

Molecular and serological investigation of *Lawsonia intracellularis* in weanling foals in Türkiye

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ABSTRACT

Equine proliferative enteropathy caused by *Lawsonia intracellularis* has been described in weanlings in many different countries including the USA, Western Europe, Israel, Korea and Brazil but not investigated in Türkiye and Eastern Europe. The objective of the study was to determine the seroprevalence and fecal shedding of *L. intracellularis* in weanling foals. A cross-sectional study was designed in randomly selected 97 weanling foals with or without clinical signs from 3 different provinces of Türkiye. Total protein and albumin levels in serum samples from 97 foals were measured using automated biochemistry analyser. Serum samples and fecal samples were also tested by bELISA and real-time PCR to detect *L. intracellularis*-specific antibodies and -DNA, respectively. Seropositivity was found to be 25.8% by bELISA. However, none of the fecal samples were positive by real-time PCR. Statistically, no significant associations were found between seropositivity and clinical signs or serum ALB/TP levels. Seropositivity indicated the exposure of weanling foal population to *L. intracellularis* for the first time in Türkiye. Further investigations are needed to better understand the epidemiology of the disease in the weanlings as well as adult horse populations and potential wildlife reservoirs in the country/region.

Introduction

Lawsonia intracellularis (*L. intracellularis*), the causative agent of equine proliferative enteropathy (EPE), is an obligate Gram-negative, intracellular bacterium. EPE has been diagnosed increasingly in horse populations in the world (6, 8, 13). *L. intracellularis* mainly infects pigs and horses but a number of other animal species were also reported to be susceptible including rabbits, rodents, foxes, wild pigs, deer, ferrets, ostriches racoon dogs, Korean water deer, non human primates and domestic dogs (11). *L. intracellularis* infected fecal materials of wildlife reservoirs and subclinically infected horses are the sources of infection (7, 13, 15, 16).

L. intracellularis typically affects weanling age group under 1 year old of horses (3, 13, 15). The agent is mainly encountered in weanling foals possibly because of declined maternal antibodies and management changes.

Separation of a foal from the dam, transportation, introduction to a new herd, deworming therapies, vaccination schedules and training can cause stress and all of these could be predisposing factors (13). EPE was often diagnosed between August and January as this period is the weanling time for most foals in the northern hemisphere (12, 13).

Clinical cases of *L. intracellularis* infection generally manifests nonspecific signs such as lethargy, high rectal temperature (≥ 38.5 °C), peripheral edema, weight loss, colic and diarrhea. (1, 10, 15) Recovery period can take weeks to months before they regain the appearance of unaffected cohorts (13, 15). *L. intracellularis* infections are located in the intestinal enterocytes and clinical cases of EPE generally lack an intestinal inflammatory response; hypoproteinemia and hypoalbuminemia are the most consistent laboratory

findings associated with EPE (13, 15). Although the actual values depend on the laboratory analyser used, total protein (TP) and albumin (ALB) concentrations are generally less than 5.0 to 5.2 g/dl and less than 3.0 to 3.1 g/dl, respectively (13).

Presumptive diagnosis of *L. intracellularis* infection is generally made based on the age of the affected animal, clinical signs, hypoproteinemia/hypoalbuminemia and presence of thickened small intestinal wall mainly in the jejunum and ileum in ultrasonography. Other ante mortem laboratory assays such as serological tests for the presence of antibodies and detection of the agent in feces by molecular assays were developed (8, 14), because *L. intracellularis* can only be grown in vitro cell culture and requires a specific atmosphere for growth (17).

To our knowledge, there is no report regarding the presence of the fecal shedding and seroprevalance of the disease in weanling foal populations in Eastern Europe and Türkiye. The goal of the present study was to determine the prevalence of *L. intracellularis* infection in weanling foal populations by means of fecal shedding of the agent and seropositivity in Türkiye for the first time.

Materials and Methods

Animals and sample collection: A cross sectional study was designed to detect the serological and molecular prevalence of *L. intracellularis* from weanling foals with or without showing any clinical signs by random sampling as some of the infected foals may not show any clinical signs. The present study was approved by the Ethics

Committee of University of Istanbul-Cerrahpasa (Report no. 2019/51). Feces and blood samples were collected randomly from 97 weanling foals from İzmit province (n=17), Bursa province (n=59) and Thrace (Silivri) region (n=21) (Figure 1) between September and January. During sampling, clinical signs including high rectal temperature (≤ 38.5 °C), diarrhea, peripheral edema, weight loss and lethargy were also recorded. Fecal samples were collected directly from the rectum and put into sterile plastic containers for detection of *L. intracellularis* DNA. Blood samples were collected by jugular venipuncture using 10 ml sterile vacutainer serum tubes for serological determination of specific anti-*L. intracellularis* antibodies. After separation of serum samples, TP and ALB levels were measured using automated biochemistry analyser. A cut off value of < 5.0 g/dl for hipoproteinemia and < 3.0 g/dl for hipoalbuminemia were used described as in the previous article (13). Fecal samples were frozen (-20 °C) until used for real time PCR analysis.

Serology (ELISA): Serum samples for antibodies against *L. intracellularis* were examined by using a commercially available blocking ELISA (bELISA) kit (Svanovir® *L. intracellularis*/ileitis-Ab, Art No: SV122275, Sweden) according to the manufacturer's instructions. In summary, serum samples were diluted 10-fold and incubated in microplate wells, precoated with whole cell *L. intracellularis* antigen, for 1 hr at 37 °C. The microplate wells were then washed three times with 300 μ l washing buffer, and 100 μ l diluted conjugate (Svanovir®, Sweden)

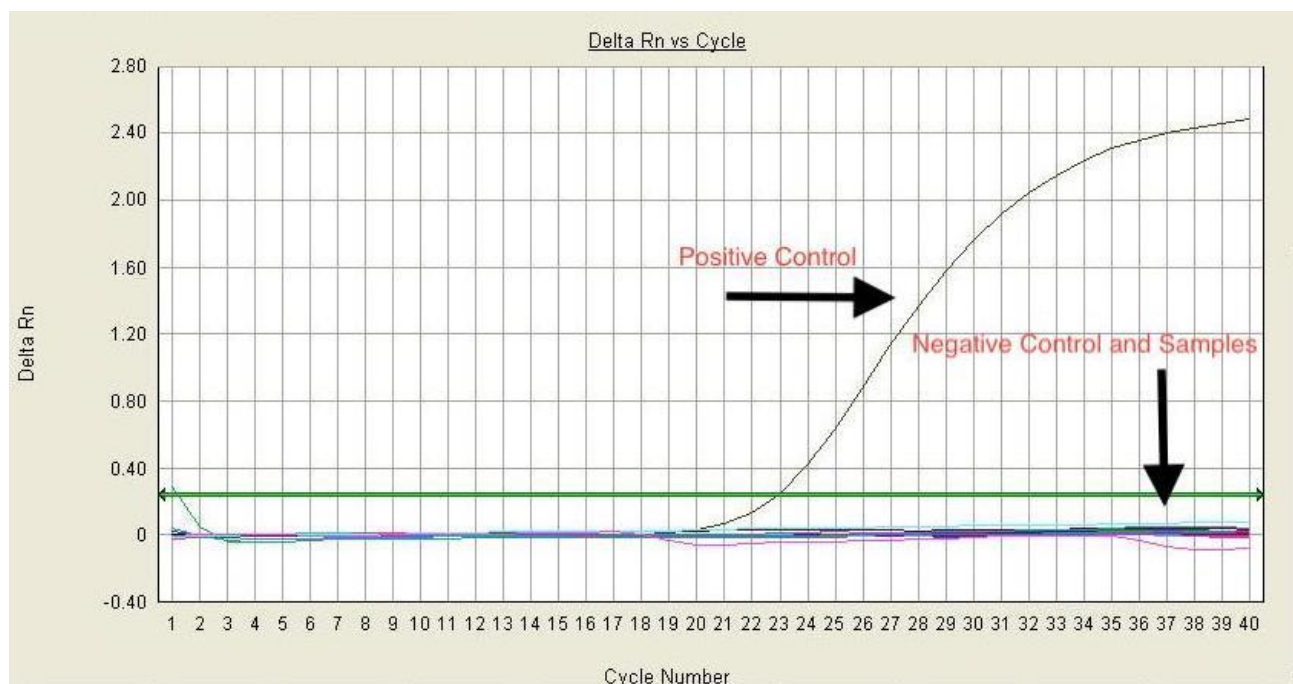


Figure 1. A representative of TaqMan probe based real-time PCR analysis in fecal samples. All samples were negative and positive control Ct value was around 23.

(1/100) was added in each well. The wells were washed again three times with 300 µl wash buffer. 100 µl of substrate solution (Tetramethylbenzidine in substrate buffer containing H₂O₂) that included in the kit was added to each microplate well, microplate wells were incubated for 10 min in dark at 22 °C. Following incubation period, the reaction was stopped by adding 50 µl stop solution to each well and gently mixing the microplate wells. The reactions were read in an ELISA reader (AllSheng AMR-100 Microplate ELISA Reader, Republic of China) at 450 nm wavelength. Negative control and positive control serum were also included as reference provided by the manufacturer. Percent of inhibition (PI) was calculated according to the formula as in the manufacturer instructions. Samples with a PI <20 % were concluded as negative, samples between 20 %-30 % inconclusive and samples >30 % positive.

Real-time PCR detection of *L. intracellularis* from fecal samples: Nucleic acid isolation and real time PCR detection assays were carried out according to the previous study with minor modifications (16). Briefly, 2 ml of sterile isotonic saline solution were added to 1 g of fecal material in a sterile centrifuge tube. Afterwards, the mixture was vortexed for 5 minutes and then centrifuged at 12500 rpm for 1 min. 150 µl of supernatant fluid was used to purify DNA by using a commercial spin column DNA isolation kit according to the manufacturer's instructions (Patho Gene-spin™ DNA/RNA Extraction Kit, Intron Biotechnology, Korea).

To increase the sensitivity of the assay, the DNA samples underwent first a DNA precipitation and PCR preamplification step according to the previous study (16). Briefly, 50 µl of sample was precipitated using 3 µl of 5 M NaCl, 5 µl of (5 mg/ml) glycogen and 150 µl absolute ethanol. The mixture was inverted several times and stored at -20°C overnight. The sample was then centrifuged at 12500 rpm for 15 min. The supernatant was removed and 100 µl of 70 % ethanol was added to the sediment and

resuspended. The last step was repeated and followed by drying the sediment for 10 min. The sediment was then resuspended in 20 µl of sterile DNase, RNase free water and stored at -20 °C until analysis. The pre-amplification step was carried out using *L. intracellularis* aspartate ammonia lyse (AAL) gene specific primer pairs (Table 1). The 20 µl PCR setup consisted of 10 µl Platinum™ II Hot-Start PCR Master Mix (2X), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 4.2 µl sterile nuclease free water and 5 µl of template DNA. Cycling conditions were initial denaturation for 2 min at 94 °C and 35 cycles of denaturation step for 5 sec at 98°C and combined annealing/extension step for 15 sec at 60 °C.

After the preamplification step, real time TaqMan® PCR in real time PCR system (Applied Biosystems 7300 Real Time PCR, U.S.A) was performed in extracted DNAs for the presence of the AAL gene of *L. intracellularis* (16). The 20 µl master mix included 10 µl of 2X AmpliGene™ qPCR Probe Mix Hi-Rox, 0.8 µl of forward primer (10 µM), 0.8 µl of reverse primer (10 µM), 0.4 µl of probe (10 µM), 3 µl of sterile nuclease free water and 5 µl of template DNA. The cycling conditions were 1 cycle for 2 min at 95 °C and 40 cycles for 5 sec at 95 °C, 30 sec at 65 °C. All amplification reactions were performed duplicate with positive DNA extracted from cell culture infected with *L. intracellularis* (kindly provided from Professor Erdal Erol, University of Kentucky, U.S.A) and negative control.

Statistical Analysis: Kappa analysis was used to determine a significant relationship between bELISA positivity and presence of clinical signs related to *L. intracellularis* infection. Chi-square test was used to determine the significance of differences in the prevalence among regions and to determine if any significant relationship was yielded in TP/ALB value and bELISA positivity. *P* values of <0.05 were considered to be significant using SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0).

Table 1. Oligonucleotide sequences of PCR primers and probe used in the study (16).

Target Gene:	Assay:	Primer/Probe:	Oligonucleotide Sequences:
<i>L. intracellularis</i> aspartate ammonia lyse gene	Preamplification/ PCR:	Forward primer	5'-AATTTGTTGTGGATTGTATTCAAGGA-3'
		Reverse primer	5'-CTTTCTCATGTCCCATTAAGCTCAA-3'
	Real Time PCR:	Same primer pairs given above.	
		TaqMan® MGB™ Probe	5'-6-FAM-CAGGGACAAGTACAAATATGAATGCTAATGAAGCAA-MGB-3'

Table 2. Province residing, breed, age, clinical signs (h.r.t.: high rectal temperature, d.: diarrhea, w.l.: weight loss, leth.: lethargy, Tb: Thoroughbred.), serum TP-ALB value and bELISA -Percent of Inhibition (PI) value of seropositive foals. The data of serum TP, albumin and ELISA % inhibition values in foals with no clinical signs were shown in range (with minimum and maximum values).

Clinical status	ELISA seropositivity	Sample no.	Province	Breed	Clinical signs	Serum TP value	Serum ALB value	ELISA PI (%)
Clinically ill	ELISA-Positives	7	Silivri	Tb.	w.l.	5.1	3.2	40.73
		39	Karacabey	Tb.	w.l.; d.	5.9	3.2	51.71
	ELISA-Inconclusives or -Negatives	1	Silivri	Tb.	h.r.t.	5.9	3.8	14.56
		3	Silivri	Arab	w.l.	5.6	3.7	20.36
		6	Silivri	Arab	w.l.	5.3	3.5	19.83
		33	Karacabey	Tb.	d.	6.8	3.52	24.48
		34	Karacabey	Tb.	w.l.; d	6.0	3.4	6.33
		56	Izmit	Tb.	w.l.; leth.	6.2	3.3	16.03
		62	Izmit	Tb.	d.	6.0	3.0	23.95
97	Izmit	Tb.	w.l.; d.	6.3	2.4	6.58		
No clinical sign	ELISA-Positives	23 samples	Karacabey and Silivri	All Tb.	-	5.7-7.8	1.7-3.8	30.82-66.50
	ELISA-Inconclusives or -Negatives	Remaining 64 samples	All three provinces	Both breeds	-	5.3-7.7	2.7-4.0	2.38-29.97

Results

The mean age (\pm standart deviation) of the foals was 6.8 (\pm 1.4) months ranging from 5 to 10 months old. Five (5.1 %) out of 97 foals were arabian and 92 (94.8 %) were Thoroughbred foals (Table 2). While a total of ten (10.3%) horses had one clinical sign (such as diarrhea, weight loss, lethargy or high rectal temperature), the remaining 87 foals did not show any clinical signs at the time of sampling (Table 2). None of the foals had peripheral edema. Mean TP and ALB values of the studied foals were 6.1 g/dl (\pm 0.5) and 3.4 g/dl (\pm 0.4), respectively.

ELISA results yielded that 25 foals were positive for *L. intracellularis* specific antibodies with a seroprevalence of 25.8 % (Table 2). Forty five (46.4%) and 27 (27.8%) of the foals' ELISAs resulted as inconclusive and negative, respectively. In the group of clinically ill foals, 2 of the foals were ELISA-positive while the remaining 8 foals were either ELISA-inconclusive or -negative (Table 2). In the group of foals with no clinical sign, 23 foals were ELISA positive; the remaining 64 foals were either ELISA inconclusive or negative (Table 2). Mean TP and ALB values in foals with a positive serology were 6.2 g/dl (\pm 0.5) and 3.3 g/dl (\pm 0.5) respectively.

The distribution of the seropositivities according to the provinces were 6.2%, 1.0% and 18.5% in Silivri, Izmit and Karacabey region, respectively (Table 2). No significant difference was found between provinces for ELISA positivity ($P= 0.117$). No significant association was found between TP ($P= 0.760$) / ALB ($P= 0.097$)

value and ELISA positivity. Statistical analysis yielded no significant relationship between presence of one or more than one clinical signs and ELISA positivity ($\kappa= 0.108$).

None of the fecal samples yielded positive results by real-time PCR (Figure 1).

Discussion and Conclusions

To the authors' knowledge, this is the first study that investigated the seropositivity and the status of fecal shedding of *L. intracellularis* by PCR in weanling foals in Eastern Europe and Türkiye. In this study, we targeted a weanling foal population (between 5 and 10 months old) as the most of the infections occur in animals under 1 year old (3, 4, 13, 15).

Different seropositivity rates were found in several previous studies. In the Netherlands, the seroprevalence was around 15 % in pre-weanling foals and seroprevalence increased significantly (to 23 %) in weanling (8). In the same study seropositivity was 89 % and 99 % in yearlings and older than 2 years old horses respectively. In Korea, seroprevalence of *L. intracellularis* was determined to be 94%, (92 % in <2 years, 96 % in 2-5 years, 94 % 6-10 years and 93 % in >10 years) by bELISA while *L. intracellularis* DNA was found to be 18% in fecal samples. Contrary to serology, the younger age was associated with the PCR positivity; 3 % in <2 years, 25 % in 2-5 years, 13 % in 6-10 years and 8 % in >10 years (11). A recent study in Belgium also demonstrated 98.8% seropositivity by bELISA and

confirmed high seropositivity rates in adult horses (mean age 12.1 years) (9). It is evident that exposure of horses to *L. intracellularis* increases by age (11). In Brazil, 9.42 % of adult mares and weanling foals were found to be positive by immunoperoxidase technique (4). In the same study, fecal shedding was found to be 3.14 % by PCR. A recent study in Brazil demonstrated similar seropositivity (5.55 %) and fecal shedding (4.32 %) (2). A recent study conducted in Israel found close results to the present study with 30.5 % and 0.7 % horses positive by ELISA and PCR, respectively (18). The differences between the present study and others could be related to differences in geography, sample size and disease status of the horses.

In the present study, although serologically positive horses were detected, none of the fecal samples contained *L. intracellularis* DNA. This strongly suggests prior exposure of *L. intracellularis* in the horse populations in Türkiye. Low sample size (and only 10 of them with clinical signs) and possible intermittent shedding are likely the major factors for not detecting *L. intracellularis* DNA in fecal samples. Even though the analytical sensitivity of the PCR method used in our study was relatively high (as low as one *L. intracellularis* organism after DNA precipitation and PCR preamplification step) (16), detection of *L. intracellularis* could be limited due to the PCR inhibitors presented in fecal samples (13). As our assay did not contain internal control, PCR inhibition was not able to be measured. This is in concordance with the previous studies that determined high seropositivity and low fecal shedding of the bacteria (4, 11, 18). Seroconversion in horses has been documented to occur approximately 14 days after experimental challenges (17) and remain seropositive for more than 6 months (19) but fecal shedding of the microorganism starts between 12-18 days postinoculation and lasted for 11 to 21 days (17).

The same bELISA kit was also used in foals and adult horses in earlier studies (8, 9, 11). In a previous study, the authors compared the bELISA with immunoperoxidase monolayer assay (IPMA), as a reference test, for the detection of antibodies against *L. intracellularis* in horses. A total of 100 serum samples were tested and found 90 % sensitivity with 94 % specificity, 88 % sensitivity with 96 % specificity and 85 % sensitivity with 98 % specificity at 1/125, 1/150 and 1/175 dilutions, respectively, and concluded as a useful alternative for diagnosis of EPE (5).

In conclusion, the present study resulted in a 25.8 % seroprevalence against *L. intracellularis* in the weanling foals with or without clinical signs for the first time in Eastern Europe and Türkiye. However, no fecal shedders were found in fecal samples of the weanling foals by real-time PCR. The results of the study strongly suggest that the weanling foal population in Türkiye is exposed to *L.*

intracellularis and therefore clinicians should be aware of this pathogen in differential diagnosis of enteric diseases. Further studies are needed for determination of fecal shedding in foals, adult horses and wildlife animals in order to better understand the status and epidemiology of *L. intracellularis* in Türkiye.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

KM, AM and EE conceived and planned the experiments. KM and AM carried out the experiments. KM, AM and EE planned and carried out the simulations. KM and AM contributed to sample preparation. KM, AM and EE contributed to the interpretation of the results. AM took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

The present study was approved by the Ethics Committee of University of Istanbul-Cerrahpasa (Report No. 2019/51).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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