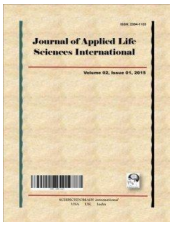

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Development of a Universal IPMA Test to Detect Lawsonia Antibodies in Multiple Species Using Protein A and G

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Authors' contributions

This work was carried out in collaboration between all authors. Author ASHH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author CJG all lab work done under her supervision and managed the analyses of the study. Author SMG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A recombinant protein combining the immunoglobulin binding sites of Proteins A and G, conjugated with horseradish peroxidase was used as a universal detection reagent for the assessment of antibodies against *Lawsonia intracellularis*. The reagent was applied in an indirect immunoperoxidase method in microplates for detection of antibodies to smooth lipopolysaccharide antigen in sera from horse and pig. Also, using protein G and protein A separately which detected from results that is species specific. An added advantage was that a universal conjugate using the proteins A and G detection reagent could be established.

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1. INTRODUCTION

Lawsonia intracellularis is an obligate intracellular bacterium causes enteritis known as proliferative enteropathy, intestinal adenomatosis, and ileitis. *Lawsonia intracellularis* infection has worldwide distribution in domestic pigs [1]. In domestic animals, other than pigs, *L. intracellularis* has been found in horses [2], rabbits [3], ferrets [4], dogs [5], and hamsters [2]. In captive wild animals, the bacterium has been detected in white-tailed deer (*Odocoileus virginianus*; [6]), rhesus macaques (*Macaca mulatta*; [7]), ostrich (*Struthio camelus*; [2]), and emu (*Dromaius novaehollandiae*; [8]). *Lawsonia intracellularis* probably caused adenomatosis in blue foxes (*Alopex lagopus*) reared in a fur farm [9].

The epidemiology of EPE has remained poorly investigated, and the transmission of Infection in foals may occur through the ingestion of feed or water contaminated with *L. intracellularis*-infected feces from infected domestic animals [10].

Diagnosis of an infection caused by *Lawsonia intracellularis* can be done by PCR on faecal or intestinal samples [11,12,13,10]. Different serological methods, such as a immunoperoxidase monolayer assay (IPMA), indirect immunofluorescence antibody test (IFAT) and a blocking enzyme-linked immunosorbent assay (bELISA) are available for detection of circulating antibodies against *Lawsonia intracellularis* [14,11].

The immunoperoxidase monolayer assay (IPMA) is a serologic enzyme immunoassay test commonly used for the diagnosis of viral infection, for instance, porcine respiratory and reproductive syndrome (PRRS) [15]. The obligate intracellular organism *L. intracellularis* infects cell line monolayers *in vitro*. Consequently, cell monolayers can be used in the same way as virus-infected cell line monolayers to perform serology with the IPMA [16].

The IPMA test has the advantages of not requiring the use of a fluorescent microscope, being easier to interpret, and having stable color reactions for several months, thereby providing a lasting record of the results that can be stored for a longer time. IPMA serologic test for *L. intracellularis* can be used as a routine technique [17].

Staphylococcus aureus Cowan strain and *Streptococcus*spp. Group G are among a group of bacteria that produce Proteins with the capacity to non-specifically attach to immunoglobulin molecules of various species. Protein A, a 42 kDa molecular weight naturally occurring product from *S. aureus* was first described by [18] as an antigen to which naturally occurring antibody was present in all human sera. However, because of the large amount of IgG in human serum capable of binding to this protein, it was realized that it was a non-specific interaction and not an antigen–antibody reaction [19]. Protein A interaction has been the topic of much research which was reviewed by [20]. Protein G was initially isolated from *Streptococcus* spp. group A [21] and later characterized by [22] and [23] as a 30 kDa molecular weight protein with an immunoglobulin binding capacity somewhat different from Protein A. It was therefore decided to prepare a chimeric molecule combining the binding characteristics of both Protein A and G [24]. The different binding characteristics of the two proteins were summarized by [25] who also reported on a gene fusion product of the two proteins which excluded an albumin binding site associated with Protein G. One of the major problems in serodiagnosis most of diseases is unavailability of specific antiglobulin conjugate. Our study focuses on validation of Protein A/G dependent IPMA in animals. Binding ability of Protein A/G-conjugate to antibodies was the Highest sensitivities in pig 100% followed by horse 85.7%. Protein A/G – IPMA proved to be sensitive and used as diagnostic tool in serodiagnosis of *lawsonia intracellularis* in wide host of animal spp. In this communication, we describe the use of recombinant Protein A/G, a reagent more universal than Protein A or G individually, labeled with horseradish peroxidase as a detection reagent for the presumptive diagnosis of lawsonia infection in horse, pig as well as for rabbit, hamster, and mice.

2. MATERIALS AND METHODS

2.1 Animals

Equine and Pig serum samples submitted to the Minnesota Veterinary Diagnostic Laboratory at the University of Minnesota, Saint Paul, Minnesota, for development of multispecies IPMA using universal conjugate protein A/G in

the detection of anti- *L. intracellularis* antibodies. We preliminary have 6 basic experiments:

The first experiment started with 12 serum samples (6 horse samples and 6 pig samples) with 3 dilution of universal conjugate protein A/G 1:1000, 1:5000 and 1: 10,000 with serum titer 1:60 and 1: 120. The second experiment with protein G using the same 6 horse and 6 pig samples with dilution 1:50, 1:500 and 1; 1000 with 1:60 and 1:120 titer. Third experiment with protein A and protein G of the same 6 serum samples used before. The protein A with 3 dilutions 1:500, 1:1000 and 1:5000 and the protein G with dilution 1:20 and 1:40 with titer 1:60 and 1:120. Fourth Experiment with using 16 serum samples with universal conjugate with 3 dilutions 1:500, 1:1000 and 1:5000 of titer 1:60 and 1: 120. Fifth experiment using universal conjugate with 4 dilution 1:250, 1:500, 1:750 and 1:1000 with serum dilution 1:60 with 24 serum samples (12 horse and 12 pig serum samples). Sixth experiment of same 24 serum samples using universal conjugate with dilution 1:250 of 3 serum dilution 1:30, 1:60 and 1:90. All serum samples were tested with IPMA.

2.2 Indirect Immuno Peroxidase Test

Acetone-fixed 96- well culture plates with McCoy cells highly infected with *L. intracellularis* was used for serology method. Wells were seeded with 5X 10^3 McCoy cells and grown for 24 hours before infection. Pure cultures of *L. intracellularis* were added to Dulbecco's modified Eagle's medium with 5% fetal bovine serum (FBS), and 100 µl of this preparation containing about 10^5*L. intracellularis* organisms was added to each well. The plates were incubated for 5 days in a gas concentration of 8.0% O2, 8.8% CO2, and 83.2% N2. Cold 50% acetone and 50% methanol solution was used to fix the cells. The plates were stored at _20 C until use [17]. Before use, the plates were rehydrated with distilled water and placed in a moist chamber in a 37ºC incubator for 10 minutes. The sera were diluted 1:30 in a plain 96-well plate using a 5% skim milk solution as a general titration for horse and pig serum samples. This was followed by preparation of dilutions at 1:60, 1:120 as done in all fourth experiments and dilution 1:60 in fifth experiment and 1:30, 1:60 and 1:90 in sixth experiment. 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 37ºC for 45 min. A different dilution of universal conjugate protein A/G or protein A and protein G alone per each experiment the conjugate is diluted with IPMA buffer. Upon completion of incubation, the sera were discarded and the plates rinsed four times with phosphate buffered saline (PBS). After the final rinse, the plate was dried on a stack of paper towels followed by the addition of 50µl/well of diluted conjugate. After incubation at 37°C for 45 min, the conjugate was discarded and the plates rinsed four times with PBS. After the final rinse, the plate was dried on a stack of paper towels followed by the addition of 100 µl of diluted AEC (3-amino-9-ethyl-carbazole) solution to each well. A 1:20 dilution of the AEC solution was made in acetate buffer (0.6 ml AEC solution in 11.4 ml acetate buffer) followed by the addition of 0.5 µl of 30% H2O2 3-5 minutes before use. The plates were incubated at room temperature for 20 min. The AEC 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.

2.3 Statistical Methods

Sensitivity and specificity for dichotomous data (positive/negative test results) were calculated using Win Episcope 2.0[1]. Agreement of categorical data (semi-quantitative evaluation scheme) was calculated using simple linear regression after checking residual plots for normality (StatsDirect statistical software Version 2.7.8). Data were transformed for CPV HI titers by calculating the $\log_2$ of 0.1x the original titer and, for CDV SN titers, by calculating the $\log_2$ of 0.25x the original titer prior to linear regression.

3. RESULTS AND DISCUSSION

The first experiment as preliminary test using known horse and pig serum samples to detect the ability of using universal conjugate protein A/G to detect the antibodies against *lawsonia intracellularis* in more than one species and the dilution used is same for both species horse and pig from the test we found that dilution 1:1000 with 100% specificity and sensitivity in both species horse and pig samples but more staining in the ground of plate dilution 1:5000 with sensitivity 50% and specificity 100% in both horse and pig but dilution 1:10,000 with 0% sensitivity and 100% specificity. 1:1000 dilution is good and promising, especially when compared with sensitivity and specificity is 100% which

mean that dilution good detect true positive by sensitivity100% and true negative by specificity 100% of the same original IPMA known serum samples. So, Protein A/G had abroad binding ability and bound sera from both species with certain dilution (Table 1).

The result of second experiment using only protein G separated than protein A. We found all samples are positive and staining with dilution 1:50. Sensitivity was 100% specificity was 0% at dilution 1:50 with spp. horse and pig, but the dilution 1:500 and 1:1000 both sensitivity was0% and specificity 100%. So this dilution 1:50 detect all samples positive with staining and not detect any negative but dilution 1:500, 1:1000 detect all samples negative by specificity 100% and no positive samples detected by 0% sensitivity. These let us thinking to do next step with protein G with new dilutions 1:20 and 1:40 (Table 2).

The result of third experiment at dilution 1:500 sensitivity for pig 100% and specificity 100% but for horse sensitivity was 50% and specificity was 100%, while at dilution 1:1000 pig sensitivity and specificity was 100% and horse 0% sensitivity, 100% specificity while at dilution 1:5000 both horse and pig samples sensitivity was 0% and specificity was100%. Plate of protein with dilution 1:20 sensitivity and specificity was 100% for horse and 0% sensitivity, 100% specificity for pig. While dilution 1:40 horse sensitivity was75% and specificity 100% but pig 0% sensitivity and 100 % specificity. Protein A strong binding to pig sera and weak binding of horse sera, on other hand protein G strong binding to horse sera and weak binding to pig sera. That is concluded that protein G alone and Protein A alone is species specific (Table 3).

The fourth experiment dilution used to overcome the staining of the first one plate Sensitivity was 50% and specificity 83% at dilution 1:500, but with horse 100% specificity and sensitivity while pig 60 sensitivity and 66% specificity 40% while dilution 1:1000 sensitivity was 20% and specificity y 100% and dilution 1:5000 with sensitivity 10% and specificity 100%. So, the detection true positive by 50% sensitivity, true negative by specificity 83% means that dilution 1:500 of universal conjugate is still Working and good, so need to detect the best dilution for using protein A/G (Table 4).

Fifth experiment using universal conjugate with 4 dilution 1:250, 1:500, 1:750 and 1:1000 with serum dilution 1:60 with 24 serum samples (12 horse and 12 pig serum samples). We found the dilution 1:250 with high sensitivity in both species horse and pig 100% and 80% specificity in horse and low in pig 40%, dilution 1:500 in pig sensitivity 85.7% and specificity 80% but in horse 57% sensitivity and 80% specificity, dilution 1:750 high specificity 100%, low sensitivity in pig 57% and with very low sensitivity in horse 28.5% but 100% specificity. On dilution 1:1000 very low

Table 1. Sensitivity and specificity of first experiment

Universal conjugate protein A/G	Dilution 1:1000		Dilution 1:5000		Dilution 1:10,000	
	Pig	Horse	Pig	Horse	Pig	Horse
True pos	4	4	2	2	0	0
True neg	2	2	2	2	2	2
False pos	0	0	0	0	0	0
False neg	0	0	2	2	4	4
Sensitivity	100	100	50	50	0	0
specificity	100	100	100	100	100	100

Table 2. Sensitivity and specificity of second experiment

Protein G conjúgate	Dilution 1:1000		Dilution 1:5000		Dilution 1:10,000	
	Pig	Horse	Pig	Horse	Pig	Horse
True pos	4	4	0	0	0	0
True neg	0	0	2	2	2	2
False pos	2	2	0	0	0	0
False neg	0	0	4	4	4	4
Sensitivity	100	100	0	0	0	0
Specificity	0	0	100	100	100	100

Table 3a. Sensitivity and specificity of protein A conjugate (Third experiment)

Protein A conjúgate	Dilution 1:500		Dilution 1:1000		Dilution 1:5000	
	Pig	Horse	Pig	Horse	Pig	Horse
True pos	4	2	4	0	0	0
True neg	2	2	2	2	2	2
False pos	0	0	0	0	0	0
False neg	0	2	0	4	4	4
Sensitivity	100	50	100	0	0	0
specificity	100	100	100	100	100	100

Table 3b. Sensitivity and specificity of protein G conjugate (Third experiment)

Protein G conjúgate	Dilution 1:40		Dilution 1:20	
	Pig	Horse	Pig	Horse
True pos	0	3	0	4
True neg	2	2	2	1
False pos	0	0	0	1
False neg	4	1	4	0
Sensitivity	0	75	0	100
specificity	100	100	100	100

Table 4. Sensitivity and specificity of fourth experiment

Universal conjúgate protein A/G	Dilution 1:500		Dilution 1:1000		Dilution 1:5000	
	Pig	Horse	Pig	Horse	Pig	Horse
True pos	3	2	1	2	1	2
True neg	2	3	2	3	2	3
False pos	1	0	1	0	1	0
False neg	2	3	4	3	4	3
Sensitivity	60	40	20	40	20	40
specificity	66	100	33	100	33	100

Table 5. Sensitivity and specificity of fifth experiment

Universal conjúgate protein A/G	Dilution 1:250		Dilution 1:500		Dilution 1:750		Dilution 1:1000	
	Pig	Horse	Pig	Horse	Pig	Horse	Pig	Horse
True pos	7	7	6	4	4	2	3	2
True neg	2	4	4	4	5	5	5	5
False pos	3	1	1	1	0	0	0	0
False neg	0	0	1	3	3	5	4	5
Sensitivity	100	100	85.7	57	57	28.5	42.8	28.5
specificity	40	80	80	80	100	100	100	100

Table 6. Sensitivity and specificity of sixth experiment

Universal conjúgate protein A/G (dilution 1:250)	Serum Dilution 1:30		Serum Dilution 1:60		Serum Dilution 1:90	
	Pig	Horse	Pig	Horse	Pig	Horse
True pos	7	6	7	6	7	5
True neg	2	5	1	5	3	5
False pos	0	1	0	1	0	2
False neg	3	0	4	0	2	0
Sensitivity	100	85.7	100	85.7	100	71.4
specificity	40	100	20	100	60	100

sensitivity in pig and horse 42.5%, 28.5% and with 100% specificity in pig and horse. So concluded that dilution 1:250 is good and decided using that dilution for universal conjugate in next experiment with 3 serum dilution 1:30, 1:60 and 1:90. Sixth experiment we found that serum dilution 1:30 with total sensitivity 92.85% and 70% specificity but in horse with high sensitivity 85.7% and 100% specificity but in pig high sensitivity 100% and low specificity 40%. Dilution 1:60 with total sensitivity 92.85% and specificity 60% on other hand high sensitivity and specificity in horse 85.7%, 100% but low specificity in pig 20% and 100% sensitivity. But dilution 1:90 with total 84.6% sensitivity and 81.8% specificity and in horse 71.4% sensitivity, 100 specificity but in pig 100 sensitivity and 60% specificity.

4. CONCLUSIONS

So concluded that dilution 1:250 is good and decided using that dilution for universal conjugate in next experiment with 3 serum dilution 1:30, 1:60 and 1:90. Sixth experiments we found that serum dilution 1:30 with total sensitivity 92.85% and 70% specificity but in horse with high sensitivity 85.7% and 100% specificity but in pig high sensitivity 100% and low specificity 40%. Dilution 1:60 with total sensitivity 92.85% and specificity 60% on other hand high sensitivity and specificity in horse 85.7%, 100% but low specificity in pig 20% and 100% sensitivity. But dilution 1:90 with total 84.6% sensitivity and 81.8% specificity and in horse 71.4% sensitivity, 100 specificity but in pig 100 sensitivity and 60% specificity.

CONSENT

As per international standard or university standard, written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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