

Tetley, L., S. M. Brown, V. McDonald and G. H. Coombs, 1998 Ultrastructural analysis of the sporozoite of *Cryptosporidium parvum*. Microbiology 144 (Pt 12)**:** 3249-3255.

The Anofel *Cryptosporidium* National Network, 2010 Laboratory-based surveillance for *Cryptosporidium* in France, 2006-2009. Euro Surveill 15**:** 19642.

Thompson, H. P., J. S. Dooley, J. Kenny, M. McCoy, C. J. Lowery *et al.*, 2007 Genotypes and subtypes of *Cryptosporidium* spp. in neonatal calves in Northern Ireland. Parasitol Res 100**:** 619-624.

Thompson, R. C., M. E. Olson, G. Zhu, S. Enomoto, M. S. Abrahamsen *et al.*, 2005 *Cryptosporidium* and cryptosporidiosis. Adv Parasitol 59**:** 77-158.

Tian, Z. C., X. C. Zhang, J. H. Li, J. G. Yin, J. Yang *et al.*, 2002 [Cloning of a species-specific gene fragment from *Cryptosporidium parvum* and the development of diagnostic PCR primers]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 20**:** 72-75.

Tian, Z. C., X. C. Zhang, J. M. Wang, J. G. Yin, J. H. Li *et al.*, 2001 [Random amplified polymorphic DNA analysis of *Cryptosporidium* species and strains]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 19**:** 100-102.

Toso, M. A., and C. K. Omoto, 2007 *Gregarina niphandrodes* may lack both a plastid genome and organelle. J Eukaryot Microbiol 54**:** 66-72.

Trembizki, E., Smith, H., Lahra, M. M., Chen, M., B. Donovan *et al.*, 2014 High-throughput informative single nucleotide polymorphism-based typing of *Neisseria gonorrheae* using the Sequenom MassARRAY iPLEX platform J Antimicriob Chemother 69: 1526-1532.

Trotz-Williams, L. A., D. S. Martin, W. Gatei, V. Cama, A. S. Peregrine *et al.*, 2006 Genotype and subtype analyses of *Cryptosporidium* isolates from dairy calves and humans in Ontario. Parasitol Res 99**:** 346-352.

Tumwine, J. K., A. Kekitiinwa, N. Nabukeera, D. E. Akiyoshi, S. M. Rich *et al.*, 2003 *Cryptosporidium parvum* in children with diarrhea in Mulago Hospital, Kampala, Uganda. Am J Trop Med Hyg 68**:** 710-715.

Tyzzer, E. E., 1907 A sporozoan found in the peptic glands of the common mouse. Proc. Soc. Exp. Biol. Med. 5.

Tyzzer, E. E., 1910 An extracellular Coccidium, *Cryptosporidium muris* (Gen. Et Sp. Nov.), of the gastric Glands of the Common Mouse. J Med Res 23**:** 487-510 483.

Tyzzer, E. E., 1912 *Cryptosporidium parvum* (sp. nov.). Arch. Protistenkd 26**:** 394-412.

Tzipori, S., 1983 Cryptosporidiosis in animals and humans. Microbiol Rev 47**:** 84-96.

Tzipori, S., K. W. Angus, E. W. Gray and I. Campbell, 1980 Vomiting and diarrhea associated with cryptosporidial infection. N Engl J Med 303**:** 818.

Tzipori, S., K. W. Angus, E. W. Gray, I. Campbell and F. Allan, 1981 Diarrhea in lambs experimentally infected with *Cryptosporidium* isolated from calves. Am J Vet Res 42**:** 1400-1404.

Tzipori, S., and J. K. Griffiths, 1998 Natural history and biology of *Cryptosporidium parvum*. Adv Parasitol 40**:** 5-36.

Tzipori, S., M. Smith, C. Halpin, K. W. Angus, D. Sherwood *et al.*, 1983 Experimental cryptosporidiosis in calves: clinical manifestations and pathological findings. Vet Rec 112**:** 116-120.

Tzipori, S., and G. Widmer, 2000 The biology of *Cryptosporidium*. Contrib Microbiol 6**:** 1-32.

Umemiya, R., M. Fukuda, K. Fujisaki and T. Matsui, 2005 Electron microscopic observation of the invasion process of *Cryptosporidium parvum* in severe combined immunodeficiency mice. J Parasitol 91**:** 1034-1039.

Ungar, B. L., 1990 Enzyme-linked immunoassay for detection of *Cryptosporidium* antigens in fecal specimens. J Clin Microbiol 28**:** 2491-2495.

United States Environmental Protection Agency, 2001a *Cryptosporidium*: Drinking Water Health Advisory. Office of Science and Technology. Office of Water March 2001.

United States Environmental Protection Agency, 2001b *Cryptosporidium*: Human Health Criteria Document. Office of Science and Technology. Office of Water.

Upton, S. J., 2000 *Suborder Eimeriorina Leger, 1911, in An Illustrated Guide to the Protozoa*. Society of Protozoologists.

Upton, S. J., and W. L. Current, 1985 The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. J Parasitol 71**:** 625-629.

Urwin, R., J. E. Russell, E. A. Thompson, E. C. Holmes, I. M. Feavers *et al.*, 2004 Distribution of surface protein variants among hyperinvasive meningococci: implications for vaccine design. Infect Immun 72**:** 5955-5962.

Valdez, L. M., H. Dang, P. C. Okhuysen and C. L. Chappell, 1997 Flow cytometric detection of *Cryptosporidium* oocysts in human stool samples. J Clin Microbiol 35**:** 2013-2017.

Valigurova, A., L. Hofmannova, B. Koudela and J. Vavra, 2007 An ultrastructural comparison of the attachment sites between *Gregarina* steini and *Cryptosporidium muris*. J Eukaryot Microbiol 54**:** 495-510.

Valigurova, A., M. Jirku, B. Koudela, M. Gelnar, D. Modry *et al.*, 2008 Cryptosporidia: epicellular parasites embraced by the host cell membrane. Int J Parasitol 38**:** 913-922.

Vetterling, J. M., H. R. Jervis, T. G. Merrill and H. Sprinz, 1971 *Cryptosporidium wrairi* sp. n. from the guinea pig *Cavia porcellus*, with an emendation of the genus. J Protozool 18**:** 243-247.

Villacorta-Martínez de Maturana, I., M. E. Ares-Mazas, D. Duran-Oreiro and M. J. Lorenzo-Lorenzo, 1992 Efficacy of activated sludge in removing *Cryptosporidium parvum* oocysts from sewage. Appl Environ Microbiol 58**:** 3514-3516.

Villacorta, I., E. Ares-Mazas and M. J. Lorenzo, 1991 *Cryptosporidium parvum* in cattle, sheep and pigs in Galicia (N.W. Spain). Vet Parasitol 38**:** 249-252.

Villacorta Martínez de Maturana, M. I., 1989 Diagnóstico, incidencia y profilaxis de la cryptosporidiosis. Infecciones experimentales., pp. 119. Universidade de Santiago de Compostela, Santiago de Compostela.

Vilogurova, A., and B. Koudela, 2005 Fine structure of trophozoites of the gregarine *Leidyana ephestiae* (Apicomplexa:Eugregarinida) parasitic in *Ephestia kuehniella* larvae (Lepidoptera). European Journal of Protistology 41**:** 209-218.

Waldron, L. S., B. Dimeski, P. J. Beggs, B. C. Ferrari and M. L. Power, 2011a Molecular epidemiology, spatiotemporal analysis, and ecology of sporadic human cryptosporidiosis in Australia. Appl Environ Microbiol 77**:** 7757-7765.

Waldron, L. S., B. C. Ferrari, C. Cheung-Kwok-Sang, P. J. Beggs, N. Stephens *et al.*, 2011b Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak in Australia. Appl Environ Microbiol 77**:** 7766-7771.

Waldron, L. S., B. C. Ferrari and M. L. Power, 2009 Glycoprotein 60 diversity in *C. hominis* and *C. parvum* causing human cryptosporidiosis in NSW, Australia. Exp Parasitol 122**:** 124-127.

Waldron, L. S., and M. L. Power, 2011 Fluorescence analysis detects gp60 subtype diversity in *Cryptosporidium* infections. Infect Genet Evol 11**:** 1388-1395.

Wang, R., L. Zhang, C. Axen, C. Bjorkman, F. Jian *et al.*, 2014 *Cryptosporidium parvum* IId family: clonal population and dispersal from Western Asia to other geographical regions. Sci Rep 4**:** 4208.

Webb, L. M., S. A. Tubach, D. C. Hunt, C. Centers for Disease and Prevention, 2014 Outbreak of cryptosporidiosis among responders to a rollover of a truck carrying calves - Kansas, April 2013. MMWR Morb Mortal Wkly Rep 63**:** 1185-1188.

Webster, K. A., 1993 Molecular methods for the detection and classification of *Cryptosporidium*. Parasitol Today 9**:** 263-266.

Webster, K. A., J. D. Pow, M. Giles, J. Catchpole and M. J. Woodward, 1993 Detection of *Cryptosporidium parvum* using a specific polymerase chain reaction. Vet Parasitol 50**:** 35-44.

Wetzel, D. M., J. Schmidt, M. S. Kuhlenschmidt, J. P. Dubey and L. D. Sibley, 2005 Gliding motility leads to active cellular invasion by *Cryptosporidium parvum* sporozoites. Infect Immun 73**:** 5379-5387.

Widmer, G., 1998 Genetic heterogeneity and PCR detection of *Cryptosporidium parvum*. Adv Parasitol 40**:** 223-239.

Widmer, G., 2004 Population genetics of *Cryptosporidium parvum*. Trends Parasitol 20**:** 3-6; discussion 6.

Widmer, G., 2009 Meta-analysis of a polymorphic surface glycoprotein of the parasitic protozoa *Cryptosporidium parvum* and *Cryptosporidium hominis*. Epidemiol Infect 137**:** 1800-1808.

Widmer, G., X. Feng and S. Tanriverdi, 2004 Genotyping of *Cryptosporidium parvum* with microsatellite markers. Methods Mol Biol 268**:** 177-187.

Widmer, G., and Y. Lee, 2010 Comparison of single- and multilocus genetic diversity in the protozoan parasites *Cryptosporidium parvum* and *C. hominis*. Appl Environ Microbiol 76**:** 6639-6644.

Widmer, G., E. A. Orbacz and S. Tzipori, 1999 Constitutive expression of small subunit ribosomal RNA transcripts in *Cryptosporidium parvum* oocysts and intracellular stages. J Parasitol 85**:** 229-233.

Widmer, G., R. Ras, R. M. Chalmers, K. Elwin, E. Desoky *et al.*, 2015 Population structure of natural and propagated isolates of *Cryptosporidium parvum*, *C. hominis* and *C. meleagridis*. Environ Microbiol 17**:** 984-993.

Widmer, G., and S. Sullivan, 2012 Genomics and population biology of *Cryptosporidium* species. Parasite Immunol 34**:** 61-71.

Widmer, G., L. Tchack, C. L. Chappell and S. Tzipori, 1998 Sequence polymorphism in the beta-tubulin gene reveals heterogeneous and variable population structures in *Cryptosporidium parvum*. Appl Environ Microbiol 64**:** 4477-4481.

Winter, G., A. A. Gooley, K. L. Williams and M. B. Slade, 2000 Characterization of a major sporozoite surface glycoprotein of *Cryptosporidium parvum*. Funct Integr Genomics 1**:** 207-217.

Wolfson, J. S., J. M. Richter, M. A. Waldron, D. J. Weber, D. M. McCarthy *et al.*, 1985 Cryptosporidiosis in immunocompetent patients. N Engl J Med 312**:** 1278-1282.

Wu, Z., I. Nagano, T. Boonmars, T. Nakada and Y. Takahashi, 2003 Intraspecies polymorphism of *Cryptosporidium parvum* revealed by PCR-restriction fragment length polymorphism (RFLP) and RFLP-single-strand conformational polymorphism analyses. Appl Environ Microbiol 69**:** 4720-4726.

Xiao, L., 2010 Molecular epidemiology of cryptosporidiosis: an update. Exp Parasitol 124**:** 80-89.

Xiao, L., C. Bern, J. Limor, I. Sulaiman, J. Roberts *et al.*, 2001a Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J Infect Dis 183**:** 492-497.

Xiao, L., R. Fayer, U. Ryan and S. J. Upton, 2004a *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbiol Rev 17**:** 72-97.

Xiao, L., R. Fayer, U. Ryan and S. J. Upton, 2007a Response to the newly proposed species *Cryptosporidium pestis*. Trends Parasitol 23**:** 41-42; author reply 42-43.

Xiao, L., and Y. Feng, 2008 Zoonotic cryptosporidiosis. FEMS Immunol Med Microbiol 52**:** 309-323.

Xiao, L., and R. P. Herd, 1993 Quantitation of *Giardia* cysts and *Cryptosporidium* oocysts in fecal samples by direct immunofluorescence assay. J Clin Microbiol 31**:** 2944-2946.

Xiao, L., M. C. Hlavsa, J. Yoder, C. Ewers, T. Dearen *et al.*, 2009 Subtype analysis of *Cryptosporidium* specimens from sporadic cases in Colorado, Idaho, New Mexico, and Iowa in 2007: widespread occurrence of one *Cryptosporidium hominis* subtype and case history of an infection with the *Cryptosporidium* horse genotype. J Clin Microbiol 47**:** 3017-3020.

Xiao, L., A. A. Lal and J. Jiang, 2004b Detection and differentiation of *Cryptosporidium* oocysts in water by PCR-RFLP. Methods Mol Biol 268**:** 163-176.

Xiao, L., J. R. Limor, L. Li, U. Morgan, R. C. Thompson *et al.*, 1999a Presence of heterogeneous copies of the small subunit rRNA gene in *Cryptosporidium parvum* human and marsupial genotypes and *Cryptosporidium felis*. J Eukaryot Microbiol 46**:** 44S-45S.

Xiao, L., U. M. Morgan, J. Limor, A. Escalante, M. Arrowood *et al.*, 1999b Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Appl Environ Microbiol 65**:** 3386-3391.

Xiao, L., U. M. Ryan, R. Fayer, D. D. Bowman and L. Zhang, 2012 *Cryptosporidium tyzzeri* and *Cryptosporidium pestis*: which name is valid? Exp Parasitol 130**:** 308-309.

Xiao, L., A. Singh, J. Limor, T. K. Graczyk, S. Gradus *et al.*, 2001b Molecular characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. Appl Environ Microbiol 67**:** 1097-1101.

Xiao, L., L. Zhou, M. Santin, W. Yang and R. Fayer, 2007b Distribution of *Cryptosporidium parvum* subtypes in calves in eastern United States. Parasitol Res 100**:** 701-706.

Xu, P., G. Widmer, Y. Wang, L. S. Ozaki, J. M. Alves *et al.*, 2004 The genome of *Cryptosporidium hominis*. Nature 431**:** 1107-1112.

Yang, R., C. Murphy, Y. Song, J. Ng-Hublin, A. Estcourt *et al.*, 2013 Specific and quantitative detection and identification of *Cryptosporidium hominis* and *C. parvum* in clinical and environmental samples. Exp Parasitol 135**:** 142-147.

Yang, R., A. Paparini, P. Monis and U. Ryan, 2014 Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples. Int J Parasitol 44**:** 1105-1113.

Yoder, J. S., R. M. Wallace, S. A. Collier, M. J. Beach, M. C. Hlavsa *et al.*, 2012 Cryptosporidiosis surveillance--United States, 2009-2010. MMWR Surveill Summ 61**:** 1-12.

Zardi, E. M., A. Picardi and A. Afeltra, 2005 Treatment of cryptosporidiosis in immunocompromised hosts. Chemotherapy 51**:** 193-196.

Zhao, W., R. Wang, W. Zhang, A. Liu, J. Cao *et al.*, 2014 MLST subtypes and population genetic structure of *Cryptosporidium andersoni* from dairy cattle and beef cattle in northeastern China's Heilongjiang Province. PLoS One 9**:** e102006.

Zhou, L., H. Kassa, M. L. Tischler and L. Xiao, 2004 Host-adapted *Cryptosporidium* spp. in Canada geese (*Branta canadensis*). Appl Environ Microbiol 70**:** 4211-4215.

Zhou, L., A. Singh, J. Jiang and L. Xiao, 2003a Molecular surveillance of *Cryptosporidium* spp. in raw wastewater in Milwaukee: implications for understanding outbreak occurrence and transmission dynamics. J Clin Microbiol 41**:** 5254-5257.

Zhou, L., C. Yang and L. Xiao, 2003b PCR-Mediated Recombination between *Cryptosporidium* spp. of Lizards and Snakes. Journal of Eukaryotic Microbiology 50**:** 563-565.

Zhu, G., J. S. Keithly and H. Philippe, 2000a What is the phylogenetic position of *Cryptosporidium*? Int J Syst Evol Microbiol 50 Pt 4**:** 1673-1681.

Zhu, G., M. J. Marchewka and J. S. Keithly, 1999 *Cryptosporidium parvum* possesses a short-type replication protein A large subunit that differs from its host. FEMS Microbiol Lett 176**:** 367-372.

Zhu, G., M. J. Marchewka and J. S. Keithly, 2000b *Cryptosporidium parvum* appears to lack a plastid genome. Microbiology 146 (Pt 2)**:** 315-321.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA


USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA