Detection of *Lawsonia intracellularis* in a dog with inflammatory bowel disease using nested PCR and serology

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ABSTRACT: A nested polymerase chain reaction (PCR) assay and serological examinations were used to detect the presence of *Lawsonia intracellularis* in a two and a half years old German smooth-coated cocker spaniel with clinical symptoms of chronic diarrhoea and histologically proven inflammatory bowel disease. Fourteen rectal swabs taken over a period of two weeks and eight biopsy specimens taken over a period of six months were used for laboratory examinations. Using the nested PCR, the DNA of *L. intracellularis* was found in a total of 2 cases, i.e. one rectal swab and one biopsy specimen of the duodenum six months later. The species specificity of the nested PCR product was confirmed by sequencing. The presence of specific IgG antibodies against *L. intracellularis* was demonstrated by the IFAT in five samples of blood serum taken over a period of seven months.

Keywords: *Lawsonia intracellularis*; nested PCR; indirect immunofluorescence; dog; chronic diarrhoea

Lawsonia intracellularis is a Gram-negative obligately intracellular bacterium (McOrist *et al*., 1995) which affects the digestive tract, and particularly the caudal portion of the small intestine – the ileum – of sensitive hosts. In dogs, changes characteristic of the presence of this intracellular bacterium (in the past called *Campylobacter*-like organisms) were reported in two cases only: in the mucous membrane of ileum (Collins and Libal, 1983) and in stomach mucosa (Leblanc *et al*., 1993). The techniques used to detect the pathogen in histological sections of affected tissues included silver staining according to Warthin-Starry, immunofluorescence and electron microscopy.

This paper describes a methodology used in the case of a dog suffering from the IBD (Husník *et al*. 2003) where the DNA of the intracellular bacteria *L. intracellularis* was detected using the nested PCR including sequencing the DNA fragment obtained, and where IgG antibodies against *L. intracellularis* in the blood serum were demonstrated.

MATERIAL AND METHODS

In the tests, specimens taken from a two and a half years old German smooth-coated cocker spaniel suffering from the inflammatory bowel disease were used. Details on the diseased dog have been given elsewhere (Husník *et al*., 2003).

Collection of specimens

1. Biopsy specimens. A total of eight mucous membrane specimens from the stomach and intestines were taken: two from the stomach and the duodenum each, and one from ileum, caecum, colon ascendens and colon descendens. The material was obtained in three endoscopic examinations on Day 40 (5 June), Day 217 (29 November) and 237 (19 December) following the first examination. The biopsy specimens for histopathological examinations were fixed in 10% neutral formalin.

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2. Rectal swabs. A total of 14 samples were gradually examined taken between Day 0 (26. 04. 2001) and Day 253 (04. 01. 2002) at intervals averaging two weeks. For their transport and storage, commercial Amies (Oxoid) transport medium was used.

3. Blood serum samples. A total of 5 blood samples were collected from the patient between Day 48 (13. 06. 2001) and Day 291 (11. 02. 2002).

Methods

1. Nested PCR

DNA extraction. Tissue specimens and rectal swabs were incubated in a lysing buffer for 1 hour, centrifuged (14 000 g/2 min), incubated with a DE-suspension (10 min) and re-centrifuged (14 000 g per 2 min) (Boom *et al*., 1990). The obtained sediment was treated with washing buffer (2×), ethanol (2×) and acetone (1×), and dried at 56°C for 15 minutes. The extracted DNA was resuspended in H_2O and stored at –20°C.

Nested PCR. The total reaction volume was 50 μl in a buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP (Top Bio, Czech Republic) and 2U Taq DNA polymerase (Top Bio, Czech Republic). Specimens of 1 μg of the extracted DNA examined were added to the reaction. In the first amplification cycle, the following parameters were obtained: initial denaturation (94°C/3 min) was followed by 35 thermal cycles (94°C/40 s; 55°C/40 s; 72°C/40 s) and concluded by a final extension (72°C/7 min). The same thermal steps and reaction times were used for re-amplification. In each reaction, control negative specimens were added to the examined ones.

Primers. Two pairs of primers were used (Jones *et al*., 1993) added to the reaction at a concentration of 1 pmol/μl. The external pair of primers LIA (5′-TAT GGC TGT CAA ACA CTC CG-3′) and LIB (5′-TGA AGG TAT TGG TAT TCT CC-3′) delimited a fragment 319 bp long. The internal pair of primers LIC (5′-TTA CAG GTG AAG TTA TTG GG-3′) and LID (5′-CTT TCT CAT GTC CCA TAA GC-3′) delineated a fragment 270 bp long.

Sequencing. A total of 10 μl of positive PCR products from the first round of amplifications were re-amplified in 100 μl reaction mixture. Nested PCR products were purified using a commercial kit (Qiagen, GmbH, Germany), sequenced in the ABI PRISM 310 Genetic Analyser (Applied Biosystem) and evaluated by the Omega version 2.0 programme (Oxford Molecular).

2. Serologic examination by the indirect immunofluorescence test

For the test, slides with fixed *L. intracellularis* cells (strain NCTC 12656^T) and 15 pits prepared by S. McOrist were used. The sera used in the tests were inactivated at 56°C for 30 min, at 1 : 10, 1 : 30 and 1 : 100 dilutions in PBS. Five μl samples were placed on slides and incubated overnight in a wet chamber at 4°C. Before the next incubation, the samples were washed six times in the PBS buffer with anti-dog IgG by conjugated FITC (PF230, Binding Site) at 1 : 30 dilution in PBS at 37°C for 30 minutes. Finally, the slides were washed for 5 min in the PBS buffer 5 times, dried and stored in a dark chamber at a room temperature. Each slide test included positive and negative serum controls. The positive serum controls were serum samples of another dog with a repeatedly positive reaction. The negative controls were serum samples from new-born pups. Test results were read by the immunofluorescence microscope at 150× to 300× magnification. Samples with clearly fluorescent *L. intracellularis* bacteria in the viewing field were considered as positive.

3. Other tests

Cultivation bacteriological tests. Rappaport-Vasiliadis medium (Oxoid) and selective XLD medium (Oxoid) were used in cultivation tests of rectal swabs for the presence of salmonellas. For the isolation of the *Campylobacter* spp., two selective media, i.e. Karmali agar (CM908, Oxoid) and Campylobacter Blood Free agar (CM739, Oxoid), were used. The samples were incubated at 37°C and 42°C for 48 hours in micro-aerophilic atmosphere with the use of CAMPYGEN (CN 25, Oxoid). In the *Helicobacter* spp. bacteria assay, the selective medium for *H. pylori* (Columbia blood agar base CM331, Laked horse blood SR48, *H. pylori* selective supplement SR147; Oxoid) was used. The cultivation was performed under anaerobic conditions at 37°C for 5 days.

For the cultivation assay of *Clostridium perfringens* in rectal swabs, a selective medium (Perfringens agar base CM587, Oxoid, Perfringens TSC selective medium supplement SR88) was used. The samples were incubated at 37°C and 42°C for 48 hours under anaerobic conditions with the use of the BR038B system and an anaerobic indicator (BR55, Oxoid).

The rectal swabs were also examined for the presence of *Helicobacter* spp. microscopically by staining

Table 1. Detection of *Lawsonia intracellularis* in intestines, rectal swabs and sera of the investigated dog by PCR and immunofluorescence (IFA)

Type of samples	Sampling time (days)															
	0	40	48	97	124	145	152	159	161	167	172	208	217	237	284	291
Rectal swab	+			–	–	–	–	–	–	–	–	–	–		–	–
Gastric mucosa		–											–			
Duodenal mucosa		–											+			
Ileal mucosa														–		
Caecal mucosa														–		
Colonal mucosa														–		
Serum			+										+	+	+	+
Results																

according to Gram and by the UREASATEST (Testline) urease test.

RESULTS

Of a total of 14 rectal swabs examined by the nested PCR assay between April 2001 and January 2002, the first sample (collected on 26. 04. 2001) was positive. Of a total of eight biopsy specimens collected from the stomach tissue (2), duodenum (2), ileum (1) and colon (2) and examined by the nested PCR assay, one of the duodenum specimens (collected on Day 217) was positive (Table 1). The nested PCR product was detected as a DNA fragment 270 bp long (Figure 1), and its specificity was confirmed by sequencing (Figure 2).

The presence of IgG antibodies against *L. intracellularis* was found in all the five samples taken in the course of the study, at dilutions of 1 : 30 and 1 : 100.

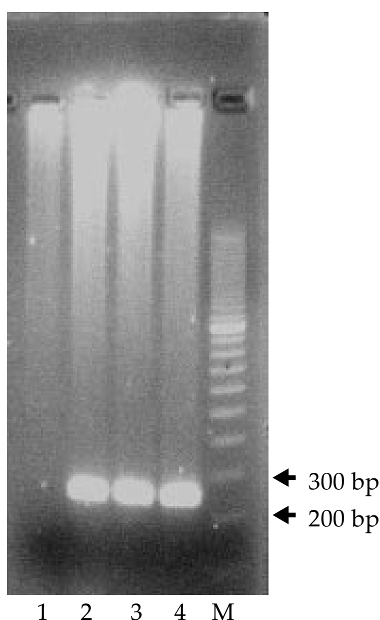

Figure 1. Nested PCR for detecting *L. intracellularis* DNA. PCR products of templates amplified with primers C and D. DNA templates were extracted from rectal swab and tissue sample (duodenum). Nested PCR products were visualised in 1 per cent agarose gel, electrophoresed in TAE buffer and stained with ethidium bromide. Lane 1 = negative control; lane 2 = positive control; lanes 3 and 4 = nested PCR positive samples; lane M = molecular size standards (Sigma)

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                            1                                                50
Lawsonia intracellularis    TTACAGGTGA AGTTATTGGG AAATATCCCT CATTAATTAC TTCATTAGCT
dog                         .......... .......... .......... .......... ..........

                            51                                              100
Lawsonia intracellularis    CAAGTTAAAC AAGCTGCAGC ACTTGCAAAC AATAAACTTG GTCTTCTTTC
dog                         .......... .......... .......... .......... ..........

                            101                                             150
Lawsonia intracellularis    TGATAAAAAA GGAGATGCTA TCTCTGCTGC ATGTAATGAA ATCATAAATG
dog                         .......... AA........ .......... .......... ..........

                            151                                             200
Lawsonia intracellularis    GAGAACTCCT TGATCAATTT GTTGTGGATT GTATTCAAGG AGGTGCAGGG
dog                         .......... .......... .......... .......... ..........

                            201                                             250
Lawsonia intracellularis    ACAAGTACAA ATATGAATGC TAATGAAGCA ATTTGTAATC GTGCTCTTGA
dog                         .......... .......... ........T. .......... ..........

                            251                270
Lawsonia intracellularis    GCTTATGGGA CATGAGAAAG
dog                         .......... ..........
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Figure 2. DNA sequences of a portion of the 16S rDNA of the intracellular agents of PE derived from the dog compared to the consensus sequences of a pig isolate of *L. intracellularis* (NCTC 12656^T). Dots indicate identical bases

All cultivation tests for the detection of *Salmonella* spp., *Campylobacter* spp., *Helicobacter* spp. and *Clostridium* spp. bacteria in rectal swab samples were negative. The *Helicobacter* spp. was also ruled out by the urease test.

DISCUSSION

At present, there is no clearly defined microbiological standard for a conclusive identification of *L. intracellularis*.

The principal molecular methods that serve as a basis for the diagnosis of the pathogen mainly in pigs include the PCR (Jordan *et al*., 1999), nested PCR (Jones *et al*., 1993) and the PCR-ELISA (Zhang *et al*., 2000). The authors believe that the most suitable method is the nested PCR assay because of its high sensitivity. This method is also considered a specific detection technique for *L. intracellularis* (Lavoie *et al.*, 2000; Lawson and Gebhart, 2000).

To our knowledge, the presence of *L. intracellularis* in dog has never been confirmed by DNA techniques. In this study, the nested PCR assay was used to examine 14 rectal swabs, and positive results were obtained in only the first sample collected, which seems to indicate a sporadic shedding of *L. intracellularis* in dogs. A positive finding in a duodenal biopsy specimen was only obtained six months later. The product of the nested PCR duodenum specimen was a fragment 270 bp long that showed a 99% homogeneity in comparison with the *L. intracellularis* (NCTC 12656^T) sequence described in pigs by McOrist *et al*. (1995).

For the assay of antibodies against *L. intracellularis*, the authors, drawing on their previous experience, chose the indirect immunofluorescence test that is usually used in pigs. All 5 serum samples collected were positive up to the titre 100. The results of serological tests closely correlate with the nested PCR assay results.

When the first blood samples were taken, i.e. 6 weeks after the bacteria in a rectal swab were found, the most intensive antibody response was obtained, which confirms the existence of a previous, non-active, infection. Blood samples taken 6

months later showed a drop in the antibody titre. Although there was no shedding of bacteria in the faeces any longer at that time, the bacteria were still found in the duodenum biopsy specimen. Our results support the theory of some authors about higher sensitivity of the IFAT test when compared with PCR assay in pigs and foals (Knittel *et al*., 1998; Lavoie *et al*., 2000).

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