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

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Abstract

Background: The genetic characterization of Cryptosporidium and Giardia has important implications for investigating their epidemiology and underpins their control. We undertook the first molecular epidemiological survey of domestic bovids in selected regions of Sri Lanka to establish whether they excreted Cryptosporidium and/or Giardia with zoonotic potential.

Methods: Faecal samples were collected from dairy calves (n = 340; Bos taurus; < 3 months of age; weekly sampling for six weeks) and water buffaloes (n = 297; Bubalus bubalis; <6 months and ≥6 months of age; one sampling) from seven different farms in Sri Lanka. Genomic DNAs were extracted from individual faecal samples and then tested for the presence of parasite DNA using a PCR-based mutation scanning-targeted sequencing-phylogenetic approach, employing genetic markers within the small subunit of nuclear ribosomal RNA and 60 kDa glycoprotein genes (designated pSSU and pgp60, respectively) for Cryptosporidium, and within the triose phosphate isomerise (ptpi) gene for Giardia.

Results: Based on pSSU sequence data, C. bovis, C. ryanae and six new genotypes that were genetically similar but not identical to C. andersoni (n = 1), C. bovis (n = 1), C. ryanae (n = 3) and C. suis (n = 1) were recorded in cattle. For pSSU, two other, new genotypes were defined in water buffalo, which were genetically most similar to Cryptosporidium genotypes recorded previously in this host species in other countries including Australia. Consistent with the findings for pSSU, no species or genotypes of Cryptosporidium with zoonotic potential were detected using pgp60. Based on ptpi sequence data, G. duodenalis assemblages A and E were detected in four and 137 samples from cattle, respectively, and assemblage E in two samples from water buffaloes.

Conclusions: The present study showed that C. parvum, the most commonly reported zoonotic species of Cryptosporidium recognised in bovine calves globally, was not detected in any of the samples from pre-weaned calves tested in the present study. However, eight new genotypes were recorded. Future studies of different host species in various regions are required to investigate the molecular epidemiology of cryptosporidiosis and giardiasis in Sri Lanka and neighbouring countries in South Asia.

Keywords: Bos taurus, Bubalus bubalis, Cryptosporidium, Giardia, Single-strand conformation polymorphism (SSCP) analysis, Restriction endonuclease fingerprinting (REF), Sri Lanka
Background

_Cryptosporidium _and _Giardia _are _two _parasitic _protists _that _mainly _infect _the _gastrointestinal _tract _and _cause _enteric _disease _in _humans _and _various _other _animals _[1,2]. _These _protozoa _are _global _in _their _distribution _and _adversely _impact _on _human _health _in _both _developed _and _developing _countries _[3,4]. _Infections _are _usually _transmitted _via _the _faecal-oral _route, _following _direct _or _indirect _contact _with _infective _stages _(oocysts _or _cysts) _[2,3]. _Although _infections _are _often _self-limiting _in _immuno-competent _individuals _[3,5], _they _can _become _severe _and _chronic _in _infants, _elderly _people _or _immuno-compromised _or _-suppressed _individuals _[6-8]. _Key _risk _factors _for _cryptosporidiosis _and _giardiasis _of _humans _include _contact _with _individuals _with _diarrhoea, _swimming _in _public _pools, _travel _to _developing _countries _and, _importantly, _direct _contact _with _animals _(e.g., _[9-12]).

Cryptosporidiosis _and _giardiasis _can _be _transmitted _from _human _to _human _(anthroponotic) _or _from _animal _(zoonotic) _[13]. _Ruminants _often _have _been _implicated _as _a _major _source _of _human _cryptosporidiosis _and _giardiasis _based _on _the _findings _of _many _molecular _epidemiological _studies _(reviewed _in _[14,15]). _For _instance, _investigations _of _outbreaks _and _case-control _studies _[16-20] _have _demonstrated _that _there _is _a _strong _link _between _human _cryptosporidiosis/giardiasis _and _the _direct _or _indirect _contact _with _cattle, _particularly _pre-weaned _calves _[15,21]. _To _date, _seven _species _(i.e. _C. _andersoni, _C. _bovis, _C. _felis, _C. _hominis, _C. _parvum, _C. _ryanae _and _C. _suis) _and _two _genotypes _of _Cryptosporidium _(i.e. _“pig _genotype _II” _and _a _new _“C. _suis-like _genotype”) _have _been _recorded _in _cattle _[22,23]. _For _Giardia _duodenalis, _assemblage _E _is _the _most _commonly _reported _genotype _in _cattle, _followed _by _assemblages _A _and _B _[24-26]. _An _appraisal _of _the _literature _shows _that _the _majority _of _molecular _studies _of _Cryptosporidium _and _Giardia _of _cattle _and _other _animals _relate _mainly _to _a _limited _number _of _countries _in _the _developed _world _[3,27], _but _there _is _little _published _information _for _developing _countries, _including _Sri _Lanka. _In _addition, _although _there _have _been _numerous _studies _of _cattle _(Bos _taurus; _< 3 _months _of _age) _and _water _buffaloes _(Bubalus _bubalis; _< 6 _months _and _≥ 6 _months _of _age) _from _seven _different _farms _(designated _AB, _NK _and _MR) _studied _were _large, _intensive _farms, _located _in _the _highland _wet _zone _(average _>2000 _mm _of _annual _rainfall _and _>900 _m _altitude), _and _maintained _Ayrshire, _Holstein _Friesian _and _Jersey _breeds, _respectively. _Faecal _samples _were _collected _weekly _for _six _weeks _from _these _three _dairy _farms. _At _the _first _visit, _faeces _were _collected _from _20 _pre-weaned _calves _(1–12 _weeks) _from _each _farm, _and _individual _calves _were _sampled _weekly _for _6 _weeks, _providing _a _total _of _340 _samples _for _molecular _testing. _In _addition, _a _total _of _297 _faecal _samples _were _collected _once _from _the _two _different _age _groups _of _water _buffalo. _The _four _riverine _buffalo _herds _(BR, _HA, _NK _and _MR) _studied _were _in _two _different _climatic _zones, _with _an _average _of _1,750-2,500 _and _1,850-5,000 _mm _of _annual _rainfall _at _altitudes _of _<300 _and _300–900 _m, _respectively.

Cattle _and _buffaloes _were _born _and _raised _on _the _farms _studied, _and _fed _whole _milk _(twice _a _day) _until _weaning _at _3 _months _of _age. _Dairy _calves _were _reared _in _individual _pens, _whereas _buffalo _calves _were _maintained _as _groups _(n = 3–10) _in _pens. _Therefore, _there _was _no _direct _contact _between _calves _and _adult _cattle _or _buffaloes. _Herd _management _practices _on _these _dairy _and _water _buffalo _farms _are _typical _of _large-scale, _intensive _farms _in _Sri _Lanka.

Sample collection

Faecal _samples _were _collected _rectally _from _pre-weaned _dairy _calves _(Bos _taurus; _< 3 _months _of _age) _and _water _buffaloes _(Bubalus _bubalis; _< 6 _months _and _≥ 6 _months _of _age) _from _seven _different _farms _(designated _AB, _NK _and _MR) _studied _were _large, _intensive _farms, _located _in _the _highland _wet _zone _(average _>2000 _mm _of _annual _rainfall _and _>900 _m _altitude), _and _maintained _Ayrshire, _Holstein _Friesian _and _Jersey _breeds, _respectively. _Faecal _samples _were _collected _weekly _for _six _weeks _from _these _three _dairy _farms. _At _the _first _visit, _faeces _were _collected _from _20 _pre-weaned _calves _(1–12 _weeks) _from _each _farm, _and _individual _calves _were _sampled _weekly _for _6 _weeks, _providing _a _total _of _340 _samples _for _molecular _testing. _In _addition, _a _total _of _297 _faecal _samples _were _collected _once _from _the _two _different _age _groups _of _water _buffalo. _The _four _riverine _buffalo _herds _(BR, _HA, _NK _and _MR) _studied _were _in _two _different _climatic _zones, _with _an _average _of _1,750-2,500 _and _1,850-5,000 _mm _of _annual _rainfall _at _altitudes _of _<300 _and _300–900 _m, _respectively.

Cattle _and _buffaloes _were _born _and _raised _on _the _farms _studied, _and _fed _whole _milk _(twice _a _day) _until _weaning _at _3 _months _of _age. _Dairy _calves _were _reared _in _individual _pens, _whereas _buffalo _calves _were _maintained _as _groups _(n = 3–10) _in _pens. _Therefore, _there _was _no _direct _contact _between _calves _and _adult _cattle _or _buffaloes. _Herd _management _practices _on _these _dairy _and _water _buffalo _farms _are _typical _of _large-scale, _intensive _farms _in _Sri _Lanka.

Isolation of genomic DNA from faecal samples, and PCR amplification

Genomic _DNA _was _isolated _from _individual _faecal _samples _using _the _PowerSoil _DNA _isolation _kit _(MoBio, _USA) _[29], _and _then _frozen _at _−20°C _until _molecular _testing. _Each _genomic _DNA _sample _was _tested _by _PCR _for_
pSSU and pgg60) within the small subunit nuclear ribosomal RNA and 60 kDa glycoprotein genes, and for *Giardia* DNA using a region (ptpi) within the triose phosphate isomerase gene [29,36]; pgg60 is employed specifically for the detection and assignment of *Cryptosporidium* species, genotypes or subgenotypes that are infective to humans (cf. [2]). Some genomic DNAs (n = 50) were also tested for inhibition of the enzymatic reaction in PCR using a DNA-spiking approach [37]. In brief, aliquots (2 μl) of individual genomic DNA samples that had been test-negative in PCR were individually spiked with 1 pg of *C. parvum* DNA and subjected to PCR-based mutation scanning to demonstrate the amplification of a specific product. There was no evidence that samples tested contained constituents inhibitory to PCR.

**Mutation scanning-based sequencing and phylogenetic analyses of sequence data**

For pSSU amplicons, single-strand conformation polymorphism (SSCP) analysis [38] was carried out as described previously [39]. For ptpi amplicons, restriction endonuclease fingerprinting was employed, using the enzyme RsaI (Promega) [40-42]. Amplicons representing each banding profile were selected and treated with exonuclease I and shrimp alkaline phosphatase (Fermentas), according to the manufacturer’s instructions, and then sequenced in both directions by direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA), using the same primers employed in secondary PCR. The quality of each sequence was assessed based on the corresponding electropherogram using the program BioEdit [43], and the sequences determined were compared with known reference sequences using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis of sequence data was performed using the Bayesian inference (BI) tree building method in MrBayes 3.1.2 [44,45]. Posterior probabilities (pp) were calculated via 2,000,000 generations, 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.

Phylogenetic analysis was conducted to assess the relationships of the sequences from the present study to those available from public databases for species or genotypes of *Cryptosporidium*, and published in the peer-reviewed literature. In brief, species were determined based on 100% sequence homology to known species of *Cryptosporidium*. Phylogenetic analysis was used to provide unequivocal support for the classification of species and genotypes of *Cryptosporidium*; new genotypes were designated using sequential numbers, according to a previous study [29]. In addition, statistical analysis of proportional difference was performed using the Fisher’s exact test [46].

**Results**

*Cryptosporidium* species/genotypes in cattle

Although pgg60 was not amplified from any of the 340 genomic DNA samples tested, the pSSU region was amplified from 211 (62.1%) of them. SSCP-based analysis of all amplicons revealed eight unique banding profiles. In total, 60 amplicons representing these eight profiles were sequenced (5–10 per profile). No sequence variation was detected among all sequences representing each SSCP profile, such that, ultimately, eight unique pSSU sequences (GenBank accession nos. KF891285-KF891292) were defined. These eight sequences differed by 72–99% upon pairwise comparison. Phylogenetic analysis (Figure 1) of these eight as well as 70 reference sequences (Additional file 1) included for comparative purposes revealed *C. bovis* (accession no. KF891286), *C. ryanae* (KF891285) and six new records (genotypes) that were genetically similar (72-99%) but not identical to *C. andersoni* (n = 1; accession no. KF891291), *C. bovis* (n = 1; KF891290), *C. ryanae* (n = 3; KF891287–KF891289) or *C. suis* (n = 1; KF891292), respectively (see Additional files 2 and 3). In the epidemiological context (Table 1), *C. bovis* and *C. ryanae* were detected in 5.6% and 7.4% of all samples only from farm AB, respectively, and the six new *Cryptosporidium* sequence types (accession nos. KF891287–KF891292) were detected in 0.3–27.6% of samples from farms AB, DY and/or NZ (Table 1).

*Cryptosporidium* species/genotypes in water buffaloes

Although pgg60 was not amplified from any of the 297 genomic DNA samples tested, the pSSU region was amplified from 29 (9.8%) of them. SSCP-based analysis of amplicons revealed three unique banding profiles. In total, nine amplicons representing these three profiles were sequenced. No sequence variation was detected among all three sequences representing each SSCP profile, such that, finally, three unique pSSU sequences (GenBank accession nos. KF891292–KF891294) were defined. These three new sequence types differed by 84-97% upon pairwise comparison. Based on the phylogenetic analysis, two sequence types (accession nos. KF891293 and KF891294) were genetically most similar but not identical to *Cryptosporidium* genotypes reported previously from water buffaloes (see Figure 1). The other sequence type defined was the same as the pSSU sequence of *Cryptosporidium* with accession no. KF891292 recorded in cattle in the present study (see section on *Cryptosporidium* species/genotypes in cattle). In the epidemiological context (Table 1), the *Cryptosporidium*
Table 1 Cryptosporidium species/genotypes and Giardia duodenalis assemblages detected in faecal samples from dairy cattle from three farms in Sri Lanka

| Farm | Total | Cryptosporidium species or genotypes* | Giardia duodenalis assemblages* |
|------|-------|-------------------------------------|--------------------------------|
| AB   | 117   | C. ryanae (KF891285) C. bovis (KF891286) | 60 |
| NZ   | 120   | Genotype 4 (KF891287) Genotype 5 (KF891288) | 39 |
| DY   | 103   | Genotype 6 (KF891289) Genotype 7 (KF891290) | 38 |
| Total| 340   | Genotype 8 (KF891291) Genotype 9 (KF891292) | 137 |

*GenBank accession nos. are given in round parentheses.
sequence types with accession nos. KF891293, KF891294 and KF891292 were detected in 2.4% (farms BR, NK and MR), 5.7% (farms HA and MR) and 1.7% (farm MR) of the 297 samples tested, respectively.

Cryptosporidium was detected on all four water buffalo farms (Table 2). Samples test-positive for Cryptosporidium were detected in 25 (8.4%) and four (1.3%) of 297 samples from animals in the categories of < 6 months and ≥ 6 months of age, respectively (Table 3). The lowest (2.4%) and highest (17.6%) percentages of samples test-positive for Cryptosporidium were detected on farms NK and HA, respectively, but there was no significant difference in numbers of test-positive samples between the two different climatic regions in which samples were collected. The total number of calves (< 6 months) that were test-positive for Cryptosporidium was significantly lower for water buffaloes than for cattle in the study population (P < 0.001).

Detection of Giardia duodenalis in cattle
All 340 faecal genomic DNA samples were subjected to genetic analysis for Giardia. The ptpi locus was amplified from 141 (41.5%) of these samples. SSCP analysis of all 141 amplicons displayed 16 distinct profiles. The direct sequencing of 60 amplicons representing all 16 profiles defined 16 distinct sequence types (GenBank accession nos. KF891295-KF891310). These 16 sequences differed by 86-99% upon pairwise comparison. All sequences (~530 bp) representing the 16 profiles were compared with publicly available sequences. One of the 16 sequences was identical to the reference sequence (accession number L02120) for G. duodenalis assemblage A. The 15 other sequences were identified as G. duodenalis assemblage E (Table 4). Three of the 15 sequences were identical to G. duodenalis assemblage E sequences with accession nos. JN162349, JN162348 and EF654688, respectively. Twelve other sequences were 99% similar to G. duodenalis assemblage E sequences with GenBank accession nos. JN162349 (n = 5), JN162348 (n = 2), JN162347 (n = 3) and GQ444455 (n = 2). A comprehensive phylogenetic analysis (Figure 2) supported their classification; the 15 and one sequences determined in this study clustered, with strong nodal support (pp = 1.00), with G. duodenalis assemblages E and A, respectively (Figure 2).

These results were then put into an epidemiological context. Giardia was detected molecularly in 141 (22.1%) samples originating from all three cattle farms. Assemblages A and E were identified in four and 137 of these samples. The highest percentage of test-positive samples was detected in cattle on farm AB (51.3%), followed by farms DY (40.8%) and NZ (32.5%). Each cattle farm had 19 calves that were test-positive for Giardia at some stage during the sampling period. Mixed infections of Cryptosporidium and Giardia were detected in 98 (28.8%) of 340 samples. Of 60 cattle from all three farms, 57 had mixed infections of Cryptosporidium and Giardia at least once during the 6-week sampling period. The total number of calves (< 6 months) that were test-positive for Giardia was significantly higher for cattle than for water buffaloes (P < 0.001).

Table 2 Cryptosporidium genotypes and Giardia duodenalis assemblage E detected in faecal samples from buffaloes from four farms in Sri Lanka

| Farm | Total | Cryptosporidium genotypes* | Giardia duodenalis assemblage E* |
|------|-------|---------------------------|----------------------------------|
|      |       | Genotype 9 (KF891292)     | Genotype 10 (KF891293) | Genotype 11 (KF891294) | (KF891311 and KF891312) |
| HA   | 51    | 0                         | 0                                 | 9                      | 1                           |
| BR   | 58    | 0                         | 4                                 | 0                      | 0                           |
| NK   | 82    | 0                         | 2                                 | 0                      | 0                           |
| MR   | 106   | 5                         | 1                                 | 8                      | 1                           |
| Total| 297   | 5                         | 7                                 | 17                     | 2                           |

*GenBank accession nos. are given within round parentheses.

Table 3 Cryptosporidium genotypes and Giardia duodenalis assemblage E detected in faecal samples collected from two different age groups of water buffaloes in Sri Lanka

| Age group | Total | Cryptosporidium genotypes* | Giardia duodenalis assemblage E* |
|-----------|-------|---------------------------|----------------------------------|
|           |       | Genotype 9 (KF891292)     | Genotype 10 (KF891293) | Genotype 11 (KF891294) | (KF891311 and KF891312) |
| <6 months | 108   | 5                         | 5                                 | 15                     | 1                           |
| ≥6 months | 189   | 0                         | 2                                 | 2                      | 1                           |
| Total     | 297   | 5                         | 7                                 | 17                     | 2                           |

*GenBank accession nos. are given in round parentheses.
Table 4 Fifteen different ptpi sequences representing assemblage E of *Giardia duodenalis* detected in cattle and buffaloes in Sri Lanka

| Farm | Host                  | Numbers of samples (accession nos.)* | Subtotal |
|------|-----------------------|-------------------------------------|----------|
| AB   | Cattle (Bos taurus)   | 47 (KF891296), 3 (KF8912967), 3 (KF891298), 2 (KF891299), 2 (KF891300), 1 (KF891302) | 60       |
| NZ   | Cattle                | 20 (KF891296), 10 (KF891297), 3 (KF891304), 2 (KF891303), 1 (KF891305), 1 (KF891306), 1 (KF891307), 1 (KF891298) | 39       |
| DY   | Cattle                | 29 (KF891298), 4 (KF891296), 2 (KF891308), 1 (KF891301), 1 (KF891309), 1 (KF891310) | 38       |
| HA   | Water buffalo (Bubalus bubalis) | 1 (KF891311) | 1         |
| MR   | Water buffalo         | 1 (KF891312) | 1         |
| BR   | Water buffalo         | 0          | 0         |
| NK   | Water buffalo         | 0          | 0         |
|      | Total                 |            | 139       |

*GenBank accession nos. are indicated in round parentheses.

Figure 2 Phylogenetic relationships of ptpi sequences of *Giardia duodenalis* based on Bayesian inference (BI) analysis. Sixteen sequences determined in the present study, and 41 reference sequences representing *G. duodenalis* assemblages A to G (accession nos. listed in Additional file 4) were included in the analysis. Sequences representing *G. ardeae*, *G. muris* and *G. microti* were used as outgroups. Accession numbers linked to sequences determined in the present study are in bold-type; the numbers of samples with particular sequence types are in parentheses. Posterior probabilities (pp) are indicated at all major nodes.
Detection of *Giardia duodenalis* in water buffalo

All 297 faecal genomic DNA samples were subjected to genetic analysis for *Giardia*. The *ptpi* locus was amplified from two (0.7%) of these samples; the two samples were from water buffaloes of <1 year of age (farms HA and MR: ≥6 months of age on both farms). The two amplicons were sequenced directly and compared with publicly available sequences. The sequences (GenBank accession nos. KF891311 and KF891312) were the same as two reference sequences (accession nos. JN162348 and JN162349) representing *G. duodenalis* assemblage E (Additional file 4). The two samples test-positive for *Giardia* also both contained a *Cryptosporidium* sequence type with accession no. KF891294.

**Discussion**

This study genetically characterised, for the first time, *Cryptosporidium* and *Giardia* from *Bos taurus* and *Bubalus bubalis* in Sri Lanka. *Cryptosporidium* was identified in 62.1% and 9.8% of 340 samples, and *Giardia* in 41.5% and 0.7% of 279 samples from these two respective bovid hosts. Overall, two *Cryptosporidium* species (*C. bovis* and *C. ryanae*) and eight new genotypes were defined based on their *pSSU* sequence (n = 5, accession nos. KF891287-KF891291 in cattle; n = 2, KF891293 and KF891294 in water buffalo; and n = 1, KF891292 in both cattle and buffalo). *C. bovis* and *C. ryanae* were detected in calves of 10 and 19 days of age, respectively, time points which are relatively consistent with the pre-patent periods reported for these species of *Cryptosporidium* [47]. Although both *C. bovis* and *C. ryanae* (previously called *Cryptosporidium* “deer-like genotype”) have been widely reported in calves from different countries throughout the world [48,49], the eight genotypes characterised here are novel. Consistent with the findings for *pSSU*, no zoonotic species or genotypes of *Cryptosporidium* were detected using *pgp60*, in the absence of any evidence of PCR inhibition.

The first new *Cryptosporidium* genotype represented by accession no. KF891287, which is distinct in *pSSU* sequence by one nucleotide substitution (G - > C at alignment position 220; Additional file 3) from *C. ryanae*, was each recorded in four and five samples, respectively, from farms AB and DY. These two genotypes differed by only one nucleotide (G - > C) from previously reported *C. ryanae* variants (accession nos. KC778534 and KC778535; [50]).

The fifth new genotype (accession no. KF891291), which differed in sequence by one nucleotide from *C. andersoni*, was identified in one sample. Phylogenetic analysis (Figure 1) revealed that the sequence representing this genotype formed a monophyletic group, with five reference sequences representing *C. andersoni* (pp = 0.88). Although *C. andersoni* occurs in adult cattle, it has been found occasionally in pre-weaned calves [51-55]. The sixth new genotype (accession no. KF891292) was identified in two faecal samples from one calf on one farm (NZ) and five samples isolated from water buffaloes from another farm (MR). This genotype is one nucleotide different from the *pSSU* sequence of a new *Cryptosporidium* genotype described previously in cattle in Australia (accession no. KC778530; [50]), Denmark (accession no. DQ182599; [56]), India (accession no. GQ345008; [57]) and the UK (accession no. HQ822134; [58]), and also in water buffaloes in Australia (accession no. KF019204; [29]). Published sequence data for the heat shock protein 70 (hsp70) and actin genes also supported the validity of this new genotype [58]. Therefore, this genotype might represent a new species, but it remains to be described.

The seventh and eighth new genotypes (represented by accession nos. KF891293 and KF891294) recorded from water buffaloes differed in sequence by a single point mutation (G - > C at position 220; Additional file 3) from genotypes 1 and 2 (accession nos. KF019202 and KF019203) described recently [29] in water buffaloes in Australia, and they were genetically most similar to those represented by accession nos. KF019202, KF019203, AB712388 and JQ002562 [29,59,60] in the phylogenetic analysis. These results indicate that these two novel genotypes of *Cryptosporidium* are buffalo-affiliated, but these genotypes are clearly different from *C. ryanae* from cattle [47]. Most samples (86.2% of 29) test-positive for *Cryptosporidium* in water buffaloes were detected in calves of <6 months of age. This result is consistent with previous studies [29,61,62], in which the occurrence of *Cryptosporidium* infection has been reported to be higher in water buffaloes of <6 months than in those of ≥6 months of age. A novel genotype represented by accession no. KF891294 was most frequently detected (58.6%) among *Cryptosporidium* test-positive samples from water buffaloes. It was also detected more frequently in samples collected from calves of <6 months of age group than those of ≥6 months age group. All of the eight novel genotypes identified here in cattle and buffaloes from Sri Lanka had a unique single-nucleotide (G - > C) substitution in *pSSU* positions 73 and 74, and a G - > C at position 220; Additional file 3) from *C. ryanae*, were each recorded in four and five samples, respectively, from farms AB and DY. These two genotypes differed by only one nucleotide (G - > C) from previously reported *C. ryanae* variants (accession nos. KC778534 and KC778535; [50]).
(Additional file 3) and appear to be autochthonous to this country.

Surprisingly, *C. parvum*, the most commonly reported zoonotic species of *Cryptosporidium* recognised in cattle throughout the world [2,15,48], was not detected in the present study in any of the samples collected from calves of 1 week to 3 months of age using a repeated sampling strategy (every week for six weeks). According to epidemiological studies conducted in many countries [16,52,55,63-67], *C. parvum* is most commonly detected in calves of <3 months of age. According to a longitudinal survey conducted by Santin and colleagues [55], *C. parvum* was detected in 85% of 503 pre-weaned calves, whereas only 1% was associated with post-weaned calves. For this reason, pre-weaned calves are recognised as the major reservoir for human cryptosporidiosis [2]. However, some recent epidemiological studies have reported an abundance of *C. bovis* infection and a limited presence of *C. parvum* in calves of 1–60 days of age in China [23,68,69], India [68] Nigeria [70] and Sweden [71]. Taken together, the present findings suggest that calves of this age group are not reservoirs for human *C. parvum* infection in the geographical regions studied here in Sri Lanka. A likely explanation for this unexpected result might relate to sound husbandry practices on farms in Sri Lanka. The three cattle farms studied were well-managed, intensive farms, and pre-weaned calves were kept and fed/watered in individual, elevated wooden calf pens with slatted floors.

*G. duodenalis* assemblage A was detected in only four samples from calves on one dairy farm (DY), whereas 97% of samples test-positive for *Giardia* in cattle related to *G. duodenalis* assemblage E. This result is consistent with the previous studies [50,72-76] reporting that 80-100% of the *G. duodenalis* isolated from cattle are in assemblage E. This assemblage was detected in calves on all three dairy farms and, at least once, in individual calves during the 6-week period of sampling. The two samples test-positive for *Giardia* in water buffaloes on farms HA and MR contained *G. duodenalis* assemblage E, although *Giardia* was not detected on the other two farms. Previous molecular studies conducted in Australia [29] and Italy [28] have also reported *G. duodenalis* assemblage E in water buffaloes. These findings support the proposal that cattle and water buffaloes in the geographical areas studied in Sri Lanka are not significant reservoirs for human infection with *Giardia*.

Both *Cryptosporidium* and *Giardia* were detected concurrently in 28.8% of 340 samples from cattle, which is in accordance with previous studies of cattle [77-79]. For each genus, the numbers of test-positive samples were higher in cattle than in water buffaloes, which seems to be consistent with a prevalence of up to 100% recorded in studies of dairy calves [22] compared with 9.5-38.3% in water buffalo [28,29,61,80-82].

**Conclusions**

Results of the present study suggest that the epidemiology of bovine cryptosporidiosis and giardiasis in Sri Lanka is distinct from those of other parts of the world. Unique nucleotide substitutions in the pSSU region appear to be specific to Sri Lanka. Expanded studies of domestic and wild bovids in various regions in Sri Lanka are required to test this proposal. The apparent absence of *C. parvum* from cattle and buffaloes and the low occurrence of *G. duodenalis* assemblage A in cattle suggest that bovids in the regions studied here have limited significance as reservoirs for human infections. Future work should be focused on large-scale studies to investigate the molecular epidemiology of cryptosporidiosis and giardiasis in a wide range of animals in Sri Lanka and in neighbouring countries in South Asia.

**Additional files**

**Additional file 1: Summary of salient information (Cryptosporidium species/genotypes, host/environmental source, country, GenBank accession nos. of sequences and associated references) pertaining to the SSU sequences used in the phylogenetic analysis of pSSU data**

| Species/Genotype | Environmental Source | Country | Accession Nos. |
|------------------|----------------------|---------|----------------|
| *C. parvum*      | Cattle               | Sri Lanka | EU410344, AY741305, EU245042, EF489038, EU331243 and AB712384 |
| *G. duodenalis* A | Water buffalo         | Sri Lanka | EU410344, AY741305, EU245042, EF489038, EU331243 and AB712384 |

**Additional file 2: Pairwise comparison of nucleotide sequence differences in the small subunit of nuclear ribosomal RNA (pSSU) among Cryptosporidium species or genotypes representing reference sequences (GenBank accession nos. EU410344, AY741305, EU245042, EF489038, EU331243 and AB712384) and those from bovids studied herein (bold-type).**

| Species/Genotype | Accession Nos. |
|------------------|----------------|
| *C. parvum*      | EU410344, AY741305, EU245042, EF489038, EU331243 and AB712384 |
| *G. duodenalis* A | EU410344, AY741305, EU245042, EF489038, EU331243 and AB712384 |

**Additional file 3: An alignment of known reference sequences representing a part of the small subunit of nuclear ribosomal RNA (pSSU) of Cryptosporidium species or genotypes (GenBank accession nos. EU410344, AY741305, EU245042, EF489038, EU331243 and AB712384) with homologous sequences derived from Cryptosporidium from bovids in the present study.**

A dot denotes a nucleotide that is identical to that in the top sequence; a dash represents a gap.

**Additional file 4: Summary of salient information (Giardia species/assemblages, host origins, country, accession nos. of sequences and associated references) pertaining to the tpi sequences used in the phylogenetic analysis of tpi data.**

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

HA carried out molecular laboratory work, data analysis, interpretation and drafting of the manuscript. KU collected samples. AW assisted with the phylogenetic analyses. HA, RBG and ARJ wrote the manuscript with critical input from RPVR and other authors. RBG and ARU conceived the project and attracted the funding. All authors read and approved the final version of the manuscript.

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