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Dual infection with *Entameoba invadens* and *Aeromonas hydrophila* in a captive anaconda (*Eunectes murinus*) leading to necrotising gastroenteritis and hepatocyte death

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Abstract: A ten-year-old male captive anaconda (*Eunectes murinus*) with a history of anorexia was found dead in Gwangju Uchi Zoo. Pathologically, this case showed multiple necrotising gastroenteritis ulcers, the death of large numbers of hepatocytes and multiple instances of chronic active interstitial nephritis. *Entamoeba invadens* and *Aeromonas hydrophila* were frequently found in these lesions. Confirmation of these pathogens was made using molecular and phylogenetic analyses. The TUNEL assay revealed the apoptotic nature of hepatocyte cell death.

Keywords: snake; *E. invadens*; *A. hydrophila*; coinfection

Entamoeba invadens (*E. invadens*) causes gastroenteritis and hepatitis and is one of the most common and serious protozoal pathogens in both wild and captive snakes, lizards and other reptiles worldwide (Geiman and Ratcliffe 1936; Jacobson et al. 1983; Jakob and Wesemeier 1995; Kojimoto et al. 2001). *Aeromonas hydrophila* (*A. hydrophila*), a Gram-negative, facultative anaerobic bacillus, is widely distributed in natural waters (Orozova et al. 2012) and exhibits a predilection for poikilothermic animals (Shotts et al. 1972). This organism has been implicated in a variety of lesions, including ulcerative stomatitis, pneumonia and septicaemia (Esterabadi et al. 1973). Although both pathogens pose a threat to captive reptiles worldwide, there is still a paucity of information about these infections in captive South Korean snakes (Jho et al. 2011). In this paper, we report dual infection of *E. invadens* and *A. hydrophila* in a ten-year-old, male, captive anaconda kept at Gwangju Uchi Zoo.

Case description

A ten-year-old male captive anaconda (*Eunectes murinus*) weighing 3 kg was found dead in the Uchi Zoo located in Gwangju city. The animal had a history of anorexia after an abrupt change in feed from fresh to frozen mice. Gross examination showed multiple discrete to coalescing ulcers throughout the gastrointestinal mucosa (Figure 1A), and multiple irregular, discrete, white foci in the liver

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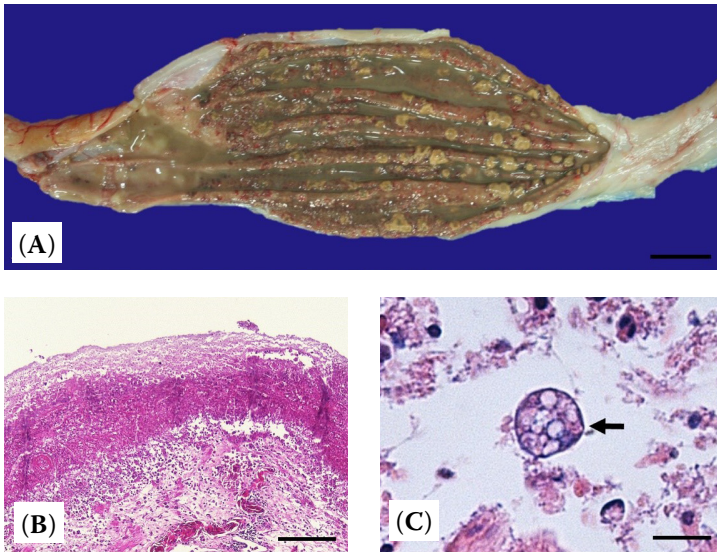

Figure 1. (**A**) Stomach showing multiple severe ulcers covered with fibrinonecrotic debris. Note the multiple instances of severe haemorrhages. Bar = 1 cm. (**B**) Stomach ulcer showing severe diffuse mucosal necrosis. Note the oedema and lymphoid cell infiltration in the submucosa. Bar = 100 µm. (**C**) A trophozoite of *Entamoeba invadens* was interspersed with the necrotic debris in the mucosa. Bar = 10 µm

(Figure 2A) and kidneys. No gross lesions were found in any other visceral organs. For histopathological observation, tissues and organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin and periodic acid-Schiff (PAS). To determine apoptotic hepatocytes, selected liver sections were used for a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labelling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Histopathologically, the ulcerative lesions observed throughout the gastrointestinal tract were transmural and covered with severely fibrinonecrotic debris intermingled with a varying number of heterophils, macrophages and giant cells (Figure 1B). The submucosa and muscular layer showed oedema, haemorrhage and an infiltration of inflammatory cells. The white foci grossly observed in the liver consisted of necrotic and apoptotic hepatocyte cell deaths; the former was characterised by rupture of swollen cell membranes with distinct nuclear changes such as pyknosis, karyorrhexis, karyolysis or nuclear absence, whereas the latter showed shrunken or fragmented cytoplasm with condensed chromatin (Figure 2B). The discrete to coalescing foci of hepatocyte cell death contained infiltration of a variable number of heterophils, lymphocytes, plasma cells, macrophages and giant cells. The TUNEL assay confirmed the presence of scattered apoptotic hepatocytes

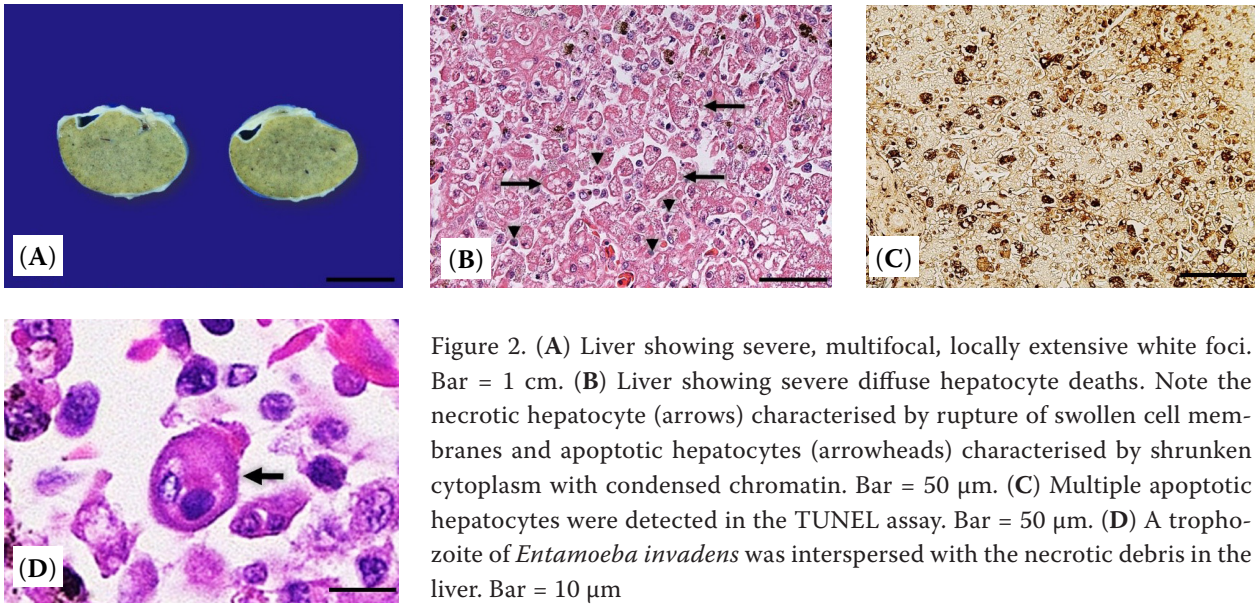

Figure 2. (**A**) Liver showing severe, multifocal, locally extensive white foci. Bar = 1 cm. (**B**) Liver showing severe diffuse hepatocyte deaths. Note the necrotic hepatocyte (arrows) characterised by rupture of swollen cell membranes and apoptotic hepatocytes (arrowheads) characterised by shrunken cytoplasm with condensed chromatin. Bar = 50 µm. (**C**) Multiple apoptotic hepatocytes were detected in the TUNEL assay. Bar = 50 µm. (**D**) A trophozoite of *Entamoeba invadens* was interspersed with the necrotic debris in the liver. Bar = 10 µm

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within the multiple cell death lesions (Figure 2C). The kidney showed multiple moderate interstitial infiltrations of lymphoid cells intermingled with heterophils, macrophages and giant cells and multiple instances of moderate linear tubular degeneration and necroses. Moreover, congestion and haemorrhages were frequently found in the gastrointestinal tract, liver and kidney. Rod-shaped bacilli, either discretely or as colonies, were found in or around the above lesions. No significant lesions except congestion were seen in other organs. Most notably, a varying number of PAS-positive, round-to-ovoid amoebic trophozoites were identified in the above lesions of the gastrointestinal track, liver and kidney (Figures 1C and 2D). The trophozoites exhibited vacuolated cytoplasm surrounded by a thick eosinophilic wall and a single centrally-to-eccentrically located, round-to-ovoid nucleus (Figures 1C and 2D). Such morphological features were suggestive of the genus *Entamoeba*.

To identify the *Entamoeba* species, DNA was extracted from the fresh tissue samples of the stomach, each region of the small and large intestines, lung and kidney using the Accuprep® Genomic DNA Extraction Kit (Bioneer, Gyeonggi-do, Republic of Korea) and amplified by polymerase chain reaction (PCR) using previously reported primer sets targeting the 16S ribosomal RNA (rRNA) gene of *E. invadens* (926 base pairs (bp)), *E. ranarum* (796 bp), *E. terrapinae* (769 bp), and *E. insolita* (677 bp) (Bradford et al. 2008). The results showed that a single band of approximately 926 bp in length was detected from the stomach, each region of the small and large intestines and the kidney using the above PCR assay only with the *E. invadens*-specific primer pair. To confirm these PCR results, one selected PCR product based on the intensity of the bands shown by agarose gel electrophoresis and ethidium bromide visualisation was eluted and purified using the GenClean II kit (BIO 101, USA) according to the manufacturer's instructions. DNA sequencing was carried out using an ABI system 3700 automated DNA sequencer (Applied Biosystems, USA). Using the DNA Basic module (DNAsis MAX, USA), the nucleotide sequence of the partial 16S rRNA gene of *E. invadens* (877 bp, devoid of primer pair sequences) was compared with other known *Entamoeba* species. Phylogenetic analysis based on the nucleotide alignments was constructed using the MEGA 6 software package (Tamura et al. 2013). Genetic distances between our strain and other reference strains were calculated using the Kimura-2 correction parameter at the nucleotide level. A phylogenetic tree was constructed using the neighbour-joining method with 1000 bootstrap replicates. Our *E. invadens* strain 16-30 showed high nucleotide identity with the *E. invadens* strains PZ (99.6%) and IP-1 (99.1%) but had low nucleotide identity with other *Entamoeba* species. The Korean *E. invadens* strain 16-30 clustered with *E. invadens* strains PZ and IP-1 but was only distantly related with other species such as the *E. terrapinae* M strain (Figure 3).

To identify the bacteria, each homogenate from the rectum, spleen and kidney were cultured on both blood and MacConkey agar plates. Three representative colonies from each homogenate sample were further propagated in Luria Broth culture. Each extract of DNA isolated from the resulting culture was then subjected to PCR analysis using a universal primer pair specific for the partial 16S rRNA genes of bacteria as described elsewhere (Park et al. 2010). Similar to the procedures for identifying *Entamoeba* species, PCR products were eluted, purified and then sequenced. Then, molecular and phylogenetic analyses were performed as described above. Nucleotide sequences from our three colonies showed 100% nucleotide identity with each other and had the highest identity with

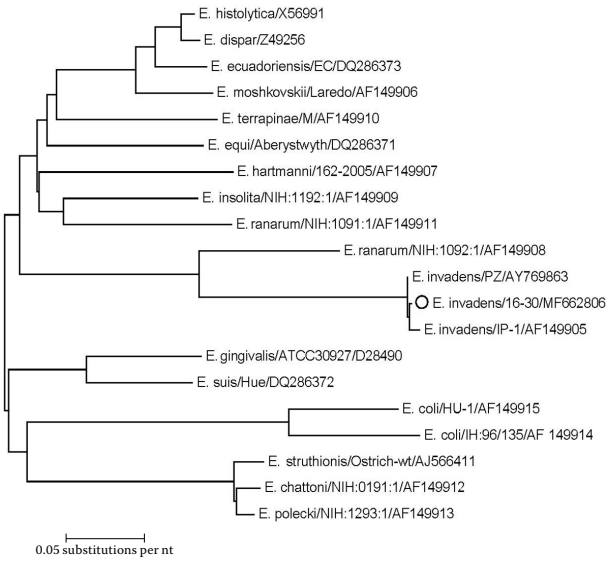

Figure 3. Phylogenetic analysis of the partial nucleotide sequences of the 16S ribosomal genes of different *Entamoeba* species. Each strain in the tree is indicated by species, strain name and GenBank accession number. The Korean strain is indicated by an empty circle

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the *A. hydrophila* strains HZN-98, MB-2, N-3-3-1 and TE090214 (99.8–100% nucleotide identity). All Korean strains were closely related with *A. hydrophila* strains but not to other *Aeromonas* species or *Listonella anguillarum*, which was used as an outgroup.

DISCUSSION AND CONCLUSIONS

Diagnosis of *Entamoeba* species has largely relied on microscopic identification (Bradford et al. 2008). However, the similar morphological appearance of pathogenic (*E. invadens*) and non-pathogenic (*E. terrapinae*, *E. insolita*, etc.) species could make a definitive diagnosis difficult (Geiman and Ratcliffe 1936; Bradford et al. 2008). The hallmark lesions observed in the present case, such as multiple severe ulcerations in the gastrointestinal tract, pronounced hepatocyte death and the presence of concurrent multiple trophozoites, are mostly linked with protozoal diseases in snakes (Page 1966). Since PCR-based methods are known to be more sensitive and specific than other classical methods for diagnosis of infection with *Entamoeba* species, we employed a PCR assay with primer pairs specific for several *Entamoeba* species (Bradford et al. 2008). Accordingly, a band of the expected size was only amplified by a primer pair specific for *E. invadens*. Thus, this case could be diagnosed as an *E. invadens* infection (Esterabadi et al. 1973). This is further confirmed by the identical nucleotide sequence of the PCR amplicon and its close phylogenetic relationship with several *E. invadens* strains.

In captive snakes, *A. hyrophila* can cause asymptomatic and symptomatic diseases; the latter include ulcerative stomatitis which may progress to septicaemia and eventually spread to other organs (Page 1966; Esterabadi et al. 1973; Gavrilescu and Denkers 2003; Orozova et al. 2012). Internal organs are usually found to be congested and/or haemorrhagic (Page 1966; Esterabadi et al. 1973; Orozova et al. 2012). In the present case, septicaemia-induced haemorrhages and congestions as well as bacterial colonies were found in the gastrointestinal tract, liver and kidney. Moreover, all isolated bacteria from those organs had high nucleotide identities with the 16S rRNA gene of *A. hydrophila* and showed a close phylogenetic relationship with *A. hydrophila* strains, confirming the bacterial etymology of this case.

Sudden dual infection of *E. invadens* and *A. hydrophila* in the present captive anaconda may be attributed to an abrupt change in feed from fresh and living to frozen mice, which may have reduced the levels of the normal mucosal commensal bacteria and thus induced pathogen growth (Kelly and Conway 2005; Baumler and Sperandio 2016).

In response to many protozoan and bacterial infections, subroutines of programmed cell death such as necroptosis and apoptosis can be induced as a host innate immune response (Gavrilescu and Denkers 2003). Although both *E. invadens* and *A. hydrophila* are known to cause necrotic cell death in snake hepatocytes (Page 1966; Esterabadi et al. 1973), its subroutines including apoptosis have not been well defined in reptiles so far. In the present study, morphological characteristics of apoptotic cell death in the anaconda liver were confirmed using the TUNEL assay.

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