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Clinical cases of zoonotic *Cryptosporidium parvum* (subtype IIdA15G1) infections in Korean goats

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Abstract: *Cryptosporidium parvum* is an enteric protozoan, which causes severe diarrhoea in a wide range of vertebrate hosts including ruminants and humans. *C. parvum* infections are responsible for immense economic losses to the livestock industry; furthermore, the zoonotic spread of the infection may lead to mortality in immunosuppressed humans. In the present study, we report two cases of severe cryptosporidiosis in goat kids in the Republic of Korea. Both cases were afflicted with severe diarrhoea upon presentation; the histopathological examinations revealed atrophied and fused intestinal villi and numerous circular basophilic organisms in the jejunum and ileum. Both cases were diagnosed with cryptosporidiosis based on the results of the histopathological analysis, amplification of the *C. parvum gp60* gene, modified Ziehl-Neelsen staining, and *C. parvum* antigen ELISA. According to the phylogenetic analysis using the *C. parvum gp60* gene for the genetic subtypes, the *C. parvum* isolates were identified as subtype IIdA15G1 with zoonotic potential. This is the first pathological report of caprine cryptosporidiosis induced by *C. parvum* subtype IId in the Republic of Korea. Considering the clinical manifestations associated with the pathological lesions and the zoonotic significance of these findings, the continuous monitoring and prevention of *C. parvum* infections in goats are essential for minimising the economic losses in ruminant farms and in maintaining public health safety standards.

Keywords: cryptosporidiosis; diarrhoea; goat; histopathology; phylogenetic analysis; zoonosis

Cryptosporidium spp. is an enteric protozoan parasite with a wide range of hosts, including humans. Cryptosporidiosis manifests in the form of severe diarrhoea in immunocompromised humans and young ruminants, primarily in the age group 5–21 days (Yu and Seo 2004; Paraud and Chartier 2012; Baroudi et al. 2018). These parasites are transmitted via the faecal-oral route of contact. In the Republic of Korea, *Cryptosporidium* spp. was detected in 9.9% of the diarrhoeal faecal samples from young calves and *C. parvum* was detected in 4.4% of the diarrhoeal faecal samples from pre-weaned Korean native calves (Lee et al. 2016a; Lee et al. 2019). Of the approximately 40 Cryptosporidium

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species reported to date, *C. parvum*, *C. ubiquitum*, and *C. xiaoi* are the most common species in small ruminants; while *C. parvum* and *C. ubiquitum* are associated with human cryptosporidiosis (Lee et al. 2016a; Baroudi et al. 2018; Enemark et al. 2020).

Based on the 60-kDa glycoprotein-encoding gene (*gp60* gene) analysis most commonly used for the identification of the subtype families, the subtypes IIa and IId have been reported to be involved in zoonotic cryptosporidiosis (Lee et al. 2016a; Baroudi et al. 2018). In the Republic of Korea, several *Cryptosporidium* spp. isolated from calf and human diarrhoeal faeces have been reported to belong to subtype IIa (Lee et al. 2016a; Lee et al. 2019; Ma et al. 2019). Although numerous field case reports, molecular biological studies, and the enumeration of the observed pathological characteristics exist on bovine cryptosporidiosis in Korea, limited information is available on cryptosporidiosis in goats (Baek et al. 2014; Baroudi et al. 2018). Also, no other subtype studies have been conducted in the Republic of Korea. Therefore, the goal of the present study was to analyse the first documented clinical cases of *C. parvum* infection in goats in the Republic of Korea and elucidate upon the genetic characteristics within the *C. parvum* isolates from diarrhoeic goat kids.

Case histories

Histories and gross pathological findings pertaining to the two cases:

In March 2019, a goat farm located in Sangju, Republic of Korea, housing 480 goats (*Capra aegagrus hircus*), reported the sudden death of nine neonatal kids (aged one week to one month) afflicted by diarrhoea. Among the dead goats, a 21-day-old goat (Cap-1) was referred to the Animal and Plant Quarantine Agency (APQA). Upon dissection, the intestinal serosa and mucosa of the small intestine with a thinned wall was hyperaemic and the blood vessels of the intestinal walls were congested (Figure 1). Furthermore, the large-intestinal contents were pasty in consistency.

In March 2018, a massive outbreak of diarrhoea was reported in neonatal native Korean goats (*Capra hircus coreanae*) on a farm located in Naju, Republic of Korea. There was a total of 800 goats on the farm. Clinically, 30% of the goat kids (aged five days to one month) on the farm manifested with anorexia, diarrhoea, and prostration. Among them, 140 kids in the herd died due to diarrhoea. Two dead goats (Cap-2), each 7 days old, were handed over to APQA for diagnostic examination. Thinned and distended intestinal walls of the ileum, with watery yellowish contents, were observed at necropsy.

HISTOPATHOLOGICAL EXAMINATIONS

After necropsy, the representative tissues were fixed in 10% neutral buffered formalin for 24 hours. The fixed representative tissues were processed routinely and 2-µm sections were stained with haematoxylin and eosin (Baek et al. 2014).

LABORATORY EXAMINATION

The faecal samples were directly collected from the rectum. A modified Ziehl-Neelsen method and a commercial enzyme-linked immunosorbent assay (ELISA) kit (*Cryptosporidium parvum* Antigen Test Kit; IDEXX Laboratories, Inc., Westbrook, ME, USA) were used to detect the Cryptosporidium oocysts and *C. parvum* antigen in the faecal samples, respectively (Yu and Seo 2004). The genomic

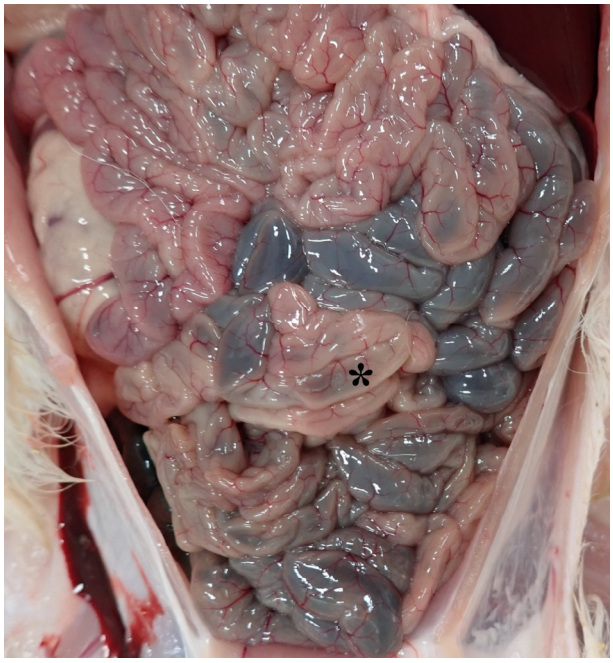

Figure 1. Gross appearance of the dead goat intestines (Cap-1)

Hyperaemic and thinned small-intestinal walls and blood vessel congestion (asterisk) of the intestinal walls

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DNA was extracted from the faecal samples and intestinal tissues using a QIAamp Mini Stool Kit and a DNA Mini Kit (Qiagen, Hilden, Germany), respectively. To detect *C. parvum* in the faecal samples and intestinal tissues, a nested polymerase chain reaction (PCR) was performed using a primer set capable of specifically amplifying the *C. parvum gp60*, as previously described (Lee et al. 2016a).

For the differential diagnosis, the intestinal contents and faecal samples were aseptically collected and then inoculated onto sheep blood agar (Asan Pharmaceutical Co. Ltd., Seoul, Republic of Korea) and MacConkey agar (Becton Dickinson, Sparks, MD, USA). To detect the major viral enteric viruses, including rotavirus, coronavirus, and bovine viral diarrhoea (BVD) virus, a LiliF-BD-Multi RT-PCR Kit (iNtRON Biotechnology, Seongnam, Republic of Korea) was utilised, in accordance with the manufacturer's instructions. An additional PCR was carried out to detect the gastrointestinal parasites, including *Giardia*, *Blastocystis,* and *Enterocytozoon*, as previously described (Lee 2007; Lee et al. 2016b; Lee et al. 2018). The faecal samples were evaluated using the standard flotation method to detect parasite oocysts, as previously described (Foreyt 2013).

PHYLOGENETIC ANALYSIS AND SUBTYPING

A phylogenetic analysis was performed using the partial region of the *C. parvum gp60*. The sequence alignment was performed using Clustal X and subsequently analysed in MEGA v7.0 (Pennsylvania State University, State College, Pennsylvania, USA). Additionally, the neighbour-joining phylogenetic tree based on *gp60* was reconstructed with Kimura's two-parameter model with 1 000 replicates (Kimura 1980). The sequences of *C. parvum* from the goats in the present study were compared with previously reported reference sequences, which were derived from various animals and countries collected from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) (Sulaiman et al. 2005; Cui et al. 2014; Lee et al. 2016a; Feng and Xiao 2017; Zahedi et al. 2018; Qi et al. 2019; Mravcova et al. 2020). Additionally, the sequence of *C. hominis* 8906 (GenBank Accession No.: AY738196) was used as an outgroup (Sulaiman et al. 2005). Subtypes were recognised based on the number of trinucleotide repeat (TCA or TCG) coding for the amino acid serine (Sulaiman et al. 2005).

HISTOPATHOLOGICAL EXAMINATIONS RESULTS

Histopathologically, villous atrophy and fusion were observed and extensive losses of villi with haemorrhaging, as well as numerous variably-sized circular basophilic organisms (approximately 1–3 μm in diameter) adherent to the mucosal surface and inside the lamina propria, were also detected in the jejunum of Cap-1. Furthermore, the lamina propria was expanded by mononuclear cells and necrotic cells with pyknotic or karyorrhectic nuclei (Figure 2A). There were no histological findings in the locations other than the small intestines.

The histopathological findings for Cap-2 were more severe than those recorded for Cap-1. Villous fusion and atrophy with haemorrhaging were noted and dilated crypts with necrotic cells were observed. Cellular infiltration involving necrotic cells, lymphocytes, plasma cells, and a few eosinophils, similar to the observations in Cap-1, was seen in the lamina propria of the jejunum. Numerous circular basophilic organisms (approximately 1–3 μm in diameter) were attached to the brush border and inside the lamina propria in the jejunum and ileum (Figure 2B).

LABORATORY EXAMINATION RESULTS

Following the modified Ziehl-Neelsen staining and *C. parvum* ELISA (IDEXX), both cases tested positive for *C. parvum* oocysts in the faeces (Figure 3). Moreover, *C. parvum gp60* was identified in the small-intestinal tissue DNA of both cases.

Additionally, *Clostridium perfringens* type D was isolated from the faecal samples of Cap-1. *C. perfringens* type A was isolated from the small intestines and caecum of Cap-2. The PCR results were negative for all viral pathogens (rotavirus, coronavirus, and BVD virus) and other gastrointestinal parasites (*Giardia*, *Blastocystis,* and *Enterocytozoon*) in both cases. With regards to the faecal flotation, no parasitic oocysts were found.

PHYLOGENETIC ANALYSIS AND SUBTYPING RESULTS

The partial sequences of the *gp60* obtained in the current study were deposited in the GenBank

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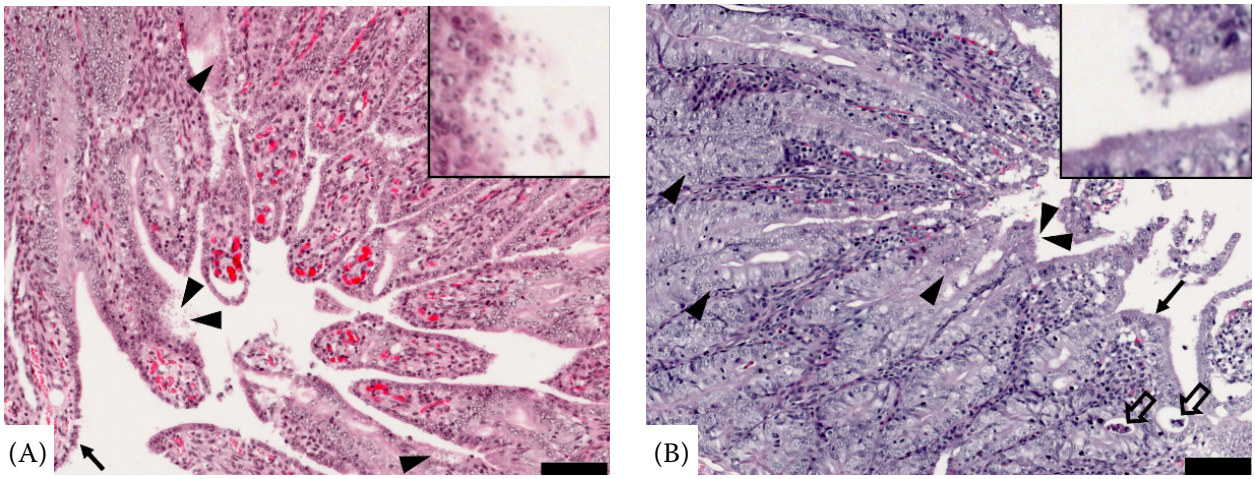

Figure 2. Haematoxylin and eosin (H&E) stain of the small intestine

(A) H&E stain of the small intestine in Cap-1. Villous fusion (arrow) and loss of the apical villi with numerous small basophilic organisms (arrowheads, inset) were observed. Inflammatory cells and debris, with associated haemorrhaging, that infiltrated into the lamina propria were identified. Scale bar = 100 µm. (B) H&E stain of the small intestine in Cap-2. Villous fusion (arrow) and detachment of enterocytes were observed. Furthermore, dilated crypts containing necrotic cells (hollow arrows) were seen and there were multiple small basophilic organisms (arrowheads, inset) attached to the brush border in the small intestine. Cellular infiltration involving necrotic cells and mononuclear cells was found. Scale bar = 100 µm

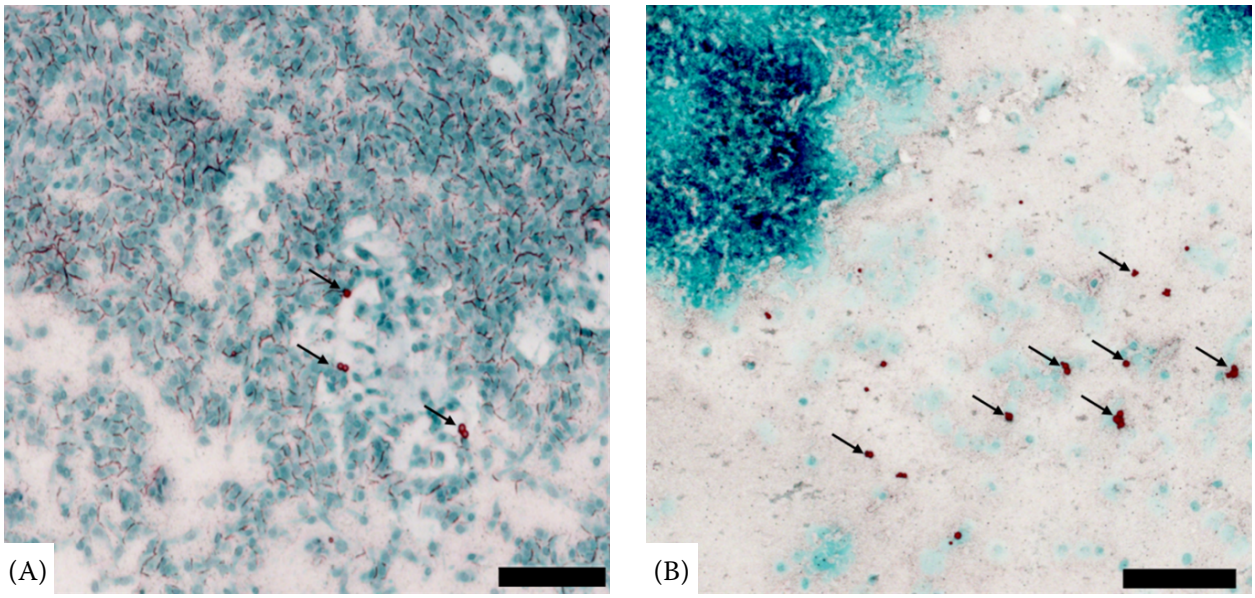

Figure 3. Modified Ziehl-Neelsen stain of the faecal samples

Cryptosporidium oocysts (arrows) in the faeces were stained a pink-red colour. (A) Faecal sample from Cap-1 and (B) faecal sample from Cap-2. Scale bar = 100 µm

database (under Accession No. MK905082 and MK905083). The sequences collected in both cases belonged to the IId group according to the phylogenetic analysis (Figure 4). Furthermore, all the sequences were identified as the IIdA15G1 subtype in accordance with a previous study (Sulaiman et al. 2005). The isolates subtyped as IIdA15G1 in the present study clustered with *C. parvum* IIdA15G1 isolates from calves, monkeys, sheep, and yaks in China; lambs in Spain and isolates obtained from humans in the Netherlands and Slovakia.

The *gp60* sequences were identical to those of the same cluster in the neighbour-joining phylogenetic tree (Figure 4).

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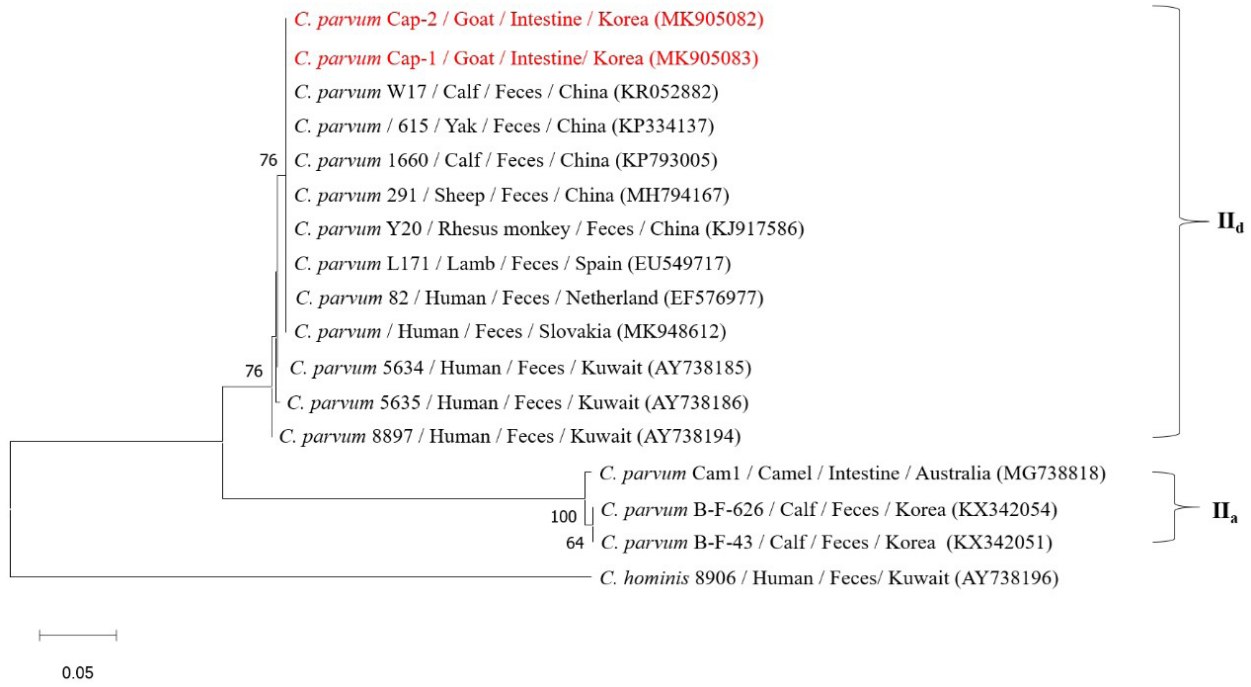

Figure 4. Phylogenetic tree of the partial *gp60* region of *Cryptosporidium* spp.
The tree was constructed using the neighbour-joining method with 1 000 replicates. The sequences isolated in the present study are indicated in red. The species, host, source, identified region, and GenBank accession number are included

DISCUSSION AND CONCLUSIONS

A Cryptosporidium infection is a significant enteric disease that causes diarrhoea among neonatal ruminants, resulting in considerable economic losses to the affected ruminant farms. In the Republic of Korea, case reports on the infection are limited to cattle and humans (Moon et al. 2013; Baek et al. 2014). To the authors' knowledge, the pathological findings associated to Cryptosporidium infections in goats have not been previously reported in the Republic of Korea.

In the present study, the histopathological lesions were limited to the small intestines in both cases. Numerous small grey basophilic organisms and inflammatory cells that infiltrated into the lamina propria with necrotic cell debris and haemorrhaging were observed. Based on the histopathological findings and laboratory examination results, both cases were diagnosed as *C. parvum*-induced villous atrophy and fusion. These findings were similar to the clinical cases of previous studies reported in Turkey and Oman (Johnson et al. 1999; Sevinc et al. 2006). In the causative examination, *C. perfringens* type D was isolated from the faeces of Cap-1 and *C. perfringens* type A was isolated from the intestines of Cap-2. Although *C. perfringens* aggravated the lesions, the isolated bacteria were regarded as commensal intestinal organisms because there were no specific pathological findings, such as enterotoxaemia and fibrinous haemorrhagic colitis associated with them (Paraud and Chartier 2012; Kim et al. 2013).

According to a previous study, numerous oocysts were excreted by *C. parvum*-infected goat kids, aged between 5 and 21 days. The morbidity and mortality rates can be elevated in *C. parvum* infected goat kids (Paraud and Chartier 2012). The three dead goats that were subjected to investigation cannot be considered representative of all the dead goats on both farms. It was, thus, impossible to investigate all the environmental factors and practiced farm management procedures that may have contributed to the lethal outcome. However, we concluded that *C. parvum* was the most likely causative agent of the diarrhoea and related lethality on the two farms, considering the pathological and laboratory findings.

According to the phylogenetic analysis, the *C. parvum* diagnosed in the present study clustered into the IId subtype family. However, a previous large-scale study on *Cryptosporidium* spp. in young Korean calves with diarrhoea showed only members of the IIa family (Lee et al. 2016a; Lee

https://doi.org/10.17221/212/2020-VETMED

et al. 2019). In the present study, all the *C. parvum* isolates were classified as IIdA15G1. This subtype has caused severe economic losses in dairy farms in northwestern China and has been isolated from various host species such as calves, monkeys, rodents, sheep, and yaks in China, as well as from human hosts in Slovakia and the Netherlands (Cui et al. 2014; Feng and Xiao 2017; Li et al. 2019; Qi et al. 2019; Mravcova et al. 2020). This subtype has been regarded as being zoonotic, transmitted between different species (Feng and Xiao 2017). For instance, a Slovakian patient, who came into contact with infected calves on a farm, suffered from diarrhoea induced by *C. parvum* IIdA15G1 (Accession No. MK948612). Considering that ruminants serve as reservoirs of *C. parvum*, we accentuate the importance of a continuous epidemiological surveillance of the *C. parvum* sub-genotype prevalence. A cryptosporidiosis outbreak (*C. parvum* IIdA15G1 subtype) in Chinese dairy cattle, due to the *C. parvum* IIdA15G1 sub-genotype was related to the possible financial collapse in ruminant farms, particularly of those housing cattle (Cui et al. 2014).

Although halofuginone lactate, not licensed against goat cryptosporidiosis, has been reported to reduce diarrhoea, there is the disadvantage that has to be administered orally for seven consecutive days (Giadinis et al. 2008). Considering the lack of efficacious medication for controlling a massive outbreak of cryptosporidiosis, surveillance and prevention is the most crucial approach towards mitigating the economic losses in the livestock industry (Paraud and Chartier 2012).

In summary, our pathological findings and subtyping results are helpful to better understand the *C. parvum* infection in goats and underscore the necessity of future investigations into further characterising goat *C. parvum* isolates.

Conflict of interest

The authors declare no conflict of interest.

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