Molecular characterization of *Cryptosporidium parvum* isolates from human cryptosporidiosis cases in Scotland

A. P. DESHPANDE[1], B. L. JONES[2], L. CONNELLY[2], K. G. POLLOCK[3], S. BROWNLIE[3] *and* C. L. ALEXANDER[2]*

[1] *Clinical Microbiology, Greater Glasgow and Clyde NHS, Glasgow, UK*
[2] *Scottish Microbiology Reference Laboratories, Greater Glasgow and Clyde NHS, Level 5, New Lister Building, Glasgow Royal Infirmary, Alexandra Parade, Glasgow G31 2ER, UK*
[3] *Health Protection Scotland, NHS National Services Scotland, Glasgow, UK*

SUMMARY

Cryptosporidium parvum (*C. parvum*) is one of the most prevalent protozoan pathogens responsible for inducing human and animal disease worldwide. In this study, the glycoprotein-60 (gp60) subtyping tool was employed to assess the molecular diversity of *C. parvum* from human feces throughout Scotland during potential outbreaks. Over a 24-month period, microscopy analysis revealed 1139 positive feces containing *Cryptosporidium* species with 256 identified by molecular methods specifically as *C. parvum*. *Cryptosporidium parvum* was shown to be more prevalent in rural areas of Scotland and subtyping of 87 isolates demonstrated the predominant family as IIa, which occurred in 94% (*n* = 82) of isolates. The IIaA15G1R1 subtype was most common, being isolated from 47% (*n* = 41) of Scottish human cases. Non-IIa strains constituted a total of 5 isolates and included subtypes from the IIc, IId and IIg families. This information contributes significantly to existing knowledge and understanding of *C. parvum* subtypes in Scotland which is vital in assisting with the management of future local and national outbreaks.

Key words: *Cryptosporidium parvum*, molecular, diversity, subtypes, Scotland, gp60.

INTRODUCTION

Cryptosporidiosis, the most common parasitic gastrointestinal disease in the Scottish population, is caused mainly by 2 protozoan pathogens, *Cryptosporidium parvum* (*C. parvum*) and *Cryptosporidium hominis* (*C. hominis*) which account for over 90% of human cryptosporidiosis worldwide (Putignani *et al*. 2010). *Cryptosporidium* species are globally distributed, having a wide host range and transmission of infective oocysts can be either zoonotic or anthroponotic. Various transmission routes of infective fecal material are possible, including direct contact or contact via contaminated food and water sources (Xiao *et al*. 2008). Cryptosporidiosis is a self-limiting infection, but can be life-threatening in immunocompromised patients who may suffer from severe, chronic infection resulting in significant losses of fluid (Chalmers and Davies, 2010; Bouzid *et al*. 2013).

Microscopy is widely used by diagnostic laboratories to establish the presence or absence of *Cryptosporidium* oocysts. However, it does not permit speciation as the morphology of oocysts from different *Cryptosporidium* species, including those which are not clinically significant, can be similar. The advent of molecular diagnostic tools permits further analysis to determine specific *Cryptosporidium* species and subtypes which is crucial for the management of outbreaks. A variety of molecular methods exist which employ 16s rRNA-based tools, 18S rRNA genotyping, classification according to the outer wall protein gene (COWP), the heat-shock protein 70 gene (HSP 70), the 60 kDa glycoprotein gene (GP60) and various polymorphic loci markers (Feng *et al*. 2013). The GP60 gene is currently the most widely used genetic marker containing a hypervariable microsatellite region for investigating *Cryptosporidium* species whilst also being one of the dominant targets for neutralizing antibody responses in humans (Strong *et al*. 2000).

The GP60 subtyping tool has shown *C. hominis* to be highly conserved (Chalmers *et al*. 2008), whilst *C. parvum* is genetically heterogeneous (Xiao, 2010) resulting in a range of differing subtypes. In Scotland, insufficient information exists on the prevalence, significance and regional molecular epidemiology of *Cryptosporidium* isolates that cause human disease. This information is essential to facilitate appropriate investigations and to manage both regional and national *Cryptosporidium* outbreaks impacting on human health. In this report, we describe the prevalence and molecular diversity

* Corresponding author: Scottish Microbiology Reference Laboratories, Glasgow Scottish Parasite Diagnostic and Reference Section, Level 5, New Lister Building, Glasgow Royal Infirmary, Alexandra Parade, Glasgow G31 2ER. E-mail: Claire.Alexander@ggc.scot.nhs.uk

of *C. parvum* subtypes from human isolates throughout Scotland. The GP60 subtyping tool was employed during a 2-year period to investigate *C. parvum* subtypes as part of the remit of the Scottish Parasite Diagnostic and Reference Laboratory (SPDRL) *Cryptosporidium* Outbreak Service in conjunction with Health Protection Scotland.

MATERIALS AND METHODS

Microscopy detection of Cryptosporidium *oocysts*

Fecal samples from patients throughout Scotland with gastrointestinal illness were submitted to local diagnostic microbiology laboratories from General Practitioners and Hospital clinicians. Feces were examined for the presence of *Cryptosporidium* oocysts by staining with either auramine phenol (AP) or modified Ziehl–Neelsen (ZN) based on the UK Standards for Microbiology Investigations (SIM Staining Procedures TP 39). Briefly air dried smears were fixed in methanol before the addition of either AP or ZN. Excess stain was removed with water before decolourizing slides using 3% acid methanol (for AP staining) or 1% acid methanol (for ZN staining). Potassium permanganate (0·1%) or malachite green (0·4%) were used as counterstains. Slides were washed, air-dried and examined for *Cryptosporidium* oocysts at ×40 objective either using a light microscope to detect ZN staining, or using a fluorescence microscope with FITC filters (690/510 nM wavelength) for AP staining.

Oocyst concentration and DNA extraction

Feces reported by diagnostic microbiology laboratories to be microscopy positive for *Cryptosporidium* oocysts, and which were deemed to be associated with potential outbreaks, were forwarded to the SPDRL for speciation and subtyping. The definitions used by Health Protection Scotland to define an outbreak are an incident in which 2 or more linked cases experience the same illness or when the observed number of cases unaccountably exceeds the expected number. On arrival at SPDRL, oocysts were concentrated by adding approximately 500 mg of feces to 7 mL of water and 3 mL of diethyl ether. The mixture was inverted for 30 s before being centrifuged at 750 $\boldsymbol{g}$ for 60 s. The sediment was transferred to a sterile tube, and 4 μL of internal control DNA (PrimerDesign, Southampton UK) added before DNA was extracted using the QIAamp DNA stool mini kit (QIAGEN, Germany).

Cryptosporidium *speciation*

To permit speciation, DNA was amplified using 2 real-time PCR assays to determine if samples were either *C. parvum* or *C. hominis* (Hadfield *et al.* 2011).

One assay detected *C. hominis* and an internal control, whilst the second assay detected *C. parvum*. The *C. hominis* assay consisted of primers CRULib13F and CRULib13RCh (CRULib13F final concentration 900 nM; CRULib13RCh final concentration 300 nM; Eurofins MWG Operon, Germany), CRULib13TMCh probe (100 nM; Eurofins MWG Operon, Germany) containing 2 μL sample DNA, 1·6 μL internal control primers/probe mix (PrimerDesign, Southampton UK) and 12·5 μL LightCycler 480 probes master mix (Roche Diagnostics, UK) in a total volume of 25 μL. The second assay to detect *C. parvum* consisted of primers CRULib13F and CRULib13RCp at a final concentration 900 nM, whilst the probe, CRULib13TMCp was at a final concentration of 150 nM. Amplification was performed on a Roche LC480 (Roche Diagnostics, UK) using cycling conditions as described (Hadfield *et al.* 2011). Negative controls (water or extraction buffer) and positive controls (DNA from *C. parvum* oocysts, IOWA isolate, Idaho, USA and previous positive clinical samples) were included in each experiment. For those isolates that were shown to be negative by the real-time PCR assays, but microscopy positive for *Cryptosporidium* oocysts, DNA amplification was performed using a previously published nested PCR assay targeting the 18S rRNA gene at Locus 1 using a modified forward primer, WR494F and reverse primer, XR1 (Nichols *et al.* 2010). The secondary PCR amplicon of approximately 435 bp was subjected to sequencing using an ABI 3500XL sequencer with Big-Dye version 3.1 chemistry.

Cryptosporidium *subtyping at the GP60 locus*

Samples identified as *C. parvum* by the real-time PCR assays were subtyped using a nested PCR approach targeting the GP60 gene (Glaberman *et al.* 2002). To account for variations in DNA concentration and the presence of potential PCR inhibitors, 2 volumes (2 and 3 μL) of DNA template were used for the first PCR amplification (PCR1) in a total volume of 50 μL containing 200 μM dNTPs (Invitrogen Ltd, UK), 400 μg mL^{-1} BSA (Sigma-Aldrich, UK), 2% Tween-20 (VWR, UK), 1% PVP pH8 (Sigma-Aldrich, UK), 6 mM MgCl$_2$ (Fisher Scientific, Loughborough, UK), 200 nM of each oligonucleotide GF1 and GR1 (Eurofins MWG Operon, Germany), 5 Units Taq polymerase (Fisher Scientific, Loughborough, UK) and 5 μL Reaction Buffer IV (Fisher Scientific, Loughborough, UK). Amplifications were performed on a 9700 GeneAmp platform, (Applied Biosystems, UK) using cycling conditions as previously described (Glaberman *et al.* 2002).

Both 2 and 3 μL were used for the second PCR amplification (PCR2) which was performed using the same conditions as PCR1 with the exception of
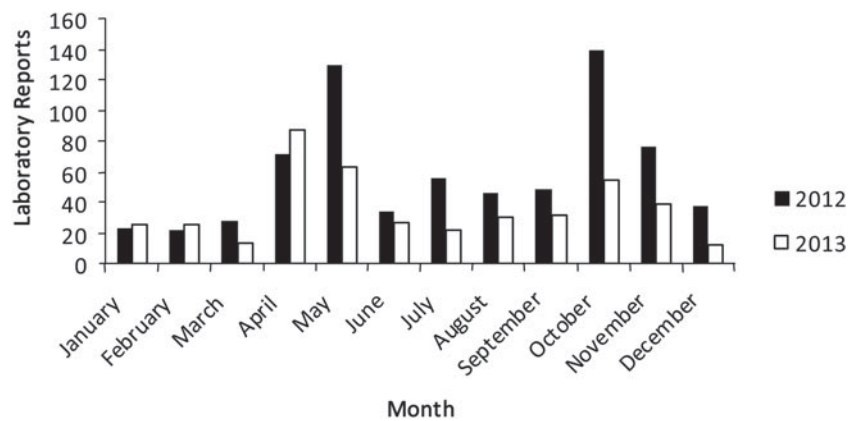
Fig. 1. Number of *Cryptosporidium* species reported in Scotland during 2012 and 2013.

oligonucleotides, GF2 and GR2 and the exclusion of PVP. Negative and positive controls as stated for the real-time PCR assays were included in every experiment. An amplicon of approximately 900 base pairs was visualized by running $10\,\mu$L of PCR2 product on a 1·4% agarose gel. Subtyping of PCR2 amplicons was performed by bi-directional sequencing using the oligonucleotides from PCR2. Bi-directional sequencing was performed on-site using an ABI 3500XL sequencer with Big-Dye version 3.1. Searching for similar sequences on the GenBank database was performed using the NCBI BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Subtypes were confirmed by manually reading through the sequences to search for predominantly trinucleotide repeats and other repeat sequences (Xiao, 2010).

RESULTS

Prevalence and seasonality of Cryptosporidium *species*

During a 2-year period, 2012 and 2013, a total of 1139 samples in Scotland were reported by diagnostic microbiology laboratories to be microscopy positive for *Cryptosporidium* oocysts. The seasonal distribution of all *Cryptosporidium* species is depicted for each year (Fig. 1). Two distinct peaks occurred; 1 during April–May ($n = 201$; i.e. 28% in 2012; $n = 150$, 35% in 2013) and the other during October–November ($n = 215$; 30% of *Cryptosporidium* species isolates in 2012; $n = 93$, 22% in 2013).

Cryptosporidium *genotypes and* C. parvum *subtypes*

Of the 1139 isolates, 445 were identified to the species level in response to potential outbreak investigations; *C. parvum* ($n = 256$); *C. hominis* ($n = 187$); *C. felis* ($n = 1$) and *C. meleagridis* ($n = 1$). Over the 2-year period, the majority of *C. parvum* isolates were sent from Grampian, Fife and Ayrshire/Arran ($n = 67$, 45 and 41, respectively), whereas the majority of *C. hominis* isolates were from Grampian, Lothian and Glasgow ($n = 53$, 26 and 26, respectively) (Fig. 2). Of the 256 *C. parvum* isolates, 158 were identified in 2012 compared to 98 in 2013. Only 2 were stated to be imported and the sex ratio was similar with 54% ($n = 140$) isolated from females and 46% ($n = 116$) from males. The median age of infected individuals was 20 years (2012 = 19 years, 2013 = 21 years). *Cryptosporidium parvum* demonstrated marked seasonality with numbers peaking during April–May ($n = 80$, in 2012; $n = 66$, in 2013) (Fig. 3).

Of the 256 *C. parvum* isolates, a total of 87 were subjected to further analysis for GP60 subtyping to assist with 5 potential outbreak investigations. Putative associations included animal contact at a visitor farm (also referred to as a petting farm), farms and consumption of contaminated food. These associations were based on limited patient information provided to local health protection teams and outbreak investigation teams, rather than being based on laboratory evidence, largely due to the challenges in obtaining samples from environmental sources. The most prevalent subtype in the Scottish population was IIaA15G2R1 which comprised of 47% ($n = 41$) of those samples subtyped (26 females: 15 males, age range 1–50) (Table 1). The second and third most common were IIaA17G1R1 ($n = 14$, 16%) and IIaA19G1R1 ($n = 11$, 9%), respectively (Table 1). Further 16 isolates were identified as belonging to the IIa family, whilst unusual non-IIa strains included; IIcA5G3 ($n = 2$), IIdA19G1 ($n = 1$), IIdA16G1 ($n = 1$) and IIgA9R1 ($n = 1$) (Table 1). Age ranges and sex ratios were similar between the different subtypes, with ages ranged from 1 to 50 years with a greater number of females than males affected in 4 of the 5 outbreaks.

The IIaA15G2R1 subtype was the most common in Fife, Grampian and Tayside ($n = 18$, 8 and 5, respectively) (Table 2), whereas one of the largest health boards in Scotland, namely Lothian was predominantly non-IIaA15G2R1 subtypes ($n = 13$), as was Ayrshire and Arran ($n = 12$). All other Health Boards had similar numbers of isolates of IIaA15G2R1 *vs* non-IIaA15G2R1 subtypes.
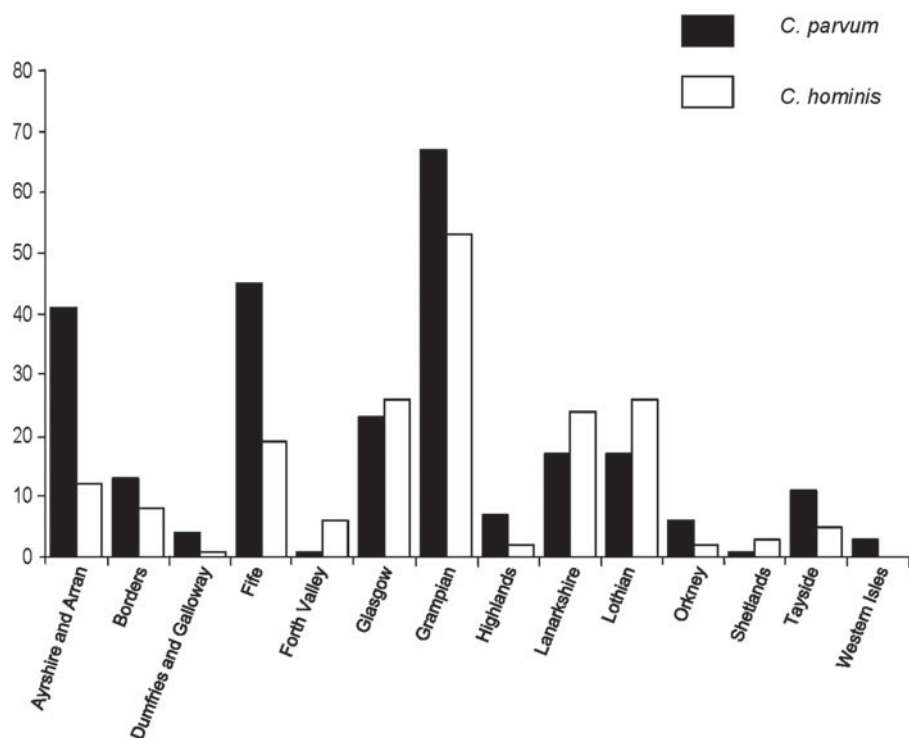
Fig. 2. Distribution of the total number of *Cryptosporidium* species per Scottish Health Board during 2012 and 2013.
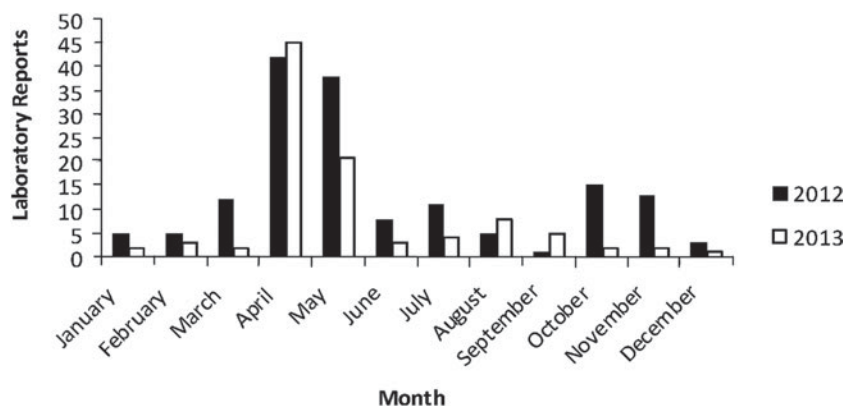

Fig. 3. Seasonal distribution of *C. parvum* in humans within Scotland during 2012 and 2013.

DISCUSSION

This is the first report to describe the molecular epidemiology of *C. parvum* subtypes which infect humans in Scotland. In addition, it highlights cryptosporidiosis as being the most frequently reported intestinal parasitic disease with more than 3 times the number of cases than the second most frequently reported intestinal parasite, *Giardia* species ($n = 191$, in 2012; $n = 167$, in 2013) (communications with Health Protection Scotland).

Cryptosporidiosis cases show seasonal fluctuations particularly during 2012 with sharp increases from April to June then later in October and November. During May 2012, the large rise in the number of *C. parvum* cases across the UK prompted enhanced surveillance and a case controlled study which identified contaminated 'ready to eat' pre-cut mixed salad leaves being implicated as the most likely cause of illness (manuscript submitted). *Cryptosporidium parvum* outbreaks have previously been associated with food sources, including salad (Pönka *et al.* 2009), contaminated fruit/juice and beef (Yoshida *et al.* 2007; Vojdani *et al.* 2008). Springtime peaks of *C. parvum* have been noted in various geographical regions, including New Zealand, Netherlands, Ireland, China and England (McLauchlin *et al.* 2000; Learmonth *et al.* 2004; Wielinga *et al.* 2008; Zintl *et al.* 2009; Wang *et al.* 2011). The springtime peak usually corresponds to an increase of infectious fecal material during the calving/lambing season. Isolates from human cases within and out-with the

Table 1. Summary of Scottish *C. parvum* outbreaks during 2012 and 2013

Health board	Potential source	Total number of cases subtyped	M:F	Predominant subtype	Other subtypes
May 2012 Lothian	Animal contact at a visitor farm	11	1M:10F	IIaA17G1R1 (*n* = 8)	IIaA15G1R1 (*n* = 2) IIaA15G2R1 (*n* = 1)
May/June 2012 Lamarkshire, Highlands, Fife, Grampian, Glasgow	Food (salad)	17	7M:10F	IIaA15G2R1 (*n* = 12)	IIdA16G1 (*n* = 1) IIgA9R1 (*n* = 1) IIaA17G2R1 (*n* = 1) IIaA17G1R1 (*n* = 1) IIaA18G2R1 (*n* = 1)
August 2012 Tayside Borders	Unknown	5	2M:3F	IIaA19G1R1 (*n* = 5)	
October 2012 Lothian, Ayrshire and Arran, Highlands and Islands, Glasgow	Unknown	10	6M:4F	IIaA15G2R1 (*n* = 3)	IIcA5G3 (*n* = 2) IIaA17G1R1 (*n* = 1) IIaA17R1 (*n* = 1) IIaA14G2R1 (*n* = 2) IIdA19G1 (*n* = 1)
April–May 2013 Fife, Ayrshire and Arran, Grampian, Tayside, Lothian, Highlands and Islands	Animal contact (on 2 different farms)	44	13M:31F	IIaA15G2R1 (*n* = 25)	IIaA19G1R1 (*n* = 6) IIaA17G1R1 (*n* = 4) IIaA18G1R1 (*n* = 3) Other IIa subtypes (*n* = 6)

UK have the same GP60 subtypes as animal sources supporting the hypothesis that ruminants are the sources of infection (Trotz *et al.* 2006; Thompson *et al.* 2007; Ng *et al.* 2008; Wielinga *et al.* 2008; Zintl *et al.* 2009; Smith *et al.* 2010). A further sharp rise in cases occurred during late summer of 2012, but in this instance, *C. hominis* rather than *C. parvum* was implicated. A similar rise in *C. hominis* cases was also noted in the Netherlands and Germany with no common source identified (Fournet *et al.* 2013).

Previous studies demonstrate the differences in prevalence rates between *C. hominis* and *C. parvum* depending on the geographical location where, in the Netherlands, a greater percentage of *C. hominis* has been described (Wielinga *et al.* 2008). In contrast, our study demonstrates that, since 2012, there were almost twice as many cases of *C. parvum* than *C. hominis*, a finding similar to previous reports from Ireland and the USA (Feltus *et al.* 2006; Zintl *et al.* 2009). Only around one-third of the isolates in Scotland are fully speciated in response to potential outbreak investigations; therefore to gain accurate data pertaining to the numbers of specific *Cryptosporidium* species in both sporadic and outbreak cases would require speciation of all isolates. However, the data achieved during 2012 and 2013 highlight a possible change in the trend within Scotland where previously, the total numbers of *C. parvum* and *C. hominis* isolates from sporadic and outbreak cases were similar (Chalmers and Pollock, 2012).

It is interesting to note the comparatively large number of *C. parvum* isolates from health boards comprising predominantly rural settings, including Grampian, Fife and Ayrshire/Arran compared with Glasgow and Lothian, which are the 2 largest urban settings in Scotland. A previous study highlighted that *C. parvum* was more prevalent in rural areas with lower population density and in those with a higher density of livestock reflecting the zoonotic nature of the infection (Pollock *et al.* 2010). Within the rural areas of Scotland, there are over 11 000 private water supplies compared to less than 10 in urban areas. Areas with a greater number of private water supplies to human inhabitants have reported a greater number of *C. parvum* cases (Pollock *et al.* 2010). Similarly, in New Zealand and Ireland, an increased prevalence of *C. parvum* in rural, lowly populated areas has been described (Learmonth *et al.* 2004; Zintl *et al.* 2009).

In this study, 3 of the Scottish outbreaks were suspected to be linked to contact with animals on farms based on the limited data available to the local Health Protection Team and Outbreak Control teams. There is strong evidence to highlight that, for *C. parvum*, contact with farm animals or their feces either through visiting or residing on a farm is associated with illness (Hunter *et al.* 2004, 2007). Worldwide evidence for potential zoonotic

Table 2. *Distribution of* C. parvum *subtypes from human isolates across Scotland*

Health board	IIaA15G2R1 subtype	Non-IIaA15G2R1 subtype	Total
Tayside	5	4	9 (10%)
Greater Glasgow and Clyde	1	2	3 (4%)
Lothian	4	13	17 (20%)
Lanarkshire	2	2	4 (5%)
Fife	17	4	21 (24%)
Ayrshire and Arran	3	12	15 (17%)
Grampian	8	8	16 (18%)
Highlands and Islands	1	1	2 (2%)
		Total	87

transmission between cattle and humans on farms also exists (Feltus *et al*. 2006; Khan *et al*. 2010).

The geographical distribution and prevalence of common *Cryptosporidium* subtypes vary and predominant subtypes identified in our analysis belonged to the IIa family which is the only one to be identified in cattle in Scotland. We report that IIaA15G2R1 is highly prevalent in the Scottish population, accounting for almost half of those isolates subtyped and this subtype is also commonly found in cattle within Scotland (personal communications, Hotchkiss, E., Moredun Institute, Midlothian); therefore it is likely that zoonotic transmission occurs. This same subtype is common in cattle in the Netherlands, Spain and the USA, (Xiao *et al*. 2007; Quilez *et al*. 2008*a*; Wielinga *et al*. 2008) and also in humans (Wielinga *et al*. 2008). It is also prevalent throughout farms in England with evidence for local transmission between farms (Brook *et al*. 2009). Chalmers *et al*. (2011) demonstrated that in England, IIaA15G2R1 and IIaA17G1R1 subtypes predominated with subtype IIaA17G1R1 being associated with human cases who had visited farms or had previous animal contact prior to the onset of any illness. IIaA17G1R1 was also commonly identified in our study but without enhanced surveillance, it was not possible to determine the degree of animal contact for each case to clearly establish an association. In contrast, in Ireland, the most prevalent subtype in human and cattle cases of cryptosporidiosis is IIaA18G3R1 (Thompson *et al*. 2007; Zintl *et al*. 2009, 2011). This subtype is also one of the most commonly identified in human cases in Australia (Ng *et al*. 2010) and has been identified in foals (Grinberg *et al*. 2008). Interestingly, no Scottish cases were identified with this subtype.

In addition to the IIa family, a small number of non-IIa strains were also identified, namely IIc, IId and IIg. The IIc subtype appears to be uncommon in Scottish human cases and has not been identified in cattle in this region providing supporting evidence

for anthroponotic transmission. In contrast, the IIc family is found relatively frequently in other regions including Portugal and Slovenia (Alves *et al*. 2003; Soba and Logar, 2008). Although in low numbers, cases of the IId family have been reported to cause human cryptosporidiosis in Portugal, the Netherlands, Ireland, Jordan, Japan, Spain, Kuwait and Australia (Alves *et al*. 2003; Sulaiman *et al*. 2005; Abe *et al*. 2006; Quilez *et al*. 2008*b*; Wielinga *et al*. 2008; Zintl *et al*. 2009; Hijjawi *et al*. 2010). Human subtypes, namely IIdA17G1 and IIdA18G1 have been isolated in a small number of cases in England (Chalmers *et al*. 2011), which differ from the 2 IId subtypes in this report. The IId family has been isolated from lambs, goats and cattle in Spain, China and Malaysia suggesting these animals as potential sources (Quilez *et al*. 2008*a*, *b*; Muhid *et al*. 2011; Wang *et al*. 2011). A third non-IIa strain, namely IIg identified in this study was first reported in Uganda (Akiyoshi *et al*. 2006) and has rarely been reported elsewhere. As neither the IIg nor the IId family have been isolated in Scottish animals, it is likely that the cases in this report have been acquired abroad however the travel history in each case was not stated.

Establishing enhanced surveillance of *Cryptosporidium* cases in Scotland and typing of both sporadic and outbreak isolates would provide valuable information to increase our understanding of potential transmission routes, clinical symptoms and the biological impact of specific subtypes. In addition, future studies are required to validate methodologies for typing of human *Cryptosporidium* isolates to ensure robust, standardized procedures are in place to assist with the management of future local and national outbreaks.

REFERENCES

Abe, N., Matsubayashi, M., Kimata, I. and Iseki, M. (2006). Subgenotype analysis of *Cryptosporidium parvum* isolates from humans and animals in Japan using the 60-kDa glycoprotein gene sequences. *Parasitology Research* **99**, 303–305.

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

Alves, M., Xiao, L., Sulaiman, I., Lal, A. A., Matos, O. and Antunes, F. (2003). Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle and zoo ruminants in Portugal. *Journal of Clinical Microbiology* **41**, 2744–2747.

Bouzid, M., Hunter, P. R., Chalmers, R. M. and Tyler, K. M. (2013). *Cryptosporidium* pathogenicity and virulence. *Clinical Microbiology Review* **26**, 115–134.

Brook, E. J., Anthony Hart, C., French, N. P. and Christley, R. M. (2009). Molecular epidemiology of *Cryptosporidium* subtypes in cattle in England. *Veterinary Journal* **179**, 378–382.

Chalmers, R. M. and Davies, A. P. (2010). Minireview: clinical cryptosporidiosis. *Experimental Parasitology* **124**, 138–146.

Chalmers, R. M. and Pollock, K. G. J. (2012). *Cryptosporidium* in Scotland 2010: reference laboratory data. *Health Protection Scotland Weekly Report* **46**, 33–35.

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.

Chalmers, R. M., Smith, R. P., Hadfield, S. J., Elwin, K. and Giles, M. (2011). Zoonotic linkage and variation in *Cryptosopridium parvum* from patients in the United Kingdom. *Parasitology Research* **108**, 1321–1325.

Feltus, D. C., Giddings, C. W., Schneck, B. L., Monson, T., Warshauer, D. and McEvoy, J. M. (2006). Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin. *Journal of Clinical Microbiology* **44**, 4303–4308.

Feng, Y., Torres, E., Li, N., Wanga, L., Bowman, D., and Xiao, L. (2013). Population genetic characterisation of dominant *Cryptosporidium parvum* subtype IIaA15G2R1. *International Journal for Parasitology* **43**, 1141–1147.

Fournet, N., Deege, M. P., Urbanus, A. T., Nichols, G., Rosner, B. M., Chalmers, R. M., Gorton, R., Pollock, K. G., van der Giessen, J. W., Wever, P. C., Dorigo-Zetsma, J. W., Mulder, B., Mank, T. G., Overdevest, I., Kusters, J. G., van Pelt, W. and Kortbeek, L. M. (2013). Simultaneous increase of *Cryptosporidium* infections in the Netherlands, the United Kingdom and Germany in late summer season, 2012. *Eurosurveillance* **18**, 1–5. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20348

Glaberman, S., Moore, J. E., Lowery, C. J., Chalmers, R. M., Sulaiman, I., Elwin, K., Rooney, P. J., Millar, B. C., Dooley, J. S., Lal, A. A. and Xiao, L. (2002). Three drinking-water-associated cryptosporidiosis outbreaks, Northern Ireland. *Emerging Infectious Diseases* **8**, 631–633.

Grinberg, A., Learmonth, J., Kwan, E., Pomroy, W., Lopez Villalobos, N., Gibson, I. and Widmer, G. (2008). Genetic diversity and zoonotic potential of *Cryptosporidium parvum* causing foal diarrhea. *Journal of Clinical Microbiology* **46**, 2396–2398.

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.

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.

Hunter, P. R., Hughes, S., Woodhouse, S., Syed, Q., Verlander, N. Q., Chalmers, R. M., Morgan, K., Nichols, G., Beeching, N. and Osborn, K. (2004). Sporadic cryptosporidiosis case-control study with genotyping. *Emerging Infectious Diseases* **10**, 1241–1249.

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.

Khan, S. M., Debnath, C., Pramanik, A. K., Xiao, L., Nozaki, T. and Ganguly, S. (2010). Molecular characterization and assessment of zoonotic transmission of *Cryptosporidium* from dairy cattle in West Bengal, India. *Veterinary Parasitology* **15**, 41–47.

Learmonth, J. J., Ionas, G., Ebbett, K. A. and Kwan, E. S. (2004). Genetic characterization and transmission cycles of *Cryptosporidium* species isolated from humans in New Zealand. *Applied and Environmental Microbiology* **70**, 3973–3978.

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.

Muhid, A., Robertson, I., Ng, J. and Ryan, U. (2011). Prevalence of and management factors contributing to *Cryptosporidium* sp infection in pre-weaned and post weaned calves in Johor, Malaysia. *Experimental Parasitology* **127**, 534–538.

Ng, J., Eastwood, K., Durrheim, D., Massey, P., Walker, B., Armson, A. and Ryan, U. (2008). Evidence supporting zoonotic transmission of *Cryptosporidium* in rural New South Wales. *Experimental Parasitology* **119**, 192–195.

Ng, J., MacKenzie, B. and Ryan, U. (2010). Longitudinal multilocus molecular characterisation of sporadic Australian human clinical cases of cryptosporidiosis from 2005–2008. *Experimental Parasitology* **125**, 348–356.

Nichols, R. A. B., Connelly, L., Sullivan, C. B. and Smith, H. V. (2010). Identification of *Cryptosporidium* species and genotypes in Scottish raw and drinking waters during a one-year monitoring period. *Applied Environmental Microbiology* **76**, 5977–5986.

Pollock, K. G., Ternent, H. E., Mellor, D. J., Chalmers, R. M., Smith, H. V., Ramsay, C. N. and Innocent, G. T. (2010). Spatial and temporal epidemiology of sporadic human cryptosporidiosis in Scotland. *Zoonoses and Public Health* **57**, 487–492.

Pönka, A., Kotilainen, H., Rimhanen-Finne, R., Hokkanen, P., Hänninen, M. L., Kaarna, A., Meri, T. and Kuusi, M. (2009). A foodborne outbreak due to *Cryptosporidium parvum* in Helsinki. *Eurosurveillance* **16**, 14.

Putignani, L. and Menichella, D. (2010). Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. *Interdisciplinary Perspectives on Infectious Diseases* **2010**, 1–39.

Quilez, J., Torres, E., Chalmers, R. M., Robinson, G., Del Cacho, E. and Sanchez-Acedo, C. (2008a). *Cryptosporidium* species and subtype analysis from dairy calves in Spain. *Parasitology* **135**, 1613–1620.

Quilez, J., Torres, E., Chalmers, R. M., Hadfield, S. J., Del Cacho, E. and Sanchez-Acedo, C. (2008b). *Cryptosporidium* genotypes and subtypes in lambs and goat kids in Spain. *Applied and Environmental Microbiology* **74**, 6026–6031.

Smith, R. P., Chalmers, R. M., Mueller-Doblies, D., Clifton-Hadley, F. A., Elwin, K., Watkins, J., Paiba, G. A., Hadfield, S. J. and Giles, M. (2010). Investigation of farms linked to human patients with cryptosporidiosis in England and Wales. *Preventative Veterinary Medicine* **94**, 9–17.

Soba, B. and Logar, J. (2008). Genetic classification of *Cryptosporidium* isolates from humans and calves in Slovenia. *Parasitology* **135**, 1263–1270.

Standards for Microbiology Investigations; Staining Procedures TP 39. http://www.hpa.org.uk/ProductsServices/MicrobiologyPathology/UKStandardsForMicrobiologyInvestigations/TermsOfUseForSMIs/AccessToUKSMIs/SMITestProcedures/

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.

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.

Thompson, H. P., Dooley, J. S., Kenny, J., McCoy, M., Lowery, C. J., Moore, J. E. and Xiao, L. (2007). Genotypes and subtypes of *Cryptosporidium* species in neonatal calves in Northern Ireland. *Parasitology Research* **100**, 619–624.

Trotz-Williams, L. A., Martin, D. S., Gatei, W., Cama, V., Peregrine, A. S., Martin, S. W., Nydam, D. V., Jamieson, F. and Xiao, L. (2006). Genotype and subtype analyses of *Cryptosporidium* isolates from dairy calves and humans in Ontario. *Parasitology Research* **99**, 346–352.

Vojdani, J. D., Beuchat, L. R. and Tauxe, R. V. (2008). Juice-associated outbreaks of human illness in the United States 1995–2005. *Journal of Food Protection* **71**, 356–64.

Wang, R., Wang, H., Sun, Y., Zhang, L., Jian, F., Qi, M., Ning, C. and Xiao, L. (2011). Characteristics of *Cryptosporidium* transmission in preweaned dairy cattle in Henan, China. *Journal of Clinical Microbiology* **49**, 1077–1082.

Wielinga, P. R., de Vries, A., van der Goot, T. H., Mank, T., Mars, M. H., Kortbeek, L. M. and van der Giessen, J. W. (2008). Molecular epidemiology of *Cryptosporidium* in humans and cattle in The Netherlands. *International Journal of Parasitology* **38**, 809–817.

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

Xiao, L. and Feng, Y. (2008). Zoonotic cryptosporidiosis. *FEMS Immunology and Medical Microbiology* **52**, 309–323.

Xiao, L., Zhou, L., Santin, M., Yang, W. and Fayer, R. (2007). Distribution of *Cryptosporidium parvum* subtypes in calves in eastern United States. *Parasitology Research* **100**, 701–706.

Yoshida, H., Matsuo, M., Miyoshi, T., Uchino, K., Nakaguchi, H., Fukumoto, T., Teranaka, Y. and Tanaka, T. (2007). An outbreak of cryptosporidiosis suspected to be related to contaminated food October 2006, Sakai City Japan. *Japanese Journal of Infectious Diseases* **60**, 405–407.

Zintl, A., Proctor, A. F., Read, C., Dewaal, T., Shanaghy, N., Fanning, S. and Mulcahy, G. (2009). The prevalence of *Cryptosporidium* species and subtypes in human faecal samples in Ireland. *Epidemiology and Infection* **137**, 270–277.

Zintl, A., Ezzaty-Mirashemi, M., Chalmers, R. M., Elwin, K., Mulcahy, G., Lucy, F. E. and De Waal, T. (2011). Longitudinal and spatial distribution of GP60 subtypes in human cryptosporidiosis cases in Ireland. *Epidemiology and Infection* **139**, 1945–1955.