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Research Brief

Prevalence and molecular characterization of *Cryptosporidium* in ostriches (*Struthio camelus*) on a farm in central Vietnam

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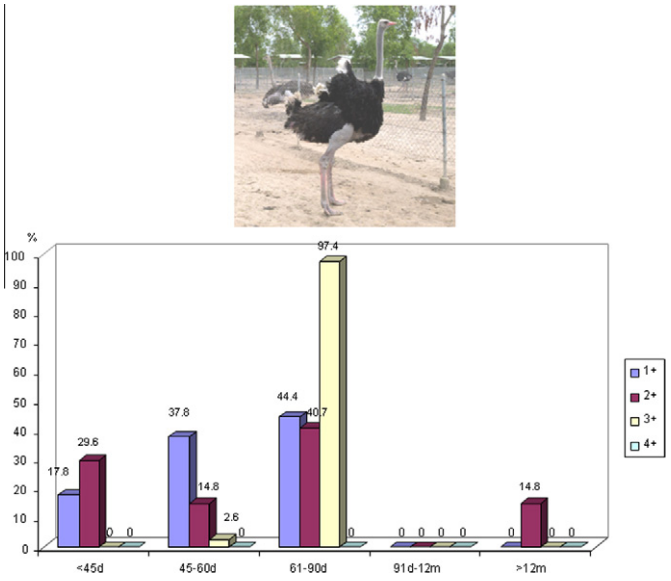
HIGHLIGHTS

- Detection of *Cryptosporidium* sp. in ostriches in Vietnam, based on microscopic and molecular methods.
- Identification of *Cryptosporidium* avian genotype II in ostriches in Vietnam, using 18S rRNA, HSP-70 and actin based genotyping tools.
- Epidemiological characrerizations of cryptosporidiosis in ostriches in Vietnam.

GRAPHICAL ABSTRACT



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ABSTRACT

This study was performed to determine the prevalence and molecular characterization of *Cryptosporidium* in ostriches on a farm in Khanh Hoa province, central Vietnam. A total of 464 ostrich fecal samples were examined *Cryptosporidium* oocysts using the modified Ziehl–Neelsen staining method, and 110 (overall prevalence 23.7%) were identified as positive by microscopy. Prevalence of *Cryptosporidium* in animals of <45 days, 45–60 days, 61–90 days, 91 days–12 months and >12 months was 23.5% (16/68), 33.3% (22/66), 35.2% (68/193), 0 and 5.8% (4/69), respectively ($p < 0.05$). The majority of positive samples scored as the 3+ level of intensity of infection were from 61 to 90 days ostriches. Molecular analysis in the 18S ribosomal RNA, 70 kDa heat shock protein and actin genes demonstrated the presence of only *Cryptosporidium* avian genotype II in ostriches in central Vietnam.

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1. Introduction

The genus *Cryptosporidium*, belonging to the phylum Apicomplexa, infects a wide range of vertebrate hosts, including birds domesticated, wild and captive (Fayer, 2010). Natural cryptosporidial infection in birds can be respiratory, enteric or renal, depending on species of birds infected and *Cryptosporidium* species involved in the infection (Lindsay and Blagburn, 1990).

Of more than 20 *Cryptosporidium* species considered valid so far, three avian *Cryptosporidium* spp. have been named: *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, *Cryptosporidium galli* (Xiao et al., 2004; Fayer, 2010), and eleven genotypes: the avian genotypes I–V, the black duck genotype, the Eurasian woodcock genotype, and goose genotypes I–IV have been reported (Fayer, 2010; Xiao, 2010). Of these, the intestinal species *C. meleagridis* is ascribed to cause sporadic cases and outbreaks of diarrhea not only in turkeys, but also in humans (Pedraza-Diaz et al., 2001; Xiao and Feng, 2008).

Previous studies have shown that cryptosporidiosis is common in ostriches. These studies, however were primarily based on microscopy of oocysts in feces and histological evidences in infected tissues (Gajadhar, 1994; Penrith et al., 1994; Jardine and Verwoerd, 1997), therefore the species/genotypes infecting ostriches were unidentified. Recently, few studies have used molecular tools to characterize the genetic structure of *Cryptosporidium* spp. in ostriches and reported the occurrence of the *Cryptosporidium* avian genotype II and *C. bailey* in surveyed ostriches (Meireles et al., 2006; Ng et al., 2006; Nakamura et al., 2009; Wang et al., 2010). Thus far, prevalence and molecular characterization of *Cryptosporidium* in ostriches are not as well known as in other vertebrates such as mammals.

The genus *Cryptosporidium* has been studied in cattle (Nguyen et al., 2007, 2012a) and pigs (Nguyen et al., 2012b) in Vietnam. However, there is no report of *Cryptosporidium* isolated from ostriches in Vietnam to date. Therefore, the aim of the present study was to determine the prevalence and genotypes of *Cryptosporidium* in ostriches in Vietnam, using molecular tools.

2. Materials and methods

2.1. Sample collection and microscopic examination

The 40 ha industrial ostrich farm, locating near Nha Trang city, Khanh Hoa province, central Vietnam, was visited during the period from January to March 2011. A total of 464 fecal samples of ostriches, comprising animals of <45 days ($n = 68$), 45–60 days ($n = 66$), 61–90 days ($n = 193$), 91 days–12 months ($n = 68$) and >12 months ($n = 69$), were collected. Fresh feces voided within 5–10 min was placed in individual plastic bags and held at 4–8 °C until processed. Samples of different consistency and color were chosen on the ground within 1–2 m^2 to avoid sampling error. Ostriches of different age groups were reared in semi-indoor pens separately. No clinical signs was observed at the time of sampling. The use of animals in this research has been approved by the Scientific Committee of Central Vietnam Veterinary Institute.

Primary screening of fecal samples was performed by the modified Ziehl–Neelsen (mZN) staining method as described previously (OIE, 2004). The intensity of infection were scored based on the number of oocysts observed under 400× magnification, according to the OIE's instructions with minor modifications, as follows: 1+ = less than 5 oocysts per slide, 2+ = 5–10 oocysts per slide, 3+ = more than 10 oocysts per slide but less than 5 oocysts per field of view, 4+ = 5 or more oocysts per field of view (OIE, 2004). Modified ZN-positive samples confirmed by sucrose flotation method (Nakai et al., 2004) were stored in 2.5% potassium dichromate ($K_2Cr_2O_7$) at 4–8 °C for further analysis.

2.2. DNA extraction and molecular analysis

Microscopically positive samples, scored as the 3+ level were selected for molecular analysis. Fecal DNA was extracted from selected samples using a FavorPrep Stool DNA Isolation Mini Kit (Favorgen Biotech Corporation, Taiwan) according to the manufacturer's instructions. Eluted DNA was dissolved in 30 μl of ultrapure water and stored at −20 °C.

For *Cryptosporidium* genotyping, the 18S ribosomal RNA (18S rRNA), 70 kDa heat shock protein (HSP-70) and actin genes were used. Fragments of the 18S rRNA (~830 bp), HSP-70 (~325 bp) and actin (~1066 bp) genes were amplified by PCR, using primers and protocols described previously (Xiao et al., 1999; Morgan et al., 2001; Sulaiman et al., 2002). Positive (*Cryptosporidium parvum* HNJ-1 strain) and negative (without template) controls were included in each PCR batch. The PCR products analyzed on 1.2% agarose gels were visualized by ethidium bromide staining and purified with ExonucleaseI/Shrimp Alkaline Phosphatase (ExoSAP-ITTM) (USB Corporation, Cleveland, Ohio). Purified products were sequenced in both directions with the same primers used in nested PCR, using an ABI Prism Big Dye Terminator Cycle sequencing kit version 3.1 (Applied Biosystems, CA, USA.) on an ABI Prism 3130 genetic analyzer automated sequencer, according to the manufacturer's instructions.

The obtained sequences were scanned against the GenBank database by BLAST suite (http://blast.ddbj.nig.ac.jp), and their species were identified. Representative nucleotide sequences in this study have been deposited in GenBank under the accession numbers AB696811-AB696816.

2.3. Statistical analysis

Data were organized in Microsoft Excel 2003 and analyzed using the EpiInfo 2011 package, version 3.5.3 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Prevalence and the 95% confidence interval (CI) were determined using the modified Wald method. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Prevalence and intensity of Cryptosporidium infection

Of the 464 ostriches examined, 110 were positive for *Cryptosporidium* oocysts, the sample prevalence within the farm was 23.7% (95% CI = 20.1–27.8%). Prevalence of *Cryptosporidium* in animals of <45 days, 45–60 days, 61–90 days, 91 days–12 months and >12 months was 23.5% (95% CI = 14.9–34.9%) (16/68), 33.3% (95% CI = 23.1–45.4%) (22/66), 35.2% (95% CI = 28.8–42.2%) (68/193), 0% and 5.8% (95% CI = 1.9–14.4%) (4/69), respectively. Significant differences were found in *Cryptosporidium* prevalences between different age groups ($\chi^2 = 50.9$, $p < 0.05$).

The intensity of *Cryptosporidium* infection was moderate, and the lowest oocyst excretion level (1+) was found in 45 (40.9%) out of 110 *Cryptosporidium* positive ostriches, the 2+ level in 27 (24.5%) positive samples, the 3+ level in 38 (34.6%) and the 4+ level was not detected. The majority of positive samples scored as 3+ level were from 61–90 days ostriches (37/38, 97.4%) (Table 1 and Fig. 1).

3.2. Genetic identification

Of the 38 positive samples scored as 3+ level, 17 PCR products were obtained for the 18S rRNA locus. The remaining *Cryptosporidium* positive samples may have insufficient DNA for PCR amplification, or