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ORIGINAL ARTICLE

Molecular investigation of *Lawsonia intracellularis* in diarrheic and healthy captive ostriches (*Struthio camelus*) in Iran

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Abstract

Lawsonia intracellularis is the causative agent of proliferative enteropathy (PE) in pigs, a disease of economic importance worldwide. The present study aimed to investigate the occurrence of *L. intracellularis* in ostriches using a sensitive and specific nested polymerase chain reaction (nested PCR). A total number of 112 fecal samples (64 healthy, 48 diarrheic) were collected from 11 ostrich farms located in four provinces in Iran (Semnan, Tehran, Gilan, Yazd). The results showed the presence of *L. intracellularis* in three diarrheic and four apparently healthy birds. The frequency of positive results was the same in both groups (6.25% healthy vs. 6.25% diarrheic). Although the results of this study did not reveal any relationship between this organism and diarrhea ($P > 0.05$), the molecular detection of *L. intracellularis* on a farm where birds were suffering from the typical clinical symptoms of PE highlights the need for evaluation of PE in ratites.

Keywords *Lawsonia intracellularis* · Ostrich · Diarrhea · Nested PCR

Introduction

Lawsonia intracellularis is an obligate, Gram-negative intracellular bacterium, belonging to the *Desulfovibrionaceae* family in the phylum proteobacteria that causes an economically important disease in pigs that is known as proliferative enteropathy (PE) (Vannucci and Gebhart 2014). The most significant finding in PE is mucosal thickening and mucosal hyperplasia in the intestine. Importantly, PE in pigs occurs in acute or chronic forms. The acute form is characterized by hemorrhagic enteropathy and is less common. The chronic form is usually subclinical and represented by mild diarrhea that causes reduced performance and weight gain in animals (Guedes 1998).

Although PE is considered as important bacterial disease in the swine industry, concerns have been increased on the possible pathogenicity of this organism in other animal species. Infection by *Lawsonia intracellularis* has been reported in a wide range of domestic and wild animal species including pigs, horses, hamsters, sheep, deer, guinea pigs, foxes, dogs,

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rabbits, ferrets, rats, ostriches, some wildlife species, and non-human primates (Hossain et al. 2016; Vannucci and Gebhart 2014). More recently, the presence of this organism was also showed in dromedary camels (Askari Badouei et al. 2014). The pathogenic capacity of this organism is best understood in pigs, horses, and hamsters (Sampieri et al. 2013; Vannucci and Gebhart 2014). One of the major reasons for the lack of evidence on the pathogenic role of this organism in other species is the challenging and difficult isolation procedure that requires strict conditions (Vannucci and Gebhart 2014). For laboratories not specialized on working with this organism, molecular approaches are more sensitive and practical means of detecting and identifying *L. intracellularis*. A nested polymerase chain reaction (PCR) has been proven to be a sensitive and specific method for detection of this organism in clinical samples (Jones et al. 1993; Nascimento Chiriboga et al. 1999).

Ostrich farming was first established in South Africa, but gradually spread to other countries in different continents (Benson 2012). Based on the latest available data, ostrich production is still dominated by countries in the tropic and sub-tropical zones with an estimated production that represents over 70% of total world production (Benson 2012). Ostrich farming has become more popular in recent years worldwide because of the low-fat meat and other valuable products such as high quality hide and feather. Since ostrich farming is relatively new in many countries, little is known about the diagnosis, prevention, and treatment of diseases (Shanawany 2014). The aim of the present study was to investigate the occurrence of *L. intracellularis* in samples from healthy and diarrheic ostriches using a molecular method for the first time.

Materials and methods

Sampling

A total number of 112 fecal samples were collected using sterile cotton swabs from fresh droppings of birds from eleven ostrich farms located in five different cities in four provinces in the major areas of ostrich farming in Iran including Garmsar (Semnan province), Shahrood (Semnan province), Varamin (Tehran province), Rasht (Gilan province), and Yazd (Yazd province). The samples were collected from the diseased birds and/or some healthy animals in each farm if possible. If animals were asymptomatic, samples were collected from the apparently healthy birds. The number of samples (diarrheic, non-diarrheic) obtained from each farm is presented in Table 1. Samples were transported to the laboratory in cold condition within 24–48 h of collection and processed immediately upon arrival as described below.

Table 1 Distribution of *Lawsonia intracellularis* positive fecal samples on the studied ostrich farms in Iran

Farm	City	Average age (months)	Retarded growth	Number of samples		No. of PCR-positive samples
				Healthy	Diarrheic	
1	Garmsar	1	+[a]	0	8	3
2	Garmsar	7	–	4	0	–
3	Garmsar	1–3	–	8	0	–
4	Garmsar	1	–	0	10	–
5	Shahrood	2–7	–	3	2	–
6	Varamin	2	–	3	3	–
7	Rasht	3	–	10	0	4
8	Varamin	1	–	28	0	–
9	Yazd	8	–	8	0	–
10	Yazd	2	–	0	14	–
11	Yazd	4	–	0	11	–
Total				64	48	7

[a] Retarded growth and mild diarrhea were the major problem in farm 1 in Garmsar

DNA extraction

Crude DNA was extracted from the fecal samples using a commercial DNA extraction kit (Cinnagen, Iran). In brief, swab tips were soaked in 500 μl PBS for 1 h at room temperature. After vigorous vortexing, the tips were discarded. The fecal suspension centrifuged at 450 × g for 5 min to settle down the large fecal materials and 100 μl of the supernatant were transferred to a new tube and the DNA extracted according to the manufacturer's instruction.

Molecular detection

The nested PCR for *L. intracellularis* was conducted in two separate steps using two sets of primer pairs as described previously (Jones et al. 1993). The first PCR was carried out using LIA (5′-tatggctgtcaaacactcc-3′) and LIB (5′-tgaaggtattggtattctc-3′) primer pairs. The nested PCR reaction was carried out using a pair of internal primers including LIC (5′-ttacaggtgaagttattgg-3′) and LID (5′-ctttctcatgtcccataagc-3′). Both PCR assays were conducted in a 25-μl volume, using 1 U *Taq* polymerase, 0.5 μM of each primer, 2 mM $MgCl_2$, 1 × PCR buffer, 200 μM dNTP mix, and ultrapure water. DNA template was 3 μl in the first step and 1 μl of the first PCR product was used in the nested reaction. Particular care was taken to avoid cross contamination between samples. The amplification conditions were as follows: 94 °C for 2 min for initial denaturation, and then 30 cycles of 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 40 s with a final extension at 72 °C for 5 min. The PCR products were electrophoresed in 1.5% agarose gels and visualized after staining with ethidium bromide.

Positive and negative controls were included in each PCR reaction. The positive control was the first sample positive for *L. intracellularis* (OS-223) confirmed by PCR and sequencing in this study. The PCR product of this sample was sequenced and deposited in GenBank after BLAST analysis to ensure the validity of the amplified product (OS-223; GenBank accession number KF199338.1). The occurrence in healthy and diarrheic groups was also compared by the chi-square method.

Results

From the total number of 112 fecal samples (48 diarrheic and 64 apparently healthy ostriches), *L. intracellularis* was detected in 7 (6.2%) samples. Three positive samples belonged to an ostrich farm in Garmsar (farm no. 1) having mild diarrhea and poor growth with approximately 20% morbidity and a few mortalities. In general, diarrhea was mild or birds showed loose and abnormal fecal consistency. The remaining four samples were obtained from an ostrich farm in Rasht (farm no. 7). All of these birds were clinically healthy birds. On this farm, ten samples were obtained, four of which were positive in molecular test (Table 1). The statistical analysis showed no relationship regarding the presence of this organism and diarrhea ($P > 0.05$).

Discussion

Reports of infection with *L. intracellularis* exist in a wide range of domestic and wild animal species worldwide (Guedes 1998). However, there is a gap in knowledge on the pathogenic capacity of *L. intracellularis* in ratites as only few studies in these birds have been reported. Proliferative enteropathy in an ostrich was first reported using multiplex-PCR and histopathology examination (using a special Warthin-Starry silver staining) in a bird showing the typical signs of PE. The affected bird was recorded as a young male ostrich that died following a few months history of poor growth (Cooper et al. 1997b). Additionally, the presence of *L. intracellularis* has been documented in a group of juvenile emus (*Dromaiusnovae hollandiae*) suffering from PE and rectal prolapse (Lemarchand et al. 1997). It is noteworthy that PE has been also reported in chickens in Japan recently (Ohta et al. 2016). Some ostrich farms in Iran have been reported to suffer from poor growth of ostrich chicks with an unknown etiological reason with occasional mortalities and mild diarrhea (personal communication with ostrich specialists). Unfortunately, the sampling time in the present study was very limited (less than 2 months) and only one farm with the typical mentioned characteristics was sampled. Three of the eight sampled birds in this farm (farm no. 1) were found to shed the organism in their feces. Additionally, four of the sampled birds in the other farm (farm no. 7) were also positive without any prior history of disease in the past months. Nevertheless, the overall rate of infection in healthy and diseased birds was similar (6.2%) in the current study. There are a number of reasons and possibilities of interpretation for this observation as will be discussed briefly.

It is important to note that *L. intracellularis* has some unique characteristics that are not commonly found in pathogenic microorganisms. Importantly, as mentioned before, this bacterium has evolved to infect a broad range of hosts. On the other hand, *L. intracellularis* shows modest genetic diversity and almost no phenotypic variations (Vannucci and Gebhart 2014). The partial sequence analysis of the 16S ribosomal DNA of strains from ferret, pig, deer, and ostrich showed 100% identity (Cooper et al. 1997a). Interestingly, recent studies have shown some degree of species-specificity in pig and horse isolates—experimental infections result in longer fecal shedding, prompt seroconversion, and greater pathological changes in animals challenged with the strains of the same species origin (Vannucci et al. 2012). Sampieri et al. (2013) showed a similar specificity for porcine and horse isolates in hamsters and rabbits, respectively. Therefore, it is concluded that for disease induction, the host should be infected with the proper host-specific strain. Secondly, due to the chronic nature of disease, PE needs substantial time to develop the typical symptoms and pathological changes (Vannucci and Gebhart 2014). Therefore, it is not unexpected to find no apparent clinical findings in animals that have been recently infected with *L. intracellularis*. Another issue is that this organism may need some additional factors provided by other gut microbiota or specific microbial community to produce disease with marked pathological changes because it was not able to induce any cell proliferation and disease in germ-free pigs (Vannucci and Gebhart 2014).

Conclusion

To our knowledge, the current study represents the first investigation on the occurrence of *L. intracellularis* in diarrheic and healthy ratites that shows its presence in ostrich farms in Iran. Although the observations in the current study are not conclusive evidence of the pathogenic role in ostriches, it shows the need for further studies on the occurrence and pathogenicity of this organism in ostriches. Future studies should be conducted using a combination of molecular and serological assays along with pathological examinations, especially on farms with poor performance and growth rate.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest to declare.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Informed consent Since there was no human participant in this study, no consent was necessary. The name of ostrich farms kept private for ethical reasons.

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