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Cryptosporidium parvum genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets

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ABSTRACT

To date, there has been no study to establish the genotypic or subgenotypic identities of *Cryptosporidium* and *Giardia* in edible shellfish. Here, we explored the genetic composition of these protists in *Mytilus galloprovincialis* (Mediterranean mussel) purchased from three markets in the city of Foggia, Italy, from May to December 2012. Samples from the digestive glands, gills and haemolymph were tested by nested PCR, targeting DNA regions within the 60 kDa glycoprotein (*gp60*) gene of *Cryptosporidium*, and the triose-phosphate isomerase (*tpi*) and *β-giardin* genes of *Giardia*. In total, *Cryptosporidium* and *Giardia* were detected in 66.7% of mussels (*M. galloprovincialis*) tested. *Cryptosporidium* was detected mostly between May and September 2012. Sequencing of amplicons showed that 60% of mussels contained *Cryptosporidium parvum* genotype IIa (including subgenotypes A15G2R1, IIaA15G2 and IIaA14G3R1), 23.3% *Giardia duodenalis* assemblage A, and 6.6% had both genetic types. This is the first report of these types in fresh, edible shellfish, particularly the very commonly consumed *M. galloprovincialis* from highly frequented fish markets. These genetic types of *Cryptosporidium* and *Giardia* are known to infect humans and thus likely to represent a significant public health risk. The poor observance of hygiene rules by vendors, coupled to the large numbers of *M. galloprovincialis* sold and the eating habits of consumers in Italy, call for more effective sanitary measures pertaining to the selling of fresh shellfish in street markets.

1. Introduction

Seafood is a major part of the culinary culture in many countries around the world, and the shellfish industry is of major economic importance in the Mediterranean area with the highest production along the coasts of Italy, Spain and France.

In Italy, current estimates indicate that 203,810 and 131,000 tonnes of fish and shellfish, respectively, are processed each year (Ismea, 2012). The most commonly farmed shellfish for human consumption is *Mytilus galloprovincialis* (Mediterranean mussel), followed by *Ruditapes philippinarum* (Manila clams). In 2011, mussel production was estimated at 98,000 tonnes (including mussels from natural benches), with >80% of the production plants located in the southern Italian regions (Ismea, 2012). Forty-six percent of consumers prefer mussels to other types of shellfish, and they are sold in street markets also in the south of Italy.

Excreta from humans and other animals are a source of a plethora of microorganisms, which are dispersed directly or, for example, via rainfall-initiated run-off from agricultural, suburban and urban lands, wastewater into rivers, streams, estuaries and coastal waters, thus contaminating the sea and its inhabitants. Bivalves filter large volumes of water and consequently can accumulate and retain particles and microorganisms; some of these organisms can be pathogenic and thus represent a potential risk to human health, particularly if eaten raw (EFSA, 2012). Pathogens of most concern in shellfish are viruses (e.g., norovirus, hepatitis A virus), bacteria (e.g., pathogenic *Escherichia coli*, *Campylobacter jejuni*, *Salmonella* spp., *Vibrio vulnificus*, *Vibrio cholerae* and *Vibrio parahaemoliticus*) and protozoans (including *Cryptosporidium*, *Cyclospora*, *Giardia* and *Toxoplasma*), particularly in young, old and/or immuno-compromised or -suppressed people (WHO, 2010).

A number of studies have shown that *Cryptosporidium* and *Giardia* are present worldwide in shellfish farmed or naturally present in lagoons and other marine environments. Several edible and inedible shellfish have been found to carry *Cryptosporidium parvum* (Gómez-Bautista et al., 2000; Fayer et al., 2002, 2003; Gómez-Couso et al., 2004, 2006a,b; Miller et al., 2005; Giangaspero et al., 2005; Li et al., 2006; Graczyk et al., 2007; Molini et al., 2007), whereas *Giardia duodenalis* assemblage A has only been reported to occur in inedible shellfish species (Graczyk et al., 1999a), although its precise identity and link to enteric disease (outbreaks) in humans had not been unequivocally established at the time.

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In spite of the major public health importance of these protists and their potential to cause zoonotic disease (Giangaspero et al., 2007; Xiao and Fayer, 2008), there has been no global study to establish the specific and/or genotypic identities of *Cryptosporidium* and *Giardia* found in shellfish. To date, *Cryptosporidium hominis* (subgenotypes IbA10G2R2, IbA9G3R2, IeA11G3T3R1), *C. parvum* (subgenotypes IIaA16G2R1, IIaA19G3R1, IIcA5G3R2) and *G. duodenalis* (assemblages A and B) have been reported to be commonly associated with human cryptosporidiosis (Xiao and Fayer, 2008; Jex et al., 2008; Jex and Gasser, 2010); these assemblages are identified mostly using PCR-based techniques employing various genetic markers (Chalmers et al., 2005; Plutzer and Karanis, 2009; Bouzid et al., 2010; Putignani and Menichella, 2010; Feng and Xiao, 2011). For *Cryptosporidium*, the SSU, *hsp*70 and/or 60 kDa glycoprotein (*gp60*) genes have been used for specific, genotypic and/or subgenotypic identification (Jex et al., 2007; Widmer, 2009; Nolan et al., 2010, 2013). For *Giardia*, markers in the *β-giardin*, *tpi*, *gdh* and/or SSU genes are most commonly used for identification to species and/or assemblages (Giangaspero et al., 2007). In the present study, we employed PCR-based sequencing of *gp60* and *tpi* and *β-giardin* to genetically classify *Cryptosporidium* and *Giardia*, respectively, from *M. galloprovincialis* from local markets in a locality in south-eastern Italy.

2. Materials and methods

2.1. Samples, and isolation of genomic DNA from mussels

Mussels (*M. galloprovincialis*) were purchased at ten different time points from each of three markets (I, II, and III) in the city of Foggia (41°28′0″N 15°34′0″E) at intervals of 6–10 days from May to December 2012, a period of time, which corresponds to the mussel commercialization (www.coopmare.com/public/relazioni/041245_RelFinale_Miglioramento%20mitili.pdf) in Southern Italy. At each time point, 500 g of mussels was purchased from one market, refrigerated at 5 °C and taken to the laboratory within 1 h. Then, for each sampling, two batches of each 15 live mussels were selected at random for subsequent processing and analyses. For each batch, haemolymph (100 μL) was aspirated from individual mussels using a needle inserted into the lateral adductor muscle, and gills and digestive glands were removed (Graczyk et al., 1999a,b; Molini et al., 2007); individual tissues were then pooled. Haemolymph was concentrated by sucrose gradient centrifugation (Graczyk et al., 1999a,b; Molini et al., 2007). Gills and digestive glands were homogenized in 1 mL of distilled water, sieved through a double layer of gauze and pelleted by centrifugation (1000 ×*g*, 4 °C, 10 min); the pellet was washed twice with TE buffer (10 mM Tris–HCl pH 8.0; 1 mM EDTA, pH 8.0) and 500 μL of this pellet (after washed twice with TE buffer (10,000 ×*g* for 15 min). were subjected to three cycles of −80 °C/5 min and 80 °C/5 min). Subsequently, genomic DNA was isolated from individual samples using the Nucleospin tissue kit (Macherey-Nagel), according to the manufacturer's instructions, and then stored at −20 °C.

2.2. PCR amplification of mussel DNA

All genomic DNA samples were first subjected to PCR-amplification of part of the 18S rRNA gene (162–196 bp) using the primer pair 1F/1R (Espiñeira et al., 2009) to assess potential inhibitory effects of molluscan components on the reaction. PCR was performed in 50 μL mixture using a standard buffer (Applied Biosystems, CA, USA), 2.0 mM $MgCl_2$ (Applied Biosystems), 200 μM dNTPs (Applied Biosystems), 100 pmol of each primer (Sigma Aldrich, Milan, Italy) and 1 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). Approximately 100 ng of genomic DNA was incorporated into each reaction, and a negative control sample (no-template) and a known positive control sample were included in each PCR run. Cycling was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) using the following protocol: 95 °C/3 min (initial denaturation), followed by 35 cycles of 95 °C/30 s (denaturation), 54 °C/30 s (annealing) and 72 °C/30 s (extension), and a final extension of 72 °C/3 min. Amplicons were then resolved in 1.5% agarose gels (Sigma Aldrich), detected using the Gel Doc XR System, and images were captured using Quantity One 4.6.3 software (Bio-Rad, CA, USA). No bands were detected in any of the no-template control samples at any stage. The sizes of amplicons were estimated by comparison with a DNA ladder (Flash Gel#100 bp— 3.0 kb, Amersham Biosciences, USA).

2.3. PCR amplification of genetic markers from Cryptosporidium and Giardia

For the genetic characterization of *Cryptosporidium*, part of the *gp60* gene (~358 bp; designated p*gp60*) was amplified using a previously described nested-PCR protocol (Sulaiman et al., 2005). The *gp60* gene was first amplified using primers AL3531 (5-ATAGTCTCCGCTGTATTC-3′) and AL3533 (5′-GAGATATATCTTGGTGCG-3′), followed by nested amplification using primers LX0029 (5′-TCCGCTGTATTCTCAGCC-3′) and AL3532 (5′-TCCGCTGTATTCTCAGCC-3′). Both PCRs were carried out in 25 μL of a standard buffer, containing thermostable polymerase and dNTPs (Ready Mix REDTaq, Sigma, St. Louis, MO) plus 100 pmol of each primer. Approximately 100 ng of genomic DNA was incorporated into each reaction, and a negative control sample (no-template) and a known positive control sample were included in each PCR run. The amplifications of *gp60* and p*gp60* were carried out using the following cycling protocol: 95 °C/3 min (initial denaturation), followed by 35 cycles of 94 °C/45 s (denaturation), 50 °C (*gp60*) or 51 °C (p*gp60*) for 45 s (annealing) and 72 °C/1 min (extension), with a final extension of 72 °C/4 min.

Two loci were used for the genetic characterization of *Giardia*: portions of the *tpi* gene (~530 bp; designated p*tpi*) and the *β-giardin* gene (~171 bp; designated p*β-g*). All PCR steps were carried out in 25 μL, including Ready Mix REDTaq (Sigma, St. Louis, MO), 100 pmol of each primer and 50–100 ng of genomic DNA or H_20 (no-template control).

The *tpi* locus was amplified using primers AL3543 (5′-AAATTATG CCTGCTCGTCG-3′) and AL3546 (5′-CAAACCTTTTCCGCAAACC-3′), followed by nested PCR using primers AL3544 (5′-CCCTTCATCGGTGG TAACTT-3′) and AL3545 (5′-GTGGCCACCACTCCCGTGCC-3′) (Sulaiman et al., 2003). For the primary amplification, the cycling protocol was 94 °C/5 min (initial denaturation), followed by 35 cycles of 94 °C/45 s (denaturation), 50 °C/45 s (annealing) and 72 °C/1 min (extension), and a final extension of 72 °C/10 min. The secondary PCR protocol (for p*tpi*) was 94 °C/5 min, followed by 35 cycles of 94 °C/45 s, 55 °C/30 s and 72 °C/1 min, with a final extension at 72 °C/10 min.

The p*β-g* region was amplified with the primers GGL (5′-AAGT GCGTCAACGAGCAGCT-3′) and GGR (5′-TTAGTGCTTTGTGACCAT CGA-3′) using the following cycling protocol: 94 °C for 4 min (initial denaturation), 40 cycles of 95 °C/1 min (denaturation), 61 °C/1 min (annealing) and 72 °C for 1 min (extension), followed by a final extension at 72 °C/7 min.

2.4. Sequencing, and analyses of sequence data

Following PCR, all amplicons were examined on ethidium bromide-stained agarose gels (no products detected in any of the no-template control samples), individually treated with the enzymes Exonuclease I (EXO I) and thermosensitive alkaline phosphatase (FAST AP, Fermentas), and then directly sequenced in both directions using a ABI PRISM BygDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) with the same primers (separately) as used in respective PCRs. Sequences were analysed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Electropherograms were inspected by eye, and consensus sequences were obtained. To investigate the species and genotypes of *Cryptosporidium* and *Giardia*, all the sequences obtained in this study

were aligned with respective sequences available in the GenBank database. All of the sequences determined showed a high similarity (98–99%) with *C. parvum* IIa or *Giardia* assemblage A sequences from GenBank. Subsequently, sequences were aligned using the ClustalW implementation of the BioEdit software (Hall, 1999), and the alignment was adjusted manually.

Phylogenetic analysis of sequence data was performed using the Bayesian Inference (BI) method in the programme MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Posterior probabilities (pp) were calculated via 2,000,000 (p*gp60*), utilizing four simultaneous tree-building chains, with every 100th tree being saved. At this point, the standard deviation of split frequencies was <0.01, and the potential scale reduction factor (PSRF) approached one. A 50% majority-rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI.

2.5. Statistical analyses

The percentage of PCR test-positive samples was calculated as the number of test-positive samples / number of samples tested × 100, using a 95% confidence interval (95% CI). The statistical significance was established using the χ^2 test, with *P* values of <0.05 being significant. Odds ratio (OR) and corresponding 95% CI values were also calculated to indicate the degree of potential risk.

3. Results

Under the present PCR conditions, there was no evidence of an inhibitory effect of molluscan components on enzymatic amplification for any of the samples tested. Sixty batches of 15 mussels each were obtained for a total of 30 samplings. Overall, 40 of 60 (66.7%) samples were PCR test-positive for *Cryptosporidium*, *Giardia*, or both *Cryptosporidium* and *Giardia* (Table 1). *Cryptosporidium* was detected in 34 of 60 (56.7%) samples, and was significantly more common in markets II and III than market I, more common than *Giardia*, and more prevalent during the period from May to September than from October to December (Table 1).

Cryptosporidium was mostly detected in one tissue, and, to a lesser extent, in two or three tissues, whereas *Giardia* was always detected only in one (gills or haemolymph) (Table 2). The p*gp60* locus was amplified from 42 (70%) of the 60 samples, including eight (13.3%) that tested positive for both *Cryptosporidium* and *Giardia*. *C. parvum* subgenotype IIaA15G2R1 was identified in 30 (83.3%), IIaA15G2 in four (11.1%) and IIaA14G3R1 in two (5.5%) of the 36 amplicons successfully sequenced. The nucleotide sequences have been deposited in the GenBank database under accession numbers KF258556–KF258573. Phylogenetic analysis of p*gp60* sequence data by BI revealed that all samples (n = 36) represented *C. parvum* genotype IIa. (Fig. 1). The p*tpi* locus was amplified from six of 60 (10%) samples tested, whereas *β-giardin* was amplified from eight (13%) samples, including six (10%) samples with *Giardia* alone, and two (3.3%) with both *Cryptosporidium* and *Giardia*. Sequence analyses of all of these amplicons revealed *G. duodenalis* assemblage A in 14 (23.3%) of 60 samples, based on perfect matches with reference sequences EU781000 (*tpi*; Lebbad et al., 2010) and EU769204.2 (*β-giardin*; Lebbad et al., 2010). *C. parvum* subgenotype IIaA15G2R1 and *G. duodenalis* assemblage A were detected in six (10.0%) of the 60 samples tested, and *C. parvum* subgenotype IIaA15G2 and *G. duodenalis* assemblage A in two samples.

4. Discussion

This is the first published report of *C. parvum* IIa (subgenotypes IIaA15G2R1, IIa15G2 and IIaA14G3R1) and *G. duodenalis* assemblage A in edible shellfish, particularly very commonly consumed *M. galloprovincialis* in highly frequented daily fish markets in a city context. These potentially zoonotic protists (*Cryptosporidium* and *Giardia*) were detected in 66.7% of the 60 samples of mussels overall tested, with genotype IIa, assemblage A and both being identified in 60%, 23.3% and 6.6% of these, respectively.

In the USA, *C. parvum* has been recorded previously in *M. galloprovincialis* (see Miller et al., 2005), in California mussels (*Mytilus californianus*), in eastern oysters (*Crassostrea virginica*) and clams (see Fayer et al., 2002, 2003; Graczyk et al., 2007), whereas *G. duodenalis* assemblage A has only been reported, to date, in inedible clams (i.e. *Macoma balthica* and *Macoma mitchelli*) (Graczyk et al., 1999b). In Mediterranean countries, *C. parvum* has been reported previously in some inedible (Gómez-Bautista et al., 2000), and edible shellfish species, including *M. galloprovincialis* (see Gómez-Couso et al., 2004, 2006a,b) in Spain, *Mytilus edulis* in France (Li et al., 2006), and *R. philippinarum* (see Giangaspero et al., 2005; Molini et al., 2007) and *Chamelea gallina* (clam) (see Giangaspero et al., 2005) in Italy; also *G. duodenalis* has been detected in clams (*C. gallina*) in Italy (Molini et al., 2004). However, all of these studies relate only to farmed or naturally occurring shellfish, while data on the presence of zoonotic protozoans in shellfish from city markets is documented only for Asian green mussel (*Perna viridis*) in Thailand (i.e. *Cryptosporidium* spp.) (Srisuphanunt et al., 2009), and the Brazilian oyster (*Crassostrea rhizophorae*) and the Guyana swamp mussel (*Mytella guyanensis*) in Brazil (i.e. *Toxoplasma gondii*) (Esmerini et al., 2010).

Here, *C. parvum* IIa was identified in half of the 30 batches of mussels purchased from all three markets. Subgenotypes within *Cryptosporidium* IIa are common and have been reported worldwide, primarily in cattle and humans and are recognized as zoonotic or anthroponotic (Jex and Gasser, 2010). In Europe, subgenotype IIaA15G2R1 has been detected

Table 1
Percentage (%) and 95% confidence intervals (95% CI) of samples test-positive for *Cryptosporidium* and/or *Giardia* in 60 samples of 15 mussels each, purchased at three markets in the city of Foggia, Italy, according to site and period of sampling.

Site and period in 2012		No. positive/ No. examined		*Cryptosporidium* % (95%CI)	No positive/ No. examined		*Giardia* % (95%CI)	No. positive/ No. examined		*Cryptosporidium* and *Giardia* % (95%CI)	No. positive/ No. examined		Totals % (95%CI)
Market I		4/20		20 (2.5–37.5)[a,b]	2/20		10 (0–23.1)	6/20		30 (9.9–50.1)	12/20		60 (38.5–81.5)
Market II		10/20		50 (28.1–71.9)[a]	4/20		20 (2.5–37.5)	2/20		10 (0–23.1)	16/20		80 (62.5–97.5)
Market III		12/20		60 (38.5–81.5)[b]	0/20		0 (–)	0/20		0 (–)	12/20		60 (38.5–81.5)
	May–Sept		18/30	60 (42.5–77.5)[c]		0/30	0 (–)		6/30	20 (5.7–34.3)		24/30	80 (65.7–94.3)[d]
	Oct–Dec		8/30	26.7 (10.8–42.5)[c]		6/30	20 (5.7–34.3)		2/30	6.7 (0–15.6)		16/30	53.3 (35.5–71.2)[d]
Total		26/60		43.3 (30.8–55.9)[e,f]	6/60		10 (2.4–17.6)[e]	8/60		13.3 (4.7–21.9)[f]	40/60		66.7 (54.7–78.6)

[a, b] Statistically significant differences ($P < 0.05$) according to site of sampling.
[c, d, e, f] Statistically significant differences ($P < 0.05$) according to period of sampling.
[a] $\chi^2 = 3.96$, $P = 0.0467$, OR = 4 (0.98–16.27).
[b] $\chi^2 = 6.67$, $P = 0.0098$, OR = 6 (1.46–24.69).
[c] $\chi^2 = 6.79$, $P = 0.0092$, OR = 4.13 (1.39–12.27).
[d] $\chi^2 = 4.80$, $P = 0.0285$, OR = 3.50 (1.11–11.02).
[e] $\chi^2 = 17.05$, $P = 0.0000$, OR = 6.88 (2.57–18.45).
[f] $\chi^2 = 13.30$, $P = 0.0003$, OR = 4.97 (2.02–12.26).

Table 2
Numbers of test-positive organ/tissue samples (N), percentages (%) and 95% confidence intervals (95% CI) of samples test-positive for *Cryptosporidium* and/or *Giardia* from in 60 samples of 15 mussels each, purchased at three markets in the town of Foggia, Italy.

Organ/tissue	*Cryptosporidium* N [% (95% CI)]	*Giardia* N [% (95% CI)]	*Cryptosporidium* and *Giardia* N [% (95% CI)]	Total N [% (95% CI)]
G	2 [3.3 (0–7.9)]	0 [0 (0–0)]	4 [6.7 (0.3–13)]	6 [10 (2.4–17.6)]
Dg	6 [10 (2.4–17.6)]	4 [6.7 (0.3–13)]	0 [0 (0–0)]	10[16.7 (7.2–26.1)]
H	12 [20 (9.9–30.1)]	2 [3.3 (0–7.9)]	0 [0 (0–0)]	14 [23.3 (12.6–34)]
G + Dg	2 [3.3 (0–7.9)]	0[0 (0–0)]	0 [0 (0–0)]	2 [3.3 (0–7.9)]
G + H	2 [3.3 (0–7.9)]	0[0 (0–0)]	4 [6.7 (0.3–13)]	6 [10 (2.4–17.6)]
G + Dg + H	2 [3.3 (0–7.9)]	0[0 (0–0)]	0 [0 (0–0)]	2 [3.3 (0–7.9)]
Total	26 [43.3 (30.8–55.9)]	6 [10 (2.4–17.6)]	8 [13.3 (4.7–21.9)]	40 [66.7 (54.7–78.6)]

Dg: Digestive glands.
G: Gills.
H: Haemolymph.

in human stool samples in Portugal (Alves et al., 2003), the Netherlands (Wielinga et al., 2008), Belgium (Geurden et al., 2009) and Italy (Del Chierico et al., 2011), and also in environmental water samples in Portugal (Lobo et al., 2009). Furthermore, it has also been isolated from people involved in an outbreak in Wales associated with a farm that was open to public visitation (Chalmers et al., 2005), supporting the proposal of zoonotic transmission. There was some variation among the p*gp60* sequences determined here, which were 98–99% similar to those representing IIaA15G2R1 derived from calves in Ireland (Thompson et al., 2007), and from humans and calves in Slovenia (Soba and Loga, 2008).

G. duodenalis assemblage A was detected in 14 samples (23.3%) from two of the three markets investigated in this study, and was the only assemblage identified using the two markers (*β-giardin* and p*tpi*). For both genetic loci, there was no sequence variation among samples classified here as assemblage A, and very limited variation compared with

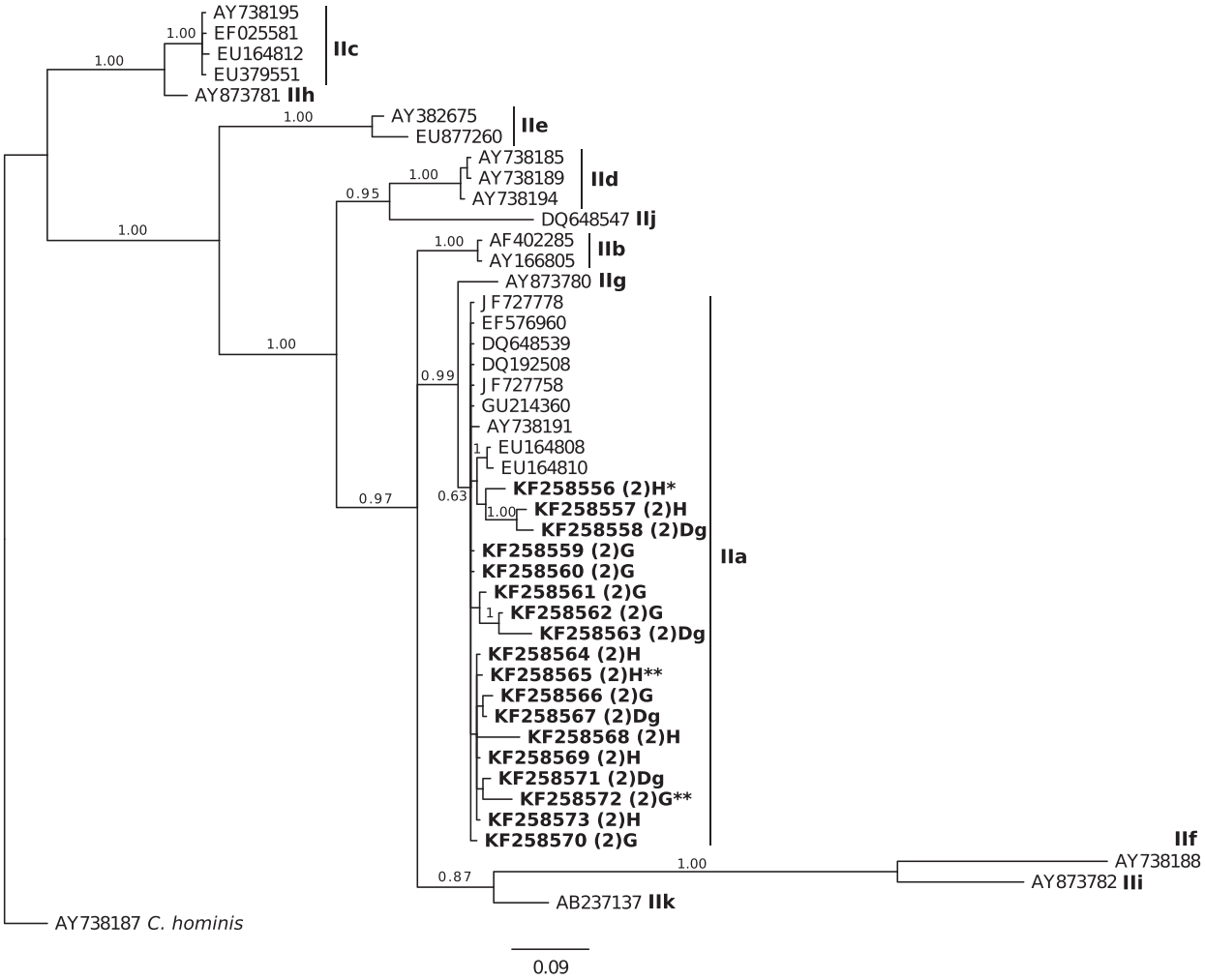

Fig. 1. Phylogenetic relationships of *Cryptosporidium parvum* based on the analysis of p*gp60* sequence data by Bayesian inference. Eighteen sequences from the present study and 26 reference sequences representing *C. parvum* genotypes IIa–IIk were included in the analysis for comparative purposes. *C. hominis* was used as an outgroup. Accession numbers of publicly available reference sequences are indicated. Each GenBank accession number in bold-type represents two identical sequences, unless otherwise indicated. Tissue locations are in brackets (Dg: digestive glands; G: gills; H: haemolymph). *IIaA14G3R1 **IIaA15G2.

those representing *G. duodenalis* assemblage A from faecal samples from humans from France (Bonhomme et al., 2011) and Sweden (Lebbad et al., 2010).

Current information for Italy indicates a relatively widespread problem with faecal-derived contamination of land and sea with *Giardia* and *Cryptosporidium* oocysts/cysts (Giangaspero et al., 2007). In this country, *Giardia* and/or *Cryptosporidium* have been detected in humans, companion animals, sheep, cattle and water buffaloes, in wastewater, surface water, and also in vegetables and shellfish. It seems that, in Italy, the role of farm animals can be significant for human infection, due to increased 'circulation' of assemblage A of *Giardia* and *C. parvum* between humans and animals, such as domesticated cats (Giangaspero et al., 2007).

Both *C. parvum* IIa and/or *G. duodenalis* assemblage A are known to infect humans and thus likely represent a significant public health risk, particularly to immuno-compromised or –suppressed people, and children. In this study, *C. parvum* was much more common than *G. duodenalis* in mussels purchased from the three markets, and there was a significant difference in the percentage of test-positive samples among seasons of the year. These findings are not entirely unexpected. Possible explanations for a higher incidence of oocyst/cyst contamination in spring/summer might be increased crowds of people at beaches and/or untreated effluent/sewage originating from hotels, camping sites and resorts. In our opinion, the present study documents an alarming level of contamination in shellfish from local markets in Foggia, particularly in spring/summer, when mussels are at their prime and when most mussels are consumed. Moreover, it needs to be taken into account that, in temperate climates, the incidence of human cryptosporidiosis peaks with an increase in environmental temperature (Jagai et al., 2009). This contamination appears to relate to a poor observance of hygiene standards and regulations by vendors, combined with lax inspections by the authorities. Indeed, mussels are often sold in bulk, exposed to direct sunlight, mixed with other types of food and often unprotected from pests (e.g., flies); and, operators handle both food and non-food articles without gloves at the same time. In addition, bivalve molluscs are immersed in water in many cases, despite European Commission Regulation 853/2004 (EC, 2004).

In the present study, there was no evidence that mussels were marked with labels, against European Commission Regulation 2065/2001; thus, individual sampling units could not be traced back to their geographical origin, or to producers or depuration plants. It was not possible to establish whether the presence of *G. duodenalis* and *C. parvum* was attributable to a possible failure in the mussel depuration/processing, whether contamination occurred after harvesting, or whether they originated from illegal farms or wild stands in contaminated waters. These proposals are somewhat supported by the condition of many consignments of mussels (varying sizes, abundance of dirt on valves) and also from information gathered that, in some areas of the Foggia Province, Italy, shellfish from authorized areas, are kept in harbour waters before being sold to retailers and wholesalers, in order to increase their weight. Such waters are often heavily contaminated with faecal coliforms (ARPA, Puglia, 2011), suggesting a likelihood of contamination also with enteric protists, such as *Cryptosporidium* and *Giardia*.

Regarding food safety, we show that mussels on sale at local markets in Foggia were highly contaminated with protozoa of particular zoonotic/anthroponotic interest, i.e. *C. parvum* IIa and *G. duodenalis* assemblage A. Although the genetic analysis of these zoonotic protists, to assess the health security of live bivalve molluscs, is still not required by current law in Italy, the magnitude of test-positivity in this study suggest a significant, indirect contamination by faecal material and a risk to the health of consumers, particularly considering that a very small number (1–30 oocysts/cysts) can initiate infection (Rendtorff, 1954; Chappell et al., 1996). Added risk factors relate also to the widespread, local habit of consuming raw or lightly steamed shellfish that contain viable and infective stages (Gómez-Couso et al., 2006c).

Although there appears to be only one documented case of cryptosporidiosis linked to the consumption of molluscs (i.e. oysters) (Baumgartner et al., 2000), the lack of epidemiological information for shellfish likely relates to inadequate diagnosis/detection or reporting, a delay or variability in the time of onset of clinical signs of cryptosporidiosis or giardiasis in affected people, because of differences in parasite factors (e.g., genotype, infectivity, virulence, infectious dose and prepatent period) or host factors (e.g., immune status and microbiome composition) and the possible transient nature of diarrhoea/enteritis (Xiao and Fayer, 2008; Chalmers and Davies, 2010; Escobedo et al., 2010; Putignani and Menichella, 2010; Feng and Xiao, 2011).

In conclusion, the findings of the present study indicate that edible mussels harbour *C. parvum* and *Giardia*, which might represent a public health hazard. The poor observance of hygiene rules by mussel vendors in the study location, the large quantities of *M. galloprovincialis* sold, and the traditional eating habits of consumers in southern in Italy all mean that public health institutions have a responsibility to implement effective sanitary checks of shellfish sold in daily street markets.

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