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Comparison of ELISA and IPMA tests for the detection ofAnti-*Lawsonia* antibodies in horses*

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ABSTRACT

The objective of this study was to compare a commercially available indirect enzyme-linked immunosorbent assay (ELISA) with an immune peroxidase monolayer test (IPMA), considered to be a reference test, for the detection of antibodies against *Lawsonia intracellularis* in horses. A total of 100 serum samples were tested with both tests at three different dilutions 1:125, 1:150 and 1:175. When sera were tested at 1:125 dilutions, the sensitivity of ELISA was 90% with 94% specifity. At a dilution of 1:150, the sensitivity was 88% and specifity was 96%. At 1:175 dilutions, the sensitivity and specificity were 85% and 98%, respectively. Based on these validation results, we believe that commercially available ELISA is a useful alternative for the diagnosis of equine proliferative enteropathy.

Keywords Enzyme-linked immunosorbent assay; ileitis; imunoperoxidase assay;*Lawsonia intracellularis*; Horses, proliferative enteropathy.

1 INTRODUCTION

Lawsonia intracellularis is an obligate, intracellular, Gram negative, curved rod, which does produce neither fimbriae nor spores (Gebhart 2006, Lavoie et al. 2000, Smith et al. 2001). Because of is obligate, intracellular nature, this bacterium needs live hosts for replication. Transmission is generally through the faecal-oral route (Dezorzova et al. 2006, Smith et al. 2001). Infection with *L. intracellularis* is commonly known as proliferative enteritis, proliferative enteropathy, proliferative ileitis or intestinal adenomatosis (Lavoie et al. 2000, Smith et al. 2001). *L. intracellularis* can infect several species but is not believed to transmit directly between species (Smith et al. 2001).

The disease is best described in pigs (Lavoie et al. 2000) in which it causes a major endemic disease with high economic losses to the swine industry (Friedman et al. 2008, Lavoie et al. 2000). Infection with *L. intracellularis* can also affect several other species such as wild boars, dogs, foxes, ferrets, rats, guinea pigs, rabbits, monkeys, ostriches, emus, sheep, deer and horses (Dauvillier et al. 2006, Deprez et al. 2005, Feary et al. 2006, Lavoie et al. 2007, Lavoie et al. 2009, Dezorzova et al. 2006, Klimes et al. 2007, Tomanova et al. 2003b). Since an infection with *Lawsonia intracellularis* affects a large number of species, the sources of contamination are probably numerous (Lawson et al. 2000). Multiple host species can be considered, free-living animals such as rodents, foxes, wild pigs and ferrets, but also domestic dogs and pigs can play a role a host (Klimes et al. 2007, Friedman et al. 2008, Dezorzova et al. 2006).

The disease in horses is called Equine Proliferative Enteropathy (EPE) and has been reported from North and South America, Australia, and Europe (Dauvillier et al. 2006, Deprez et al. 2005, Feary et al. 2006, Lavoie et al. 2007). In horses, *L. intracellularis* most commonly affects foals (Bihr 2003, Feary et al. 2006, Lavoie et al. 2000, Pusterla et al. 2008a, Pusterla et al. 2008b, Schumacher et al. 2000) causing symptoms such as diarrhoea, profound dullness, fever, rapid weight loss, colic, rough hair coat, and ventral oedema due to severe hypoproteinemia (Bihr 2003, Feary et al. 2006, Kimberley et al. 2007, Lavoie et al. 2000, Pusterla et al. 2008b, Schumacher et al. 2000). Since hepatic and renal values of weanlings infected by *L. intracellularis* are within range, it is believed that hypoproteinaemia is caused by protein-loosing enteropathy (Bihr 2003, Feary et al. 2006).

DIAGNOSIS OF AN INFECTION BY *L. INTRACELLULARIS* CAN BE MADE BY PCR ON FECAL OR INTESTINAL SAMPLES (FRAZER ET AL. 2008, GUEDES ET AL. 2002, JACOBSON ET AL. 2004, PUSTERLA ET AL. 2008A, PUSTERLA ET AL. 2009A). SEROLOGICAL METHODS, SUCH AS IMMUNOPEROXIDASE MONOLAYER ASSAY (IPMA), INDIRECT IMMUNOFLUORESCENCE ANTIBODY TEST (IFAT), AND A BLOCKING ENZYME-LINKED IMMUNOSORBENT ASSAY (BELISA) ARE AVAILABLE FOR THE DETECTION OF CIRCULATING ANTIBODIES AGAINST *L. INTRACELLULARIS* (BOESEN ET AL. 2005, FRAZER ET AL. 2008, GUEDES ET AL. 2002, HAMMER 2003, KELLER ET AL. 2005, LEE 2006). SEVERAL MANAGEMENT FACTORS, SUCH TRANSPORTATION, MIXING AND ANTIBIOTIC ADMINISTRATION MAY CONTRIBUTE TO A OUTBREAK OF EPE IN BREEDING FARMS AS WELL (LAVOIE ET AL. 2000). ALTHOUGH A GOOD THERAPY WITH GOOD PROGNOSIS IS AVAILABLE, AN INFECTION WITH *LAWSONIA INTRACELLULARIS* REMAINS AN IMPORTANT CAUSE OF MORBIDITY AND MORTALITY IN FOALS (FEARY ET AL. 2006). IN THE NETHERLANDS, *L. INTRACELLULARIS* INFECTION IN HORSES WAS FIRST REPORTED BY BUTLER ET AL. IN 2006 (BUTLER ET AL. 2007). IN A 2008 STUDY ON THE PREVALENCE OF *L. INTRACELLULARIS*, 23% OF THE TESTED FOALS HAD POSITIVE *L. INTRACELLULARIS* ANTIBODIES (REE 2008). ALL DAMS OF FOALS THAT TESTED POSITIVE OR INCONCLUSIVE AT PRE-WEANING SCREENING WERE TESTED ALSO AFTER WEANING OF THEIR FOAL. ALL OF THESE DAMS WERE TESTED POSITIVE FOR *L. INTRACELLULARIS* (REE 2008). THE AIM OF THE PRESENT STUDY WAS TO DETECT OF ANTIBODIES AGAINST *LAWSONIA INTRACELLULARIS* IN HORSE IN MINNESOTA

2 PROCEDURE FOR PAPER SUBMISSION

2.1 Review Stage

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MATERIAL AND METHODS

Animals: Serum samples were submitted to the Veterinary Diagnostic Laboratory at University of Minnesota for disease diagnosis. Samples (n=100) submitted fromJanuary 2008 to July 2010 were included in this study. All serum samples were tested with both processed with immune peroxidase test and Bioscreen Ileitis antibody ELISA. All samples were tested at three different dilutions e.g., 1:125, 1:150 and 1:175.

Indirect ImmunoPeroxidase Test: The Lawsonia antigen was coated in 96-well microtiter plates as described previously. Before use, the plates were rehydrated with distilled water and placed in a moisture chamber in a 37°C incubator. The sera were diluted 1:30 in a plain 96-well plate using a 10% skim milk solution. This was followed by preparation of another four dilutions at 1:60, 1:120, 1:240, and 1:480. The distilled water was discarded and the plate was blotted dry on a stack of paper towels. Known negative and positive sera were added as controls. Diluted serum was added at 50 µl per well and the plate incubated at 37°C for 45 min. A 1:4,000 dilution of anti-horse IgG HRP conjugate in IPMA buffer was then added. Upon completion of incubation, the diluted sera were discarded and the plates and rinsed four times with PBS. After final rinse, the plate was dried on a stack of paper towels followed by the addition of 30 µl/well of diluted anti-horse IgG HRP conjugate. The places were re-incubated at 37°C for 45 min. The anti-horse IgG conjugate was discarded and the plates rinsed with PBS four times. After the final rinse, the plate was dried on a stack of paper towels followed by the addition of 100 µl of diluted AEC-DMF solution to each well. A 1:20 dilution of the AEC-DMF solution was made in acetate buffer (0.6 ml AEC-DMF solution in 11.4 ml acetate buffer) followed by the additon of . 5 µl of 30% H_2O_2 3-5 minutes before use. The plates were incubated at room temperature for 20 min. The AEC-DMF solution was discarded and the plates rinsed with distilled water four times. After the final rinse, the plate was dried on a stack of paper towels followed by drying at 37°C for at least 20 min. The plates were then read under an inverted microscope.

ELISA: Sample dilutions, nagative serum control, and positive serum control were added to appropriate wells of the 96-well microtiter plate supplied with the kit. The amount added was 100 µl per well. The plates were coered with a lid and incubated for 1 hour at 37°C.The contents of the plates were discarded and the wells filled with the wash solution supplied with the kit. A total of three washes were given. The conjugate was diluted 1:100 with the conjugate diluent followed by the addition of 100 µl of diluted conjugate in each well. The plate was covered with the lid and incubated for 1 hour at 37°C. The plate was washed thrice with the wash solution. The wells were emptied, 100 µl of buffered peroxidase substrate (PS) was added per well, incubated for 10 min at 21°C away from the light. "Stop solution" was added at 50 µl per well. The underside of the plate was carefully wiped and the plate read at 450 nm and 630 nm after photometer was first blanked on air. Per cent inhibition (PI) for each sample was calculated as follows:

Average OD of NC – OD of sample/ Average OD of NC X 100

PI of ≥ 30% was considered positive, PI of 20-30% as questionable/suspect, and PI of < 30% as negative.

RESULTS:

Indirect ImmunoPeroxidase test result (IPMA): 57(57%) out of the 100 samples are positive and 43 (43%) are negative and compared with lleities antibody Elisa found that at the 1:125 dilution 53 (53%) is positive and 47(47%) is negative while in1:150 dilution 50 (50%) are positive and 50(50%) are negative and 1:175 dilution 47 (47%) are positive and 53(53%) are negative these dilution are used after testing original 20 horse serum samples with dilution 1:50/1:160, 1:180,1:100,1:100,1:150 and 1:1200 .These original tested horse samples with high specifity and sensitivity at dilution 1:150and these dilution used for testing 100 horse samples compared with IPMA..

Sensitivity and specificity of the ELISA:

To determine the optimal cut-off value for lleities intracellularis ELISA, horses were examined for L.intracellularis specific antibody levels. The sensitivity of the assay was evaluated by

Examining 100 serum samples collected from horses following natural infection with

L. intracellularis. In Table (1).

DISCUSSION:

PCR on fecal samples is the most effective test for diagnosis of an infection caused by *Lawsonia intracellularis* (Jacobson et al. 2004). However, since the bacterium is not shed continuously, repeated sampling and testing of a large number of samples is essential (Keller et al, 2005). Studies in Denmark showed that 75% of the examined pigs had a positive PCR-assay on faecal samples (Stege et al. 2004). However, 93,7% of the pigs in randomly selected herds had positive *Lawsonia intracellularis* antibodies tested with serology (Boesen et al. 2005). Serology is a useful tool for detection of antibodies against *Lawsonia intracellularis*. The disadvantage of serology is that detectable antibodies are not seen earlier than 2 weeks after the onset of infection (Gebhart 2006, Jacobson et al. 2004). Thus it only provides historical information on exposure to the bacterium (Gebhart 2006, Guedes et al. 2003, Guedes 2004) and it is not correlated to clinical disease (Jacobson et al. 2004). Frazer (2008) tested positive antibody results in horses in which typical signs of EPE had not been observed. An advantage of serology is that it is suitable for testing large numbers of samples (Guedes 2004).

Several techniques can be used for detecting antibodies against *Lawsonia intracellularis*. Immunoperoxidase monolayer assay (IPMA) and indirect immunofluorescence antibody test (IFAT) are specific and sensitive (Boesen et al. 2005). A great disadvantage of this techniques is the fact that each sample require a microscopically evaluation by a skilled diagnostician, which making these test subjective and limits the number of samples that can be handled (Boesen et al. 2005, Keller et al. 2005).

An ELISA test can easily be used for large sample numbers (Boesen et al. 2005). Keller et a**l.** (2005) tested in his study that the bELISA had at least the same sensitivity as indirect immunofluorescence antibody test (IFAT). Serum as well as plasma can be used in the Enterisol Ileitis ELISA (Keller et al. 2006). A novel ELISA is also tested as a specific and sensitive method for detection of *Lawsonia intracellularis* antibodies (Boesen et al. 2005).subclinically infected animals (Jacobson et al. 2009). Since there is no "gold standard" diagnostic method available for detecting antibodies against *Lawsonia intracellularis* in horses, there is no comparison

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for the bELISA (Boesen et al. 2005, personal communication van Maanen, Animal Health Services Deventer).

Several isolates of the bacterium are known (Gebhart 2006, Lee 2006, Wattanaphansak et al. 2009). Gebhart (2006) showed that isolates of pig *Lawsonia intracellularis* showed >98% 16S-rDNA similarity to isolates of *Lawsonia intracellularis* in different animal species (Lavoie et al. 2009, Pusterla et al. 2009b), and that there are only minor differences between isolates (Gebhart 2006). Another study in pigs showed that although the coating antigen of the bELISA kit is prepared with a European isolates, it can be used for detection of antibodies in Korea (Lee 2006). Since there are no specific horse values available for ELISA the values used in this study are the same as used by testing antibodies with ELISA in pigs. Serum samples can be tested with other test principles like immunoperoxidase monolayer assay (IPMA) to confirm the results.

Most reported cases of a *Lawsonia intracellularis* infection involve animals aged < 1 year (Frazer et al. 2008, Guimarães et al. 2009). However, a study by Guimarães et al. (2009) revealed to infected horses aged 13 and 16 month who were shedding the organism through feces. Still *Lawsonia intracellularis* infection commonly occurs more in young horses because of the decline in maternal antibodies and management changes that could cause stress and predispose them to disease (Frazer et al. 2008). After a primary infection no re-infection occurred with fecal excretion or with clinical signs (Boesen et al. 2005, Riber et al. 2009).

Seroconversion occurs not sooner in pigs than 2 weeks post-challenge (Gebhart 2006). Hammer (2003) suggested that exposure builds until the dose of *Lawsonia intracellularis* becomes high enough to induce seroconversion. Several studies have been done about the persistence of antibodies against *Lawsonia intracellularis*. Guedes et al. (2003) found that pigs remain seropositive for 13 weeks after exposure. A persistence of antibodies can even be found up to 4 months (Boesen et al. 2005, Stege et al. 2004). Dogs remain serologically positive over a period of 291 days (Tomanova et al. 2003b).Foals, positive for *Lawsonia intracellularis*, remained seropositive for more than 6 months (Dauvillier et al. 2006).

Guedes (2004) suggest that the level of antibodies against *Lawsonia intracellularis* decays gradually after reaching its peak. So, the higher peak serum titres, the longer detectable antibodies persists (Guedes 2004). When this peak is reached is not clear. Pusterla et al. (2009a) showed that the seroprevalence of the mares on a endemic farm increased with each foaling month, 33% in January up to 100% in may. Another study showed that the prevalence in young horses was 60,6%, but in horses over one year in age the prevalence was 87,1% (Klimes et al. 2007). Boesen et al. (2005) suggests that the increasing antibodies in their study within natural infected pigherds are the result of the pigs being boosted by *Lawsonia intracellularis* in the herd. In foals a increase in antibodies was shown, 14% showed positive titer before weaning and 23% after weaning (Ree 2008).

In pig's subclinical infection with *Lawsonia intracellularis* appears to be common in growing pig al. 2005, Kimberley et al. 2007, Kroll et al. 2005, Lee 2006). It is difficult to identify because the pigs don't show clinical signs (Gebhart 2006, Kroll et al. 2005). The only signs seems to be, lack of uniformity in weight (Guedes 2004, Keller et al. 2005). As well as pigs, horses with no signs of EPE can test positive for *Lawsonia intracellularis* antibodies also (Feary et al. 2006, Frazer et al. 2008, Guimarães et al. 2009). The subclinical form of an infection with *Lawsonia intracellularis* is easily missed, because the animal does not show clinical signs which belong to a infection with the bacterium (Guedes 2004). The present study revolved a mean antibody titer of 98,3%. In yearling it was 89%, in horses older than 2 years it was 99%. The seropositivity of the horses means that all positive tested horses have had an exposure to *Lawsonia intracellularis*. Guimarães et al. (2009) proposed that seropositivity in horses aged > 13 months implies subclinical late infection, a constant exposure situation or long term persistence of serum antibodies against *Lawsonia intracellularis* in horses. However, because the horses had never had symptoms from EPE the conclusion of the longitudinal study suggested that the findings indicated a long term persistence of antibodies against *Lawsonia intracellularis* (Guimarães et al. 2009). Further studies must be done to know more about the different isolates of *Lawsonia intracellularis*, as there may be a difference in pathogenicity between the different isolates of *Lawsonia intracellularis*.

ELISA and IPMA. However, both tests are equally able to detect the antibodies in animal exposure to *L. intracellularis* in horses specially that study one of the main studies for using ELISA as bio screen test in serum sample of horse.. There are several advantages to using ELISA

Compared with the current IPMA: 1) ELISA carries less risk of other bacterial contamination, as

the plates need not be cultured with live bacteria for assay preparation; 2) IPMA requires highly skilled readers to interpret the outcome, while ELISA gives more objective results due to automation of the reading process.

Presently, the only serologic test used in the United States for *L. intracellularis* is IPMA. This present study shows that ELISA can be used as an alternative to IPMA for the screening of sera for the presence of *L. intracellularis* antibodies. The results based on the standard validation protocol, the specificity, repeatability, detection of seroconversionand test agreements validation indicate that ELISA is a reliable and precise test for the detection of *L. intracellularis* antibodies in horses.

Table (1).Compared results of ELISA and IPMA in the first study.

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Pig samples	Elisa PI%	ELISA result	IPMA re-sult
1	-22	NEG	NEG
2	75	POS	POS
3	4.7	NEG	NEG
4	66	POS	POS
5	4.7	NEG	NEG
6	77	POS	POS
7	79	POS	POS
8	18	NEG	NEG
9	2	NEG	NEG
10	80	POS	POS
11	61	POS	POS
12	13	NEG	NEG
13	7.3	NEG	NEG
14	73	POS	POS
15	5.4	NEG	NEG
16	82	POS	POS
17	71	POS	POS
18	4.8	NEG	NEG
19	42	POS	POS
20	1.8	NEG	NEG

Tale (2).Multiple dilution of Elisa compared with IPMA in the second study:

Elisa dilution (1:50)	Elisa dilu-tion(1:60)	Elisa dilution (1:80)	Elisa dilution (1:100)	Elisa Dilu-tion (1:150)	Elisa dilution (1:200)	(0
S	S	S	S	N	N	
P	P	P	S	N	N	
S	S	N	N	N	N	
P	P	P	P	P	P	
P	P	P	P	P	P	
P	P	P	P	P	P	
P	S	P	P	N	N	
N	S	S	S	N	N	
N	S	S	N	N	N	
S	S	S	N	N	N	
P	P	P	P	S	N	
N	N	N	N	N	N	
N	N	N	N	N	N	
P	P	P	P	P	S	
P	P	P	P	P	N	
P	P	P	P	P	P	
P	P	P	P	P	P	
P	P	P	P	P	P	
P	P	P	P	P	N	
P	P	P	P	P	P	

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Table (3). Compared result of Elisa and IPMA in the third study.

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COMPARED RESULTS OF Elisa and IPMA				
RESULT	TITRE1:125	TITRE1:150	TITRE1:175	IPMA
POS	53	50	47	57
NEG	47	50	53	43
FalseNEG	6	7	8	
FalsePOS	3	2	1	

Table (4). The sensitivity and specivity of the ELISA results in the first study.

Elisa result		
Sample results	Pig samples	Horse samples
Neg	10	5
Pos	10	15
False neg	0	0
False pos	0	4
Sensitivity	100%	100%
Specivity	100%	55.6%

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Table (5). The sensitivity and specivity of the ELISA results in the second study .

Elisa result						
Sample results	Dilution 1:50	Dilution 1:60	Dilution 1:80	Dilution 1:100	Dilution 1:150	Dilution 1:200
Neg	7	8	7	8	10	13
Pos	13	12	13	12	10	7
False neg	0	0	0	0	0	2
False pos	2	2	3	2	0	0
Sensitivity	100%	100%	100%	100%	100%	77.8%
Specivity	77.8%	80%	70%	80%	100%	100%

Table(6). The sensitivity and specivity of the ELISA in the third study.

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	Elisa test		
	TITRE1:125	TITRE1:150	TITRE1:175
POS	53	50	47
NEG	47	50	53
FalseNEG	6	7	8
FalsePOS	3	2	1
Sensitvity	90	Sensitivity 88	Sensitivity 85
Specifity	94	Specifity 96	Specifity 98

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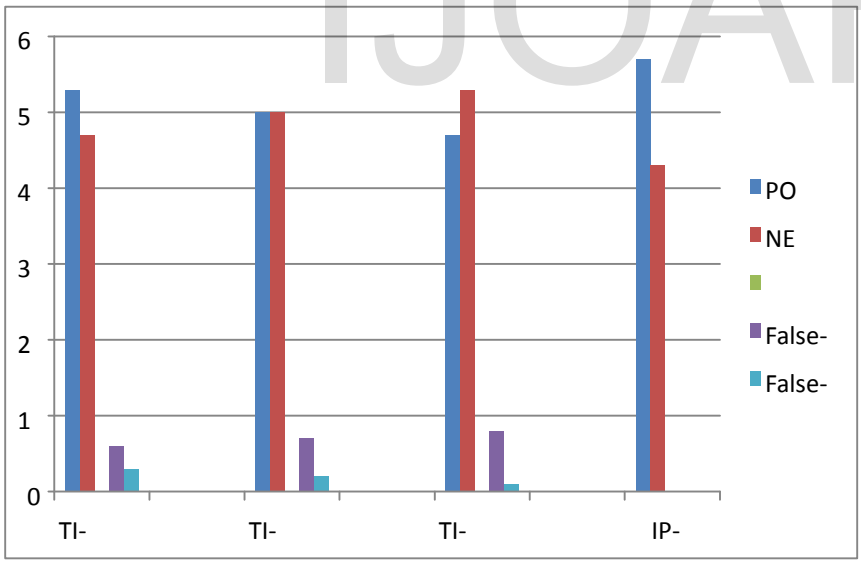

Fig(1).Compartive result of Elisa and IPMA of the third study.

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