Performance of a *Mycoplasma hyopneumoniae* serum ELISA for antibody detection in processing fluids

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Summary

The diagnostic performance of a commercial *Mycoplasma hyopneumoniae* (MHP) serum enzyme-linked immunosorbent assay (ELISA) was evaluated for MHP antibody detection in processing fluids (n = 494) using samples from three commercial swine farms. Based on historical monitoring, one farm was considered MHP positive and two were considered MHP negative. Samples were tested at a 1:10 dilution and diagnostic sensitivities and specificities estimated for specific ELISA sample-to-positive (S:P) cutoffs. At S:P ≥ 0.40, diagnostic sensitivity and specificity were estimated as 97.6% and 100.0%, respectively. Overall, the results suggest that processing fluids can be used for MHP antibody surveillance in breeding herds.

Keywords: swine, *Mycoplasma hyopneumoniae*, processing fluid, enzyme-linked immunosorbent assay, diagnostic performance

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Resumen - Comportamiento de un ELISA para suero de *Mycoplasma hyopneumoniae* para la detección de anticuerpos en fluidos de proceso

Se evaluó el comportamiento diagnóstico de un ensayo inmunoabsorbente ligado a enzimas (ELISA) en suero de *Mycoplasma hyopneumoniae* (MHP) comercial para la detección de anticuerpos contra MHP en fluidos de proceso (n = 494) utilizando muestras de tres granjas porcinas comerciales. Con base en el monitoreo histórico, una granja se consideró positiva para MHP y dos negativas para MHP. Las muestras se analizaron a una dilución de 1:10, y las sensibilidad y especificidad de diagnóstico se estimaron para los puntos de corte específicos de muestra a positivo (S:P) de ELISA. Con S:P ≥ 0.40, la sensibilidad y especificidad diagnóstica se estimaron en 97.6% y 100.0%, respectivamente. En general, los resultados sugieren que los fluidos de proceso se pueden utilizar para la vigilancia de anticuerpos MHP en hatos reproductores.

Résumé - Performances d'un ELISA sérique pour la détection d'anticorps envers *Mycoplasma hyopneumoniae* dans les fluides de procédures

Les performances diagnostiques d'une épreuve immuno-enzymatique (ELISA) sérique commerciale envers *Mycoplasma hyopneumoniae* (MHP) ont été évaluées pour la détection d'anticorps MHP dans les fluides de procédures (n = 494) à l'aide d'échantillons provenant de trois fermes porcines commerciales. Sur la base de la surveillance historique, une ferme a été considérée comme positive au MHP et deux ont été considérées comme négatives au MHP. Les échantillons ont été testés à une dilution de 1:10 et les sensibilités et spécificités diagnostiques ont été estimées pour des seuils ELISA spécifiques échantillon-à-positif (S:P). À S:P ≥ 0.40, la sensibilité et la spécificité diagnostiques ont été estimées à 97.6% et 100.0%, respectivement. Dans l'ensemble, les résultats suggèrent que les fluides de procédures peuvent être utilisés pour la surveillance des anticorps MHP dans les troupeaux reproducteurs.

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Mycoplasma hyopneumoniae (MHP), the etiological agent of enzootic pneumonia[1] and a major player in the porcine respiratory disease complex,[2] is one of the most economically important pathogens of swine, costing the US swine industry approximately $400 million annually.[3] Sow herd stability is key to the reduction of MHP losses in growing pigs because piglets are born MHP-free and become infected by contact with sows shedding the microorganism.[4] For this reason, control programs typically focus either on enhancement of sow herd immunity (vaccination or intentional gilt exposure) or complete elimination of MHP. Regardless of the approach, testing for MHP-specific DNA or antibody is needed to establish the status of the breeding herd population.[5,6] Because each diagnostic approach has its advantages and disadvantages, the choice is determined by which best fits the farm's MHP control strategy and yet is practical in terms of sampling and testing.

Processing fluid (PF), the serosanguineous fluid recovered from testicles and tails at the time of piglet processing (3-5 days of age), is an easily collected specimen with high diagnostic utility.[7-10] Sow herd surveillance using PF was first reported[11] in 2010 and has been widely adopted by the industry, eg, the Iowa State University Veterinary Diagnostic Laboratory performed approximately 395 diagnostic tests on processing fluids in 2017; 11,790 tests in 2018; 22,411 tests in 2019; 22,163 tests in 2020; and 26,075 tests in 2021 (Dr Giovani Trevisan, DVM, email, January 15, 2022). Although Boettcher et al[11] reported the detection of MHP-specific (colostral) antibody in PF collected from piglets ≤ 7 days of age, there are no reports substantiating or expanding upon this initial report. Therefore, the purpose of this study was to evaluate the diagnostic performance (ie, sensitivity and specificity) of a commercial enzyme-linked immunosorbent assay (ELISA) for the detection of MHP antibodies using PF samples.

Methods

Design

Processing fluid samples (n = 494) from 3 commercial farms were tested for the presence of MHP antibodies using a commercial MHP indirect serum antibody ELISA at a 1:10 dilution. Based on intervention program and historical monitoring, one farm was considered MHP positive (246 PF samples) and two farms were considered MHP negative (248 samples). Receiver operating characteristic (ROC) curve analysis was used to analyze diagnostic performance using farm MHP status as a proxy of sample status. From this analysis, diagnostic sensitivity and specificity and 95% CI were estimated over a range of cutoffs.

PF samples

Samples were collected from 3 commercial swine farms from 2018 through 2020 for the purpose of monitoring porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV). The criteria to establish MHP status corresponded to the MHP status of gilts used for the original stocking at each farm. The status of the MHP-negative farms was established based on their stocking history (ie, stocked with confirmed naïve gilts) and syndromic and routine surveillance. The latter consisted of monthly serum collection tested by MHP ELISA. Neither MHP-negative farm implemented MHP vaccine for piglets, gilts, or sows. The MHP-positive herd was stocked with MHP-positive gilts (confirmed at stocking via MHP ELISA on serum) that received commercial MHP vaccine at weaning (4 weeks of age; 2 mL Circumvent PCV-M; Merck Animal Health USA) and again at prebreeding (20 weeks of age; 2 mL Circumvent PCV-M). There was no mass vaccination of the sow herd and the piglets did not receive any MHP vaccine prior to weaning. Clinical signs of MHP in that herd were only identified sporadically in the gilt development unit in gilts 15 to 20 weeks of age, including mild coughing for 2 to 3 weeks with no noticeable performance impact (no mortality or average daily gain concerns).

Sample collection was performed by farm personnel using procedures previously described.[9] In brief, PF samples were collected at the time of piglet processing (ie, castration and tail docking) by placing testicle and tail tissues on gauze suspended over the top of a plastic container, thereby allowing the tissue exudate to pool in the bottom of the container. Each PF sample included tissues from 14 to 56 litters of 3- to 5-day-old piglets. At the end of processing, the liquid was transferred to a tube, stored at approximately 4°C, sent to the Iowa State University Veterinary Diagnostic Laboratory for PCV2 and PRRSV PCR testing, and then stored at -20°C until tested for MHP antibody.

MHP indirect antibody ELISA

The MHP ELISA (*M hyo* Ab test; Idexx Laboratories Inc), an assay designed to detect anti-P46 antibodies in serum, was used in the study. Samples were thawed, allowed to reach room temperature, and briefly vortexed. Thereafter, samples were tested for the presence of MHP antibodies following the instructions provided by the manufacturer with the exception that samples were tested at a 1:10 dilution rather than the 1:40 dilution described for serum.

To perform the test, samples were diluted 1:10 by adding 15 µL of sample to 135 µL of kit diluent in a dilution plate. Thereafter, 100 µL of diluted samples were transferred to plate wells, after which the plates were incubated (30 minutes, 22°C) on a plate heater (16-Position Microtiter Plate Heater; J-KEM Scientific) and then washed four times with 350 µL of wash solution on a plate washer (ELx405 Biotek Instruments Inc). Then 100 µL of kit conjugate was added to each well and the plate incubated (30 minutes, 22°C). The wash cycle was then repeated, 100 µL of 3,3',5,5'-Tetramethylbenzidine substrate was added to each well, the plates incubated (15 minutes, 22°C), and then 100 µL of stop solution was added into each well. Plates were read on an ELISA reader (EMax Plus Microplate Reader; Molecular Devices) using SoftMax pro 7.0 Software (Molecular Devices) and optical density (OD) results converted to sample-to-positive (S:P) ratios:

$$\text{MHP ELISA S:P} = \frac{\text{(Sample OD – Negative control mean OD)}}{\text{(Positive control mean OD – Negative control mean OD)}}$$

Statistical analysis

Diagnostic sensitivities and specificities for specific ELISA S:P cutoffs were estimated by ROC analysis using R software[12] (version 4.0.3; The R Foundation) and pROC package.[13] To perform the analysis, MHP ELISA S:P results with negative values were truncated to zero and sample status (positive, negative) was assumed to match farm status (MHP positive or MHP negative). Estimation of 95% CI for diagnostic sensitivity and specificity for every ELISA S:P cutoff was performed using a nonparametric stratified bootstrapping method with 10,000 iterations.[13,14]

Results

A frequency distribution of MHP ELISA S:P responses by farm status is given in Figure 1 and a summary of test responses by farm and year is given in Table 1. Among all samples from the two MHP-negative farms (n = 248), 246 (99.2%) had S:P values < 0.20 and all 248 (100%) had S:P values < 0.40. Among samples from the MHP-positive farm (n = 246), 240 (97.6%) had S:P values ≥ 0.40. Table 2 lists the diagnostic sensitivity and specificity estimated for specific MHP ELISA S:P cutoffs and 95% CI.

Discussion

Routine surveillance based on DNA and antibody detection is crucial for tracking MHP in commercial herds.[15] In sow herds, serum antibody testing is a common approach, but serum-based MHP surveillance is constrained both by the labor required for collecting blood samples and the number of samples required for statistically valid surveillance.[15] However, other specimens have been described to contain detectable levels of MHP antibody and could potentially be used for surveillance, eg, colostrum, milk, muscle tissue exudates (meat juice), and processing fluids.[11,16-18] In this regard, processing fluids are of particular interest because they are easily collected[10] and achieve better detection at the population level at a lower cost than individual pig sampling.[7,10,19]

The use of processing fluid antibody testing for sow herd surveillance was first reported in 2010 and has since been described for the nucleic acid- or antibody-based surveillance of a variety of pathogens, including hepatitis E,[7] influenza A virus,[11] MHP,[11,20] PRRSV,[9,11,21,22] PCV2,[8,22,23] porcine delta coronavirus,[22] and *Salmonella enterica*.[11]

On a diagnostic timeline, processing fluids were preceded by use of meat juice samples and the two are similar in derivation, ie, both are tissue exudates. Like processing fluids, meat juice contains detectable antibodies against a variety of pathogens, eg, *Toxoplasma gondii*,[24] pseudorabies virus,[25] *Salmonella enterica*,[26] PRRSV,[27] porcine epidemic diarrhea virus,[28] *Yersinia enterocolitica*, and *Actinobacillus pleuropneumoniae*.[17] Pertinent to the current study, Meemken et al[17] reported a 91% diagnostic sensitivity and 96% specificity for MHP antibody detection in meat juice when compared to serum antibody.

Processing fluids and meat juice differ in the source of the antibody in the sample. Antibody in meat juice is derived from the pig from which the sample was collected and indicates that the pig had been infected by, or vaccinated for, the pathogen of interest.[29] In contrast, antibody in processing fluids from 3- to 5-day-old piglets primarily represents circulating maternal antibody (primarily IgG). That is, colostral IgG is transported from the piglet's intestinal tract and into the lamina propria by nonselective endocytosis, then enters the intestinal lymphatic system, and finally, the circulatory system.[30] Therefore, antibody detection in processing fluid samples provides the means to surveil sow herd MHP antibody status - not the piglet humoral immune response against MHP infection.

Consistent with the report by Boettcher et al,[11] the commercial MHP ELISA used in this study was performed using a processing fluid sample dilution of 1:10 rather than the 1:40 dilution used in serum testing. The initial study of MHP antibody in 181 sows and processing fluids from their litters described strong agreement in MHP ELISA results at the herd level.[11] However, surveillance requires the use of assays with known diagnostic performance. The present study determined that the manufacturer´s recommended cutoff (S:P ≥ 0.40) provided 97.6% (95% CI, 95.5%-99.2%) and 100.0% (95% CI, 100%-100%) diagnostic sensitivity and specificity, respectively. However, since

Figure 1: Frequency distribution of MHP antibody ELISA (IDEXX Laboratories Inc) S:P responses by farm MHP status. MHP = *Mycoplasma hyopneumoniae*; ELISA = enzyme-linked immunosorbent assay; S:P = sample-to-positive ratio.

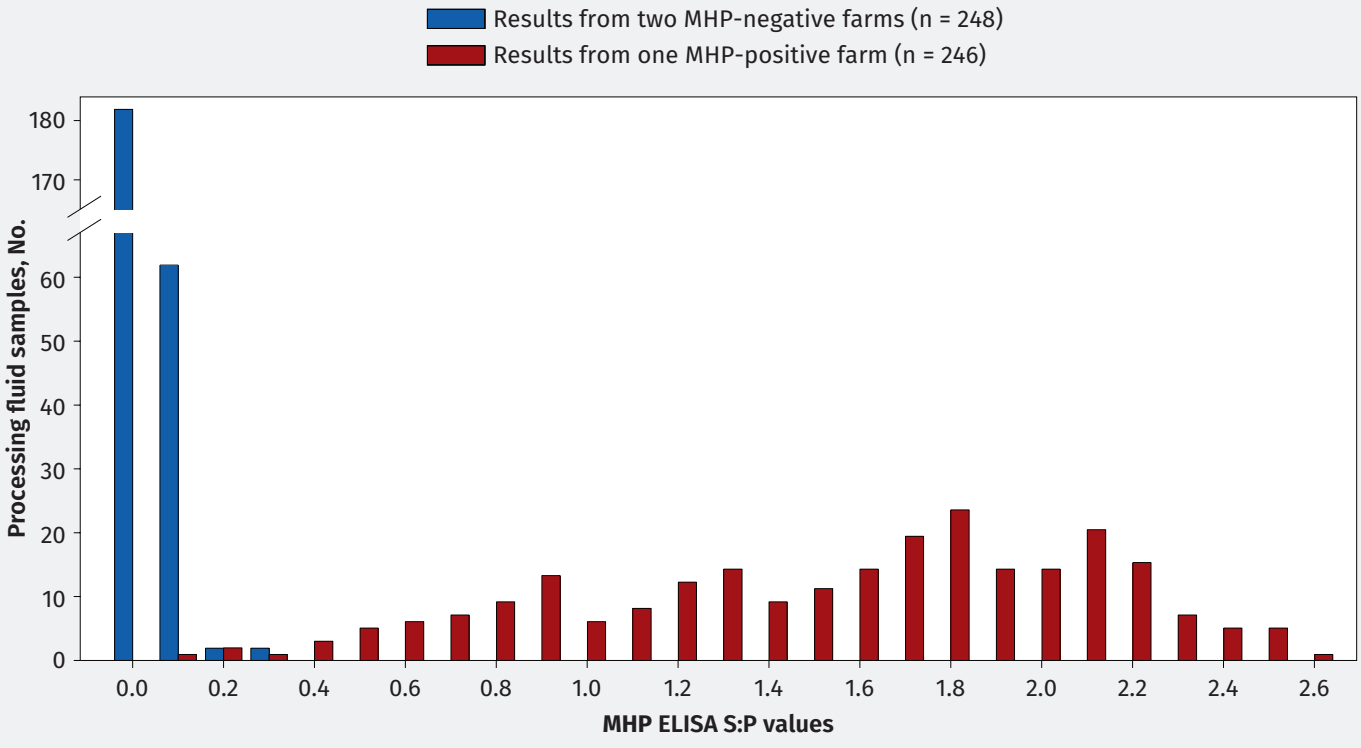

Table 1: Summary of MHP antibody ELISA* processing fluid sampling and testing data by farm

Farm (MHP status)	Year	No. samples	MHP ELISA mean S:P (min, max)
1 (positive)	2018	39	0.80 (0.10, 1.40)
	2019	143	1.51 (0.24, 2.61)
	2020	64	2.11 (1.18, 2.73)
	Total	**246**	**1.55 (0.10, 2.73)**
2 (negative)	2018	33	0.01 (0.0, 0.06)
	2019	122	0.03 (0.0, 0.28)
	2020	49	0.03 (0.0, 0.14)
	Total	**204**	**0.03 (0.0, 0.28)**
3 (negative)	2018	38	0.03 (0.0, 0.13)
	2019	6	0.05 (0.02, 0.09)
	Total	**44**	**0.03 (0.0, 0.13)**

* *M hyo* Ab test (IDEXX Laboratories Inc) with processing fluid samples tested at a 1:10 dilution.

MHP = *Mycoplasma hyopneumoniae*; ELISA = enzyme-linked immunosorbent assay; S:P = sample-to-positive ratio.

Table 2: Processing fluid MHP antibody ELISA* diagnostic sensitivity and specificity by S:P cutoff†

S:P cutoff	Sensitivity, % (95% CI)	Specificity, % (95% CI)
0.1	99.6 (98.8-100)	94.4 (91.5-97.2)
0.2	**99.2 (98.0-100)**	**99.2 (98.0-100)**
0.3	**98.8 (97.2-100)**	**100 (100-100)**
0.4	97.6 (95.5-99.2)	100 (100-100)
0.5	95.5 (92.7-98.0)	100 (100-100)
0.6	93.9 (90.7-96.7)	100 (100-100)
0.7	90.7 (87.0-93.9)	100 (100-100)
0.8	88.6 (84.6-92.3)	100 (100-100)
0.9	83.7 (78.9-88.2)	100 (100-100)
1.0	79.7 (74.8-84.6)	100 (100-100)

* *M hyo* Ab test (IDEXX Laboratories Inc) with processing fluid samples tested at a 1:10 dilution.

† Diagnostic sensitivity and specificity point estimates derived from ROC analysis using R software[12] (version 4.0.3) and pROC package.[13] A 95% CI was calculated using a nonparametric stratified bootstrapping method with 10,000 iterations.[13,14]

MHP = *Mycoplasma hyopneumoniae*; ELISA = enzyme-linked immunosorbent assay; S:P = sample-to-positive ratio.

near-perfect diagnostic specificity to minimize false-positive results is important for surveillance,[31] users may elect to use a higher cutoff using the cutoffs and associated diagnostic sensitivities and specificities provided in Table 2.

One limitation of the study was that sample classification was based on farm status rather than individual sow status. Two distinctly different MHP antibody response patterns were observed in samples from MHP-negative vs MHP-positive farms, but it is possible that samples from the MHP-positive sow herd were negative for MHP antibodies. Notably, four samples from the MHP-positive herd had S:P values < 0.40 (Figure 1). The overall impact of this small number of misclassified samples on the analysis would be to slightly underestimate the diagnostic sensitivity of the ELISA, but this will have little impact on the utility of this population-based surveillance tool. Still, the lack of detection in the MHP-negative dataset suggests a high level of specificity of this sample type and test. The MHP ELISA cannot differentiate between vaccine or acquired antibodies. Thus, positive processing fluid samples used from this study may have resulted from the use of vaccine in the breeding herd and not maternal antibodies derived from natural infection. This point will need to be considered for routine surveillance of vaccinated but antigen-free herds.

Overall, this study demonstrated that processing fluids could be used for detection of MHP-specific antibodies. The diagnostic performance of the sample type in known status samples revealed a high level of accuracy. The convenience and low-cost nature afforded by processing fluids, combined with its potentially high herd sensitivity, make it highly promising for monitoring naïve herds. Future investigation would need to determine the sensitivity of this sample type compared to serum or deep tracheal swabs for timely detection of MHP antibodies in MHP-naïve herds.

Implications

Under the conditions of this study:

- The MHP antibody ELISA discriminated between negative and positive sow herds.
- An S:P cutoff ≥ 0.40 provided 98.8% sensitivity and 100% specificity.
- Processing fluids could be used for surveillance of MHP-naïve herds.

Acknowledgments

Conflict of interest

Dr Zimmerman serves as a consultant to Idexx Laboratories, Inc. The terms of the consulting arrangement have been reviewed and approved by Iowa State University in accordance with its conflict of interest policies. No other conflicts reported.

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* Non-refereed references.

Conversion tables

Weights and measures conversions

Common (US)	Metric	To convert	Multiply by
1 oz	28.35 g	oz to g	28.35
1 lb (16 oz)	0.45 kg	lb to kg	0.45
2.2 lb	1 kg	kg to lb	2.2
1 in	2.54 cm	in to cm	2.54
0.39 in	1 cm	cm to in	0.39
1 ft (12 in)	0.3 m	ft to m	0.3
3.28 ft	1 m	m to ft	3.28
1 mi	1.6 km	mi to km	1.6
0.62 mi	1 km	km to mi	0.62
1 in^2	6.45 cm^2	in^2 to cm^2	6.45
0.16 in^2	1 cm^2	cm^2 to in^2	0.16
1 ft^2	0.09 m^2	ft^2 to m^2	0.09
10.76 ft^2	1 m^2	m^2 to ft^2	10.8
1 ft^3	0.03 m^3	ft^3 to m^3	0.03
35.3 ft^3	1 m^3	m^3 to ft^3	35.3
1 gal (128 fl oz)	3.8 L	gal to L	3.8
0.26 gal	1 L	L to gal	0.26
1 qt (32 fl oz)	0.95 L	qt to L	0.95
1.06 qt	1 L	L to qt	1.06

Temperature equivalents (approx)

°F	°C
32	0
50	10.0
60	15.5
61	16.1
65	18.3
70	21.1
75	23.8
80	26.6
82	27.7
85	29.4
90	32.2
102	38.8
103	39.4
104	40.0
105	40.5
106	41.1
212	100.0

°F = (°C × 9/5) + 32
°C = (°F - 32) × 5/9

Conversion calculator available at: amamanualofstyle.com/page/si-conversion-calculator

Conversion chart, kg to lb (approx)

Pig size	Lb	Kg
Birth	3.3-4.4	1.5-2.0
Weaning	7.7	3.5
	11	5
	22	10
Nursery	33	15
	44	20
	55	25
	66	30
Grower	99	45
	110	50
	132	60
Finisher	198	90
	220	100
	231	105
	242	110
	253	115
Sow	300	136
	661	300
Boar	794	360
	800	363

1 tonne = 1000 kg
1 ppm = 0.0001% = 1 mg/kg = 1 g/tonne
1 ppm = 1 mg/L