

SWINE HEALTH

Title: Evaluation of diagnostic assays for *Mycoplasma hyopneumoniae*: Their potential role in eradication success – npb #05-006

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Abstract

Mycoplasma hyopneumoniae (Mhyo) is an important pathogen in the swine population worldwide. Diagnosis of infection with this organism on an individual pig and herd basis continues to be challenging due to its slow spread within a herd, delayed seroconversion after infection, and genetic differences between isolates. All of these factors play important roles in the often difficult process of accurately detecting Mhyo, especially for eradication purposes. To evaluate the sensitivity and specificity of ELISA and PCR assays and also sampling sites for PCR, a time-course study was performed with groups of pigs challenged with field isolates of Mhyo, *M. flocculare*, *M. hyorhinitis* or *M. hyosynoviae*. Forty-eight pigs were divided into eight challenge groups. Four groups received different Mhyo isolates, two received different *M. flocculare* isolates, and one group each received *M. hyorhinitis* or *M. hyosynoviae*. Two pigs were necropsied at each of the following time points: 28, 70 and 95 days post challenge (DPC). Serum and nasal swabs were collected throughout the study and bronchoalveolar lavage fluid (BAL) and bronchial swabs were collected at necropsy. Three different ELISA assays, DAKO, IDEXX and Tween 20, were performed and all were found to be 100% specific for Mhyo. The DAKO ELISA appeared to be the most sensitive, detecting pigs earlier (14 DPC) and more consistently than either the Tween 20 or IDEXX ELISAs, which did not detect any positives until the 28 DPC time point. The Tween 20 and IDEXX ELISAs overall performed similarly to each other in this study, but differed 24% of the time on individual results from 28-95 DPC. In addition, the Tween 20 and IDEXX ELISAs appeared to detect lower levels of antibodies from one of the Mhyo isolates compared to the other 3 Mhyo isolates. Real-time PCR was run on the nasal and bronchial swabs and BAL collected. BAL was found to be the most reliable sample for detection of Mhyo.

Additionally, a panel of isolates of Mhyo, *M. flocculare*, *M. hyorhinitis* and *M. hyosynoviae* were compiled and tested against a number of mycoplasma-specific PCR assays. All Mhyo PCR assays tested were 100% specific, but several assays showed decreased sensitivity and did not detect all field isolates of Mhyo. A new real-time PCR assay was developed that appears specific and detects 100% of the Mhyo isolates in our collection. In summary, detection of infected animals on a herd basis by serology or PCR early in *M. hyop* infection is unpredictable and results should be interpreted with care. It may be beneficial to perform a combination of the DAKO assay with either the Tween 20 or IDEXX assays to increase diagnostic sensitivity, especially early in infection. Sampling site and PCR assay selection also have a significant impact on test outcome.

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Introduction

It is of increasing interest to swine producers and veterinarians to improve the health status of their herds by eradicating various pathogens including porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* (Mhyo). A key factor in a successful eradication program is the ability to accurately diagnose the presence, or perhaps more importantly, the absence of the pathogen of interest. Accurate determination of the absence of a pathogen also becomes critical during replacement animal selection to prevent the re-introduction of the disease into a herd. Mhyo, the cause of enzootic pneumonia, and an integral component of the porcine respiratory disease complex (PRDC), is one of the most important causes of respiratory disease-associated losses in swine production.

When assessing the criteria for selecting the appropriate diagnostic assay to use for Mhyo, the pathogenesis and epidemiology of the organism must be considered. Mhyo colonizes the ciliated epithelial cells of the lower respiratory tract and does not invade lung tissue. Mhyo grows slowly both in the pig and under culture conditions. As a result of its location and slow growth characteristics, Mhyo is poorly recognized by the immune system of the pig resulting in slow antibody production systemically. A number of techniques are currently used to detect the presence of Mhyo in the host and include: culture, serology, and polymerase chain reaction (PCR) assays. Typically, isolation and culture of an organism is considered the “gold” standard for the diagnosis and confirmation of its presence in the host, and technically this would be true for Mhyo. However, isolation of Mhyo is difficult due to the requirement for specialized media with Mhyo-antibody negative serum, and the organisms’ fastidious nature. Due to its slow growth properties, it typically requires 4 to 8 weeks to reach measurable levels and contamination from other bacteria and mycoplasmas that grow more rapidly are problematic and frequently preclude attempts to obtain pure cultures of the organism (9). Furthermore, presumptive identification has to be confirmed using various techniques such as PCR, immunofluorescence or microbe-specific antibodies (1). The use of PCR assays has increased our ability to detect Mhyo. However, many of the current assays are based on a limited number of isolates collected in the 1970s and 1980s (3, 5, 6, 11, 16, 22). A recent study by Dubosson, et al. found that detection of Mhyo on Swiss farms required a combination of two real-time PCR assays specific for different portions of the Mhyo genome in order to detect all of their isolates (7). However, little is known about the ability of current PCR assays used in the US to detect different Mhyo isolates.

Serology is the most common and convenient tool used to determine the presence or absence of an organism within a herd. However, as with most diagnostic assays associated with Mhyo infection, interpretation of serological results may be challenging (9, 12, 13, 14, 21). Currently Mhyo serum antibodies are detected in the US by three enzyme linked immunosorbent (ELISA) assays; the Tween 20 ELISA (4), the HerdCheck® *M. hyo* ELISA (IDEXX Laboratories, Westbrook, ME), and the DAKO *Mycoplasma hyopneumoniae* ELISA (DAKO Corporation, Carpinteria, CA). Recent research in our laboratory has demonstrated that these tests have excellent specificity with very few false positives. Conversely, sensitivity was relatively poor with a high rate of false negative results observed, especially during the early phases of infection (8). These findings are in agreement with others (14, 15, 17). As a result, the use of serology alone as a diagnostic tool for eradication schemes is questionable. It is also important to remember that the antibodies detected in blood to Mhyo by ELISAs do not directly correlate to protection against disease.

This study sought to assess the ability of current diagnostic assays to detect a number of isolates of Mhyo at various time points following infection. Of special concern was early infection, prior to the onset of clinical disease and pneumonia. Animals in the early stages of infection in which the organism is not detected have the most potential for infecting naïve herds. Confirmation of negative herd status for the organism remains problematic in many cases as the presence of the organism alone is not always correlated with disease or pneumonia. The development of PCR assays has greatly enhanced our ability to detect Mhyo, however due to the genetic variability of the organism the ability of the current assays to detect all isolates remains unknown.

Objectives

1. Evaluate the ability of current serological assays to detect antibodies to new and old isolates of Mhyo at various time points following infection.
2. Assess the ability of current PCR assays to detect newer Mhyo isolates both *in vitro* and *in vivo* from pigs using nasal swabs, bronchial swabs and bronchoalveolar lavage.

3. Evaluate assay cross-reactivity with other mycoplasma species and their antibodies.
4. Develop a real-time PCR assay that detects Mhyo genetic variants.

Materials and Methods

In vivo study

An experimental challenge study was performed using 48, 10-12 day-old pigs from a commercial herd previously determined to be negative for Mhyo, PRRSV and SIV. Pigs were divided into eight groups of six and housed in separate rooms in an animal isolation facility. At approximately one month of age, the pigs were challenged on three consecutive days with log phase culture (10 mL intratracheally for Mhyo and *M. flocculare* groups, or 5 mL intranasally for *M. hyorhinis* and *M. hyosynoviae* groups). The groups included two Mhyo isolates from 1995 and two from 2000. There were two *M. flocculare* groups and one group each of *M. hyorhinis* and *M. hyosynoviae*. Two pigs were necropsied at each of the following time points: 28, 70 and 95 DPC. Pigs were bled up to seven times: prior to challenge, and at 14, 28, 42, 56, 70 and 95 days post challenge (DPC). Nasal swabs were collected approximately 7, 14, 21, 28, 42, 56, 70 and 95 DPC and bronchoalveolar lavage fluid (BAL) and bronchial swabs were collected at necropsy.

For serology, the IDEXX and DAKO ELISAs were performed as per manufacturer's instructions and the Tween 20 ELISA was performed as previously described (3). The swabs and BAL were tested using previously published real-time PCR assays (7)

In vitro study

DNA was extracted from a collection of Mhyo, *M. flocculare*, *M. hyorhinis* and *M. hyosynoviae* isolates and used to assess the sensitivity and specificity of various Mhyo PCR assays. Genomic DNA was obtained from cultures using the QIAamp DNA mini kit (Qiagen, Valencia, CA). A survey of diagnostic labs and Mhyo researchers around the world was used to identify the most commonly used PCR assays for Mhyo. These assays were then used to test the mycoplasmal DNA (6, 7, 11, 15, 18, 19, 20, 22). The primer pairs from nested-set PCRs were run as individual assays (not as nested-sets) to determine the ability of each pair to form products in the Mhyo isolates. Based on these results a real-time PCR to a target different than the two published real-time assays was developed using PrimerQuestsm software (Integrated DNA Technologies, Iowa City, IA).

Results

Serology

The DAKO, IDEXX and Tween 20 ELISAs detected antibodies in pigs in all four of the Mhyo challenge groups. All pigs were seronegative for Mhyo antibodies prior to challenge. Seroconversion occurred as early as 14 days post challenge (DPC) in 22 of 24 pigs as measured by the DAKO ELISA. None of the Mhyo-infected pigs were seropositive at 14 DPC using the IDEXX or Tween 20 ELISAs. By 28 DPC all pigs in all challenged groups were seropositive using the DAKO ELISA and remained positive until necropsy. At 28 DPC the Tween 20 ELISA detected antibody positive or suspect values in 11 of 23 (48%) of the Mhyo-infected pigs, while the IDEXX ELISA detected antibodies in 16 of 23 (70%) pigs. By 42 DPC the IDEXX and Tween 20 ELISAs detected Mhyo antibodies in 14 of 15 (93%) of the Mhyo-infected pigs. Among pigs that were kept longer than 28 DPC, all were found positive at one time point by the DAKO and IDEXX ELISAs, while one pig remained seronegative throughout the trial based on the Tween 20 ELISA .

One Mhyo isolate (M hyo 1) induced lower antibody levels as measured by optical densities using the IDEXX and Tween 20 ELISAs (Figures 1-2). No difference was observed in any of groups by the DAKO assay (Figure 3). The lower antibody levels detected by the IDEXX and Tween 20 assays did not appear to be due to reduced pneumonia in that group of pigs compared to the other 3 Mhyo-infected groups.

PCR

Several of the previously published PCR assays did not detect all Mhyo isolates in our panel (Table 1). Standard PCRs targeting the 16S ribosomal RNA gene were 100% sensitive, as was the target gene sequence and primers developed by Sorensen, et al. (15). Although the two real-time assays (A and D, Table 1) did not

detect all isolates as has been previously reported (7). One isolate (4, Table 1) was not detected by either real-time PCR.

Real-time PCR assays were performed on the nasal swabs, bronchial swabs and BAL from the challenge study. Of the three samples, BAL was the most consistently positive, followed by bronchial swab, then nasal swab (Table 2). The nasal swabs required more rounds of replication in the real-time assay to be detected compared to either BAL or bronchial swabs indicating lower amounts of Mhyo DNA in the nasal swab samples (data not shown).

Specificity

The DAKO, IDEXX and Tween 20 ELISAs detected antibodies in pigs challenged with all four of the Mhyo isolates. In contrast, no antibodies were detected by the Mhyo assays from the sera of pigs challenged with *M. flocculare*, *M. hyorhinitis* or *M. hyosynoviae*. This gave all three Mhyo ELISA assays a specificity of 100% for these three tests. All of the PCR assays tested were specific to the mycoplasma species for which they were designed (data not shown).

Real-time PCR

A Mhyo-specific real-time PCR was developed that detected all Mhyo isolates in our collection. The assay uses primers based on a different gene sequence than was targeted by the currently published real-time assays (7). The assay was specific for all samples in our panel of Mhyo isolates (N, Table 1) and did not detect any of the isolates in our panels of *M. flocculare*, *M. hyorhinitis* or *M. hyosynoviae* (data not shown).

Discussion

The purpose of this study was to assess the ability of a number of currently available diagnostic assays to detect swine mycoplasmas. Pigs were experimentally infected with isolates of the major swine mycoplasmas enabling us to assess the specificity of the various assays by looking for cross-reactivity. No apparent cross-reactivity appeared between the different species of mycoplasmas in any of the assays assessed. However, it is important to note that we only evaluated the diagnostic capability of the assays for a limited number of mycoplasma isolates, which may not accurately reflect their ability to detect all isolates under field conditions.

Antibodies to Mhyo can take weeks to months to develop in infected animals, and the sensitivity of the three assays has been shown to vary at different stages of infection (2,7,13). Two of the ELISAs used in this study, the Tween 20 and IDEXX, are indirect assays using membrane or whole cell preparations as antigen. As a result the antibodies assessed recognize membrane or cellular proteins specific to the isolates used as antigen. The DAKO ELISA is a competitive inhibition assay that uses a monoclonal antibody to an internal Mhyo protein. In the DAKO assay, a monoclonal antibody competes with antibodies generated from exposure to the organism. The presence of serum antibodies prevents the monoclonal antibody from binding and as a result, once the level of serum antibodies reaches the positive level, quantification of antibody levels is difficult. It is important to remember that the virulence factors of Mhyo are not defined, and there has been no correlation between the presence of serum antibodies detected by any of the assays and protection against disease.

The DAKO ELISA appeared to be the most sensitive assay in this study as it detected antibodies in pigs at the earliest time point (14 DPC) and more consistently than either the Tween 20 or IDEXX ELISA. Neither the Tween 20 nor the IDEXX ELISA detected antibodies until 28 DPC. The Tween 20 and IDEXX ELISAs performed similarly to each other in this study overall, but differed 24% of the time on individual results from 28-95 DPC. In a study comparing the DAKO to the IDEXX, sera collected under field conditions identified sera that were positive by the DAKO ELISA, but negative by the IDEXX ELISA, and vice versa (2). Discrepancies between the assays were resolved using an immunoblot assay as a confirmatory test and that found that a positive result by either ELISA was a true positive (2). Therefore, it is important to recognize that the study performed here used only four Mhyo isolates and the results may not reflect the ability of the assays to detect all isolates in the field. However, this study confirmed earlier studies that if antibodies are detected by any of the assays, infection has occurred, regardless of the other test's result.

Variation in antibody levels to different isolates of Mhyo as determined by the Tween 20 and IDEXX ELISAs were demonstrated in this study. These differences may be important as serological results are

interpreted from samples collected under field conditions. The difference in antibody levels was not due to reduced disease. These results further confirm the differences in antigenic recognition of Mhyo isolates by the immune system of the pigs. Because the DAKO assay does not allow quantification of antibody levels, it is impossible to determine if similar results would have been detected with that assay.

Reliable detection of serum antibodies early in Mhyo infection under field condition may be more variable and results should be interpreted with care when the point of exposure is uncertain. It is also possible that the challenge dose may impact the rate of seroconversion so multiple time points for bleeding may be necessary to detect seropositive pigs under field conditions. Serology for Mhyo is best used on a herd basis rather than on an individual pig basis. Under field conditions, use of a combination of assays such as the DAKO with either the Tween 20 or IDEXX would increase detection sensitivity, especially early in infection. Based on the research, a positive result with any of the three ELISA assays should be considered positive. It is important to note that none of these ELISAs should be used as a confirmatory assay as none of the antigens used in the assays are known to be conserved between all isolates. No antibodies that cross-reacted with Mhyo were detected from serum from pigs infected with the other species of mycoplasma making all three ELISA assays 100% specific for Mhyo.

As has been previously demonstrated (11), BAL is the most sensitive sample for the detection of Mhyo by PCR analysis. Because Mhyo does not colonize the upper airways efficiently, nasal swab samples have lower amounts of DNA and so require extremely sensitive tests to detect positive results. While bronchial swabs and BAL samples are more difficult to obtain, requiring anesthesia or euthanasia of the pig, they are more sensitive at detecting Mhyo.

Several of the PCR assays tested in the study did not detect all isolates of Mhyo in our collection. It is unknown how this translates to detection of isolates in the field. It is extremely difficult to obtain pure Mhyo cultures, so only a limited number are available for assessment. The lack of universal detection of Mhyo isolates is problematic if eradication of the organism is the goal. One researcher found that using two real-time assays improved their ability to detect isolates of Mhyo (7). This may be an effective strategy to reduce false negatives; however, it is not known whether the assays proposed will detect all isolates of Mhyo in the field. In addition, we identified one Mhyo isolate in our study that was not detected by either of the two published real-time assays, further confirming these concerns. In an attempt to improve diagnostic capabilities, we have developed a real-time PCR assay for Mhyo that appears to have a very high sensitivity for Mhyo. However, further studies are underway in collaboration with other mycoplasmologists to investigate the ability of this assay to detect more isolates.

Lay Interpretation

Respiratory disease induced by *Mycoplasma hyopneumoniae* (Mhyo) is a problem recognized by the swine industry world-wide. Due to the significant production costs associated with Mhyo-induced respiratory disease, eradication is a strategy of interest to many producers. To implement a successful eradication strategy for an organism, effective diagnostic assays capable of accurately detecting infected animals is critical. The purpose of this study was to investigate the ability of common diagnostic assays to detect of Mhyo infection. Of special interest was determining the ability of serological assays to detect early infection. Also investigated was the ability of current polymerase chain reaction (PCR) assays to detect different Mhyo isolates.

A collection of isolates of Mhyo, *M. flocculare*, *M. hyorhinis*, and *M. hyosynoviae* was used to determine if previously published PCR assays were able to correctly detect their targets. In addition, pigs were infected with isolates of common swine mycoplasmas including Mhyo, *M. flocculare*, *M. hyorhinis*, and *M. hyosynoviae*. From these pigs the ability of serum antibody assays (ELISAs) and PCR assays to identify infection with the various isolates was investigated.

All assays were specific for Mhyo and did not detect either antibodies to or DNA from the other swine mycoplasmas. However, the ability of the assays to either detect serum antibodies or DNA varied between the Mhyo isolates. Based on the results of this study a combination of diagnostic assays may be required to accurately assess Mhyo infection under field conditions. Improved diagnostics are needed to accurately assess Mhyo infection status. The need for these diagnostic assays increases with the interest in eradication of Mhyo.

Careful interpretation of lab results is required for accurate diagnosis of the infection status of a herd in relation to Mhyo.

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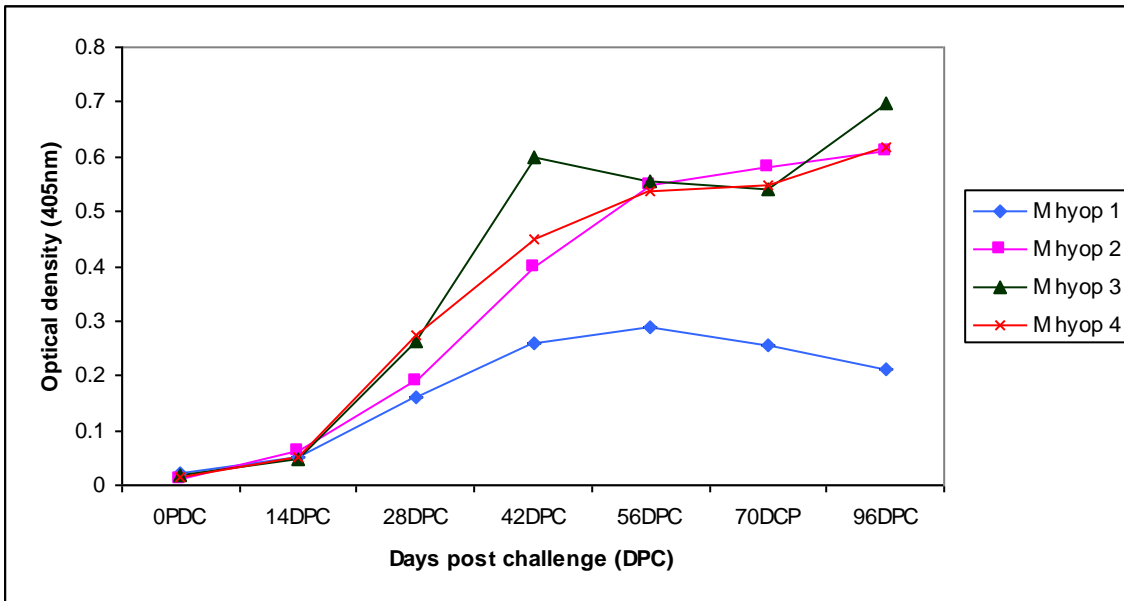


Figure 1. Comparison of group mean antibody levels (OD) in Mhyo-infected groups over time by Tween 20 ELISA.

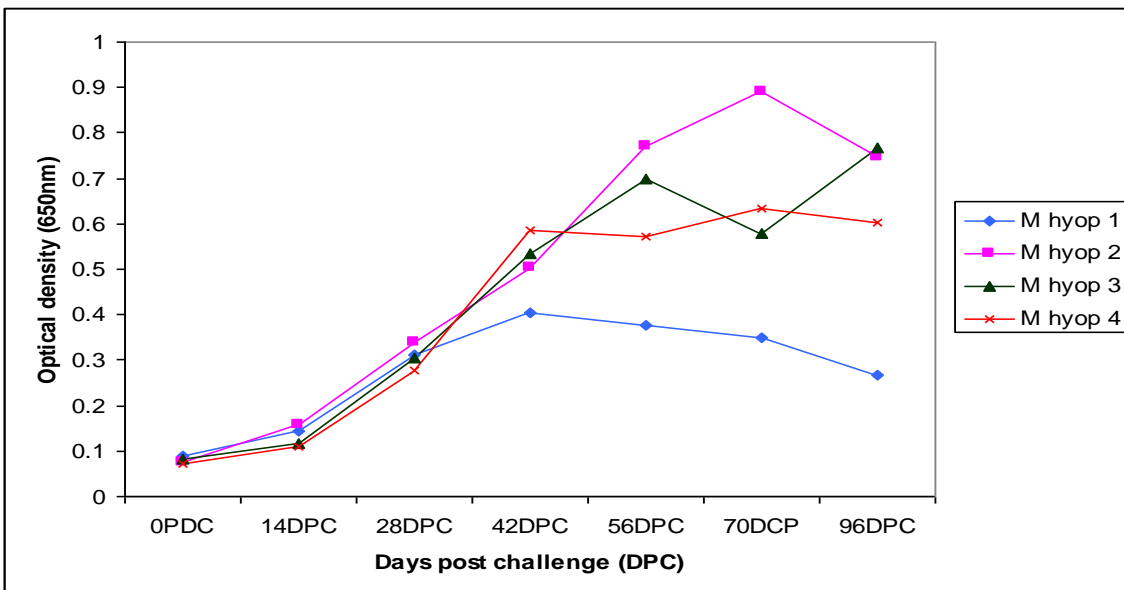


Figure 2. Comparison of group mean antibody levels (OD) in Mhyo-infected groups over time by IDEXX ELISA.

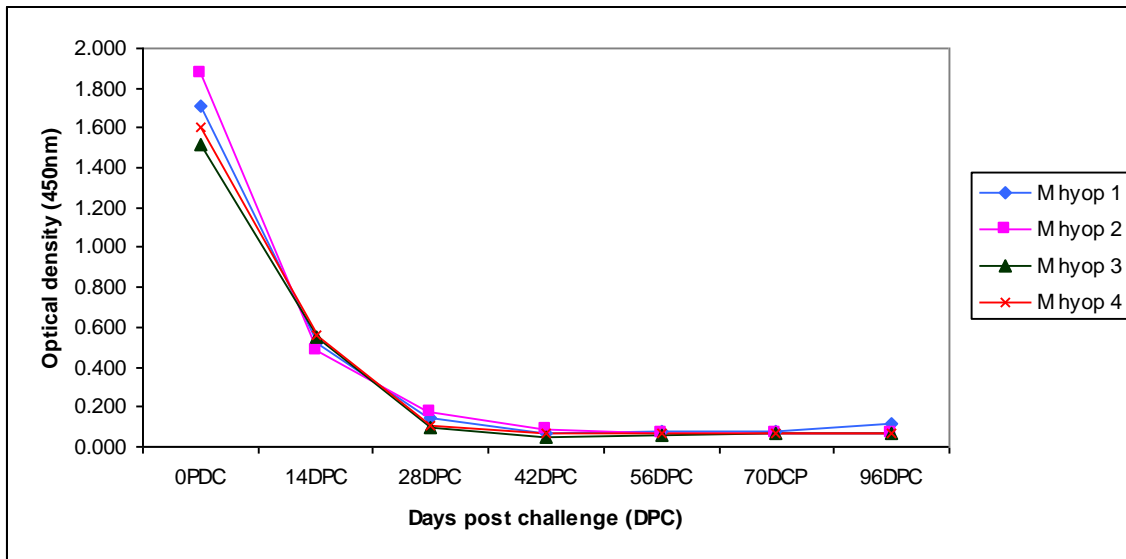


Figure 3. Comparison of group mean antibody levels (OD) in Mhyo-infected groups over time by DAKO ELISA.

Mhyo isolates	PCR assays												
	A	B	C	D	E	F	G	H	I	J	K	L	M
1	+	+	+	-	-	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	-	+	+	+	+	+	+	-	+
5	+	+	+	-	-	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	-	+
10	+	+	+	-	-	+	+	+	+	+	+	+	+
11	-	-	-	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	-	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	-	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	-	-	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+	+	+	+	+
27	+	+	+	+	+	+	+	+	+	+	+	-	+
28	+	+	+	-	-	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+	+	+	-	+
30	-	-	-	+	+	+	+	+	+	+	+	-	+
31	+	+	+	-	-	+	+	+	+	+	+	-	+
32	-	-	-	+	+	+	+	+	+	+	+	+	+
33	+	+	+	-	-	+	+	+	+	+	+	-	+
34	+	+	+	+	+	+	+	+	+	+	+	-	+
35	+	+	+	+	+	+	+	+	+	+	+	-	+
36	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 1. Summary of PCR assay results using a panel of Mhyo isolates. A-REP assay (ref 7), B-outer nested set (ref 19), C-inner nested set (ref 19), D-ABC assay (ref 7), E-inner nested set (ref 22), F-multiplex assay (ref 18), G-(ref 15), H-outer nested set (ref 6), I-inner nested set (ref 6), J-(ref 20), K-outer nested set (ref 11), L-inner nested set (ref 11), M-newly developed real-time PCR.

Pig #	Group	Days post challenge													
		7	14	21	28	28	28	42	56	70	70	70	95	95	95
		NS	NS	NS	NS	BS	BAL	NS	NS	NS	BS	BAL	NS	BS	BAL
153	1	+	-	+	+			+	-	NA	+	+			
157	1	+	+	+	+			+	-	NA	-	+			
163	1	+	+	+	+			+	-	NA			NA	-	-
169	1	-	+	+	+	+	+								
170	1	+	+	+	+			+	+	NA			NA	+	+
172	1	-	+	+	+	+	+								
158	2	-	-	+	+	+	+								
159	2	+	+	+	+			+	-	NA	-	-			
160	2	+	+	+	+			+	+	NA	+	+			
161	2	+	+	+	+			+	+	NA			NA	-	+
162	2	-	+	+	+	+	+								
164	2	-	+	+	+			+	-	NA			NA	+	+
752	3	+	+	-	-			-	-	+			-	-	-
755	3	+	-	+	+	+	+								
762	3	-	+	+	-	+	+								
764	3	-*													
767	3	-	-	+	+			+	-	-			-	-	+
773	3	+	+	+	+			-	-	+	+	+			
751	4	+	+	-	+			+	+	+			-	+	+
757	4	+	+	+	-			-	-	+	+	+			
759	4	+	+	+	+			+	-	-	+	+			
768	4	+	+	+	+	+	+								
770	4	+	+	+	+			+	+	+			+	+	+
772	4	+	+	+	+	+	+								

Table 2. Summary of real-time PCR assay results from DNA obtained on nasal swabs (NS), bronchial swabs (BS) and bronchoalveolar lavage (BAL) from pigs experimentally infected with Mhyo isolates. NA-not available.

* Pig 764 euthanized for medical reasons after this time point. Where boxes are blank samples were not to be collected from that pig at that time point.