Field Trial Assessment of Two Commercial Vaccines Against *Mycoplasma Hyopneumoniae*

Marcial Marcos (marcial.marcos@merck.com)
MSD Animal Health https://orcid.org/0000-0002-0352-3080

Javier Rabanal
PROGATECSA S.A.

David del Pico
PROGATECSA S.A.

Jesús Bollo
MSD Animal Health

Alfredo Romero
MSD Animal Health

Maria Luisa Pérez-Breña
MSD Animal Health

Marta Jiménez
MSD Animal Health

Rut Menjón
MSD Animal Health

Teresa Tejedor
Universidad de Zaragoza Facultad de Veterinaria

Hanny Swam
Center for Diagnostic Solutions, MSD AH Boxmeer

Research article

Keywords: Mycoplasma hyopneumoniae, porcine respiratory disease complex, control, vaccination, intradermal, efficacy

Posted Date: September 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-67592/v1

Abstract

Background: *Mycoplasma hyopneumoniae* (*M.hyo*) is one of the most prevalent pathogens causing respiratory disorders in swine populations as well as primary agent of porcine respiratory disease complex (PRDC). The aim of this trial is to compare the efficacy of two commercial vaccines under field conditions, applied via different routes of administration, as a tool to control disease.

Results: 3 groups each consisting of 440 animals, were included in this longitudinal study; one group vaccinated against *M.hyo* with an intradermal vaccine (group I), a second group vaccinated with an intramuscular vaccine 2940 strain (group H) and finally a third group, not vaccinated as a control (group N). All animals were individually weighed at weaning; upon entry into the fattening unit and just prior to movement to the slaughterhouse. Pigs from each group were periodically blood sampled to evaluate the immunological status against other pathogens involved in PRDC. Tracheobronchial swabs were collected from 30 pigs per treatment group during the final weighing. Slaughterhouse lung lesion evaluations were developed to assess levels of enzootic pneumonia-like lesions (EP) and pleuritis (P).

Statistical evaluation of all test parameters indicated a better performance of the intradermal vaccinated group (group I):

· in terms of weight (kg) just prior to movement to the slaughterhouse, (I= 97.45; H = 93.44; N = 93.05).

· The number of positive tracheobronchial swabs, as well as *M.hyo* load detected individually (I = 36.7% positive samples with log10 Ct average value 1.48; H = 100% with log10 Ct 1.40; and N = 96.7% with log10 Ct 1.40),

· EP prevalence (I = 35.1%, H = 47.0%, N = 50.5%) and

· Pleurisy prevalence (I = 22.4%, H = 29.7%, N = 39.3%).

In terms of the involvement of other PRDC pathogens, the serological study could lead us to think that the agents studied were present at same level in the all 3 vaccination groups.

Conclusion: In this study, intradermal vaccination provided better performance than unvaccinated control group and intramuscular vaccine, in terms of weight increase (versus intramuscular group); reduction of enzootic pneumonia and pleurisy lesions, and tracheobronchial *M.hyo* prevalence at the end of the finishing period.

Background

Mycoplasma hyopneumoniae (*M. hyo*) is one of the most widespread pathogens worldwide that affects pigs [1], it causes significant losses in growth [2] and, therefore, economic losses [1–3]. It is considered to be the primary causative agent of enzootic pneumonia (EP), as well as one of the main responsible agents for the onset of the porcine respiratory disease complex (PRDC) [4]. It is responsible for the appearance of a dry, chronic, non-productive cough due to its adhesion to the pseudostratified ciliated epithelium that covers almost the entire respiratory system, thus compromising its defensive capacity, in addition to causing an important inflammatory reaction at the pulmonary level [1]. It is a bacterium that does not penetrate the organism of the individual, as other pathogens can, but remains on the surface of the mucosa of the respiratory system, destroying the histological structure of the epithelium. Consequently, it “dismembers” the host defence mechanisms; and as a result, it facilitates the infection of other concomitant agents [4, 5, 6], not only by its direct action but by an indirect effect.

There are several factors we can consider in the control of M.hyo infections [4, 5]. Firstly, the optimisation of management practices and housing conditions will have a positive impact on disease control. Various parameters within this section must be considered: the size of the farm, livestock density in the pens, the production system, the purchase and introduction of animals, biosecurity, and the optimisation and improvement of housing conditions. Another point of control could be antibiotic therapy, numerous works have been published with the aim of demonstrating the varying efficacy of different types of treatment to minimise the effects of *M.hyo* [7, 8]. Additionally, we have vaccination as a control mechanism. Previous studies report that in some countries approximately 70% of swine production is vaccinated against *M.hyo* every year, a trend that increases as the

sector becomes professionalised [4]. The vaccines available on the market are effective, in that they prevent the onset of clinical signs and lung lesions, in addition to the reduction, frequency and severity of such lesions [4]. Differences in efficacy among commercial vaccines for disease control have been described in published papers [9]. However, according to the authors' knowledge, no publication so far has been able to demonstrate the role of vaccination in reducing colonisation [10, 11], although vaccination has been proven to be efficient for disease control [12]. Within the wide range of vaccine products available, all of them are for intramuscular administration, except one vaccine for intradermal application. Studies by Martelli et al., [12], Tassis *et al*., [13], and Beffort *et al*., [14] have demonstrated, not only the greater efficacy of this product against other vaccines for intramuscular use (in terms of average daily weight gain (ADWG), reduction of clinical signs and lung lesions) but also an increase in the levels of local protection at the pulmonary level with respect to some vaccines, measured in the form of IgA and levels of Il-10.

Therefore, the aim of this field trial is the comparison of two commercial *M.hyo* vaccines, intramuscular administration versus intradermal application, using different strains as antigen, against an untreated control group, to evaluate the possible differences in the growth of pigs on a commercial fattening farm, as well as the control of associated lung lesions.

Results

1. Results of the growth study: a study of pig weights at various rearing stages.

The mean ages of the animals at first weighing (post-weaning, WEIGHT 1) were: 22.8 ± 1.57 (SD) days, 22.4 ± 1.23 (SD) days and 21.8 ± 0.84 (SD) days respectively, for groups H, I and N. The mean ages of the animals at the second weighing (pre-fattening, WEIGHT 2) were: 57.3 ± 1.83 (SD) days, 57.4 ± 1.23 (SD) days and 57.0 ± 1.39 (SD) days, respectively for groups H, I and N. The mean ages of the animals at the third weighing (pre-slaughterhouse, WEIGHT 3) were: 160.9 ± 2.47 (SD) days, 161.4 ± 1.33 (SD) days and 162.3 ± 1.52 (SD) days, respectively for groups H, I and N.

Three groups of piglets were generated depending on the cycle of the sow from which they came (SOW CYCLE (grouped cycle): primiparous, farrows 2 to 6, and farrows 7 to 9); and 3 groups of piglets depending on the vaccine against *M. hyo* they received before weaning (TREATMENT GROUP: vaccinated intramuscularly (H), vaccinated intradermally (I), and not vaccinated (N)).

Table 1 shows the results obtained.

TABLE 1: Results of the three weighings (kg)

Cycle	Treatment	Weight 1			Weight 2			Weight 3		
		Number	Mean	SD	Number	Mean	SD	Number	Mean	SD
Primiparous	H	107	6.10	.828	107	16.71	2.777	77	91.74	10.684
	I	98	6.36	.790	98	17.17	2.572	61	96.49	10.396
	N	95	5.57	.696	95	15.84	2.300	76	92.08	8.474
	Total	300	6.01	.837	300	16.58	2.615	214	93.21	10.038
Cycles 2 to 6	H	259	6.07	.820	259	16.32	2.748	167	93.01	10.355
	I	251	6.26	.845	251	16.59	2.666	182	97.60	10.235
	N	255	5.82	1.059	255	15.89	2.708	187	92.58	10.072
	Total	765	6.05	.931	765	16.27	2.720	536	94.42	10.451
Cycles 7 to 9	H	58	6.45	.950	58	17.73	2.836	45	97.93	12.237
	I	78	6.35	.944	78	16.91	2.763	59	97.98	11.763
	N	67	6.30	.943	67	16.70	2.349	50	96.26	9.681
	Total	203	6.36	.942	203	17.07	2.677	154	97.41	11.232
Total	H	424	6.13	.849	424	16.61	2.802	289	93.44	10.901
	I	427	6.30	.851	427	16.78	2.668	302	97.45	10.557
	N	417	5.84	.993	417	16.01	2.577	313	93.05	9.720
	Total	1268	6.09	.919	1268	16.47	2.703	904	94.64	10.568

(Number: effectively analysed; SD: standard deviation)

A statistically significant interaction of the sow's cycle and treatment was detected on WEIGHT 1, ($p = 0.005$). Therefore, a simple main effects analysis was performed for treatment with statistical significance. Statistically significant differences were detected in the mean WEIGHT 1 between the treatment groups for primiparous sows, ($p < 0.001$) and for cycles 2 to 6, ($p < 0.001$) but no significant differences were detected between the different treatments groups for cycles 7 to 9 ($p = 0.650$).

For the study of WEIGHT 2, WEIGHT 1 was incorporated as a covariate in the two-way ANOVA. Once the highly significant effect of WEIGHT 1 on WEIGHT 2 was corrected, ($p < 0.001$), no statistically significant interaction of the sow's cycle and treatment was detected on WEIGHT 2, ($p = 0.332$), nor significant differences between treatment groups, ($p = 0.262$). However, significant differences were detected between the cycles with respect to WEIGHT 2, ($p = 0.019$). For multiple comparisons the Bonferroni correction was performed; the primiparous sows showed a mean WEIGHT 2 significantly higher than those of cycles 2 to 6 ($p = 0.020$). No other significant differences were detected.

Finally, for the study of WEIGHT 3, both WEIGHT1 and WEIGHT 2 were incorporated as covariates in the two-way ANOVA. No significant effect of WEIGHT1 was detected on WEIGHT 3, (p = 0,269), though there was a significant effect for WEIGHT 2 on WEIGHT 3, ($p < 0.001$). Once the highly significant effect of WEIGHT 2 on WEIGHT 3 was corrected, no statistically significant interaction of the sow's cycle and treatment was detected on WEIGHT 3, ($p = 0.511$). However, significant differences were detected between the cycles with respect to WEIGHT 3, ($p = 0.003$), and also between treatment groups, ($p = 0.001$). For multiple comparisons, the Bonferroni correction was performed. Regarding the cycles, the mean WEIGHT 3 in primiparous sows was significantly lower than the mean in cycles 7 to 9 ($p = 0.002$). Regarding the treatments, the mean WEIGHT 3 was significantly higher in group I with respect to group H ($p = 0.001$). No other significant differences were detected.

2. Results of the tracheobronchial swab test.

The results of this part of the trial were divided into two sections. Firstly, the comparison of the percentage of positive samples between the treatment groups (TABLE 2) was carried out. Subsequently, taking into account only those samples that had tested positive, the degree of positivity (numerical value of Ct) was assessed. The Ct value refers to the number of cycles required in the real-time PCR technique for a sample to be identified as positive, i.e. lower values correspond to more positive samples. In addition, a logarithmic transformation (log10) was applied to the test results to solve the problems of the absence of normality presented by the original variable. Note: it is only necessary to compare positive scores, because all negative scores have the same value (> 38).

TABLE 2. Results of the tracheobronchial swab test.

Variable	Treatment			*p*
	H	I	N	
Positive samples	30/30 (100%)[a]	11/30 (36.7%)[b]	29/30 (96.7%)[a]	<0.001
Log10 positivity degree	1.40±0.060[a]	1.48±0.077[b]	1.40± 0.090[a]	0.005

[a,b:] a different superscript in the same row indicates significant differences. The results for Log10 positivity degree are mean ± SD.

The Pearson Chi-square test detects highly significant differences between the treatments in terms of the percentage of positives (p> 0.001). As shown, the lowest percentage of positives corresponds to treatment group I; among treatment groups H and N no significant differences are detected. Regarding the degree of positivity, it is demonstrated that the mean in the treatment group I is significantly higher than in groups H and N, corresponding to a lower *M. hyo* bacteria load in this treatment group. There are no significant differences in any other case.

3. Results of the slaughterhouse assessment.

In TABLE 3, the results of lung assessments at the slaughterhouse are shown. The mean disease index values for treatment groups H, I and N were 0.7, 0.5 and 0.8, respectively. The mean APPI index values for groups H, I and N were 0.7, 0.4 and 0.7, respectively. Note: we only have 2 data sets per group for I and N, and one data set in the case of group H, therefore the power of the statistical study is very low and consequently, there are no significant differences, although there are numerical differences. Considering prevalence data for EP we can see that statistical differences appear in favour of treatment group I (35.1%) compared to H (47.0%) and N groups (50.5%). Also, considering prevalence data for pleuritis we can see a statistical difference between treatment group I (22.4%) over control group N (39.3%). No more significant differences were found.

TABLE 3. Results of the slaughterhouse assessment.

Variable	Treatment			*p*
	H	I	N	
Disease index	0,7	0,5	0,8	0.368
EP prevalence	87/185 (47.0%)[a]	138/393 (35.1%)[b]	153/303 (50.5%)[a]	<0.001
Maximum lesion	1/185 (0.5%)	2/393 (0.5%)	6/303 (2.0%)	0.123
Prevalence of pleuritis	55/185 (29.7%)[a,b]	88/393 (22.4%)[b]	119/303 (39.3%)[a]	<0.001
APPI Index	0,7	0,4	0,7	0.331

[a,b:] a different superscript in the same row indicates significant differences.

4. Results of the serological study

Table 4 shows the number of positive animals tested individually for antibodies against PRRS (ELISA-PRRS IDEXX), *M. hyo* (ELISA-MHYO IDEXX), and PCV2 (ALPHALISA PCV orf2).

TABLE 4. Result of positive serology

Sampling (weeks of age)	ELISA-PRRS (IDEXX) Positives			ELISA-MHYO IDEXX Positives			ALPHALISA-PCV type2 Positives		
	H	I	N	H	I	N	H	I	N
1st (3 weeks)	16/16	14/16	16/16	0/16	3/16	0/16	15/16	15/16	16/16
2nd (6 weeks)	8/16	9/16	11/16	7/16	1/16	0/16	16/16	16/16	16/16
3rd (9 weeks)	8/16	11/16	15/16	8/16	0/16	0/16	16/16	16/16	15/16
4th (13 weeks)	15/15	16/16	14/15	7/15	0/16	0/15	15/15	16/16	14/15
5th (17 weeks)	16/16	16/16	15/15	10/16	0/16	0/15	13/16	16/16	10/15
6th (21 weeks)	15/15	15/15	15/15	15/15	1/15	1/15	11/14	11/15	13/15

Regarding the App analysis (ELISA-APXIV IDEXX®) at 21 weeks, both H and I treatment groups detected 15/16 positives and 1/16 suspect, all serum samples were positive for group N. In the analysis of molecular biology (pool samples), no PCR positive for PRRS was detected in the 3rd week of life, at the time of vaccination. In the 6th week, in both H and N treatment groups 2/4 positives compared to 3/4 positives for treatment group I, were detected. Between weeks 9 and 13 all pools were positive in all groups. For PCV2, all the pools were negative, except in week 21, where groups H and N presented 2/4 positives, while in group I there was only 1/4 positive. All the positives found for PCV2 had a value of less than 3 (based on logarithm base 10), which is not associated with clinical symptoms of circovirosis.

Discussion

The trial was developed as a longitudinal study in which weaned piglets were included for 6 consecutive weeks. The reason for choosing this protocol, instead of a protocol in which different treatment groups were mixed in the same week, was to eliminate the effect of the interaction of treatments within the same week. Additionally, the protocol used in this trial has allowed us to avoid the possible interaction between the different treatment groups if they were to have temporarily cohabited. As seen in other trials, when cohabiting, the control group may benefit from the decrease in infection pressure, fostered by the presence of vaccinated groups; alternatively, cohabiting may mask the lesser effect of a vaccine with reduced protection by mixing with other animals vaccinated with another more effective vaccine [21]. This protocol also facilitated the slaughterhouse assessment. This type of sample design may indeed have other drawbacks, which we will discuss later on.

1. Growth study discussion:

First, it should be noted that, despite the great care that was taken at the time of designing the trial and selecting the animals to make all the groups as homogeneous as possible, differences between them were detected at the time of the first weighing (WEIGHT 1). Obviously, at this moment there is no point in assuming any possible vaccination effect against *M. hyo* since the piglets had only been vaccinated for 3 days. It would be possible that this kind of trial, to assess the performance of different *M. hyo* vaccines, which begins only once the animals enter the fattening unit, could have its final result affected by such an undetected variable. Therefore, throughout the statistical study carried out in the different stages of animal growth

(comparisons at the time of WEIGHT 2 and WEIGHT 3), the effect of the initial weight has been considered when assessing possible statistically significant differences between treatments.

In the case of the data obtained in WEIGHT 2, both WEIGHT 1 and SOW CYCLE affected the weight obtained at that time, around 57 days of age. This shows the great influence that weaning weight has on the weight of piglets just 5 weeks later, before moving on to the fattening units [22,23]. Likewise, the cycle of the sow from which they were farrowed has an influence, being only in this case restricted to piglets born to primiparous sows, which are significantly larger than piglets born to sows of cycles 2 to 6. However, once the highly significant effect of WEIGHT 1 has been corrected, the influence of the interaction SOW CYCLE*TREATMENT or the TREATMENT GROUP is no longer detected, which seems to indicate that the vaccine used, at this time of the trial, acts in the same way in all cycles and that the influence of the cycles on the mean weight is similar in all treatment groups. This could lead to the hypothesis that, until this stage in development, *M. hyo* infection is not important in the population.

Finally, as for the growth study, there is one last weighing (WEIGHT 3), carried out around 161-162 days of age for all the animals that kept their individual identification. The analysis of the weights recorded leads us to assertain that the effect of weight at weaning (WEIGHT 1) does not influence WEIGHT 3, additionally, WEIGHT 1 does not affect the weight just before leaving for the fattening units (WEIGHT 2), which in turn, affects growth at the time of WEIGHT 3, perhaps because there may have been little time between the two weighings. Once the highly significant effect of WEIGHT 2 has been corrected, the effect of the interaction SOW CYCLE*TREATMENT GROUP is not detected, but an important influence is maintained separately from both the SOW CYCLE and, for the first time in the trial, from the TREATMENT GROUP. Regarding the SOW CYCLE, we may conclude that the piglets born to primiparous sows, show a mean weight significantly lower than that of cycles 7 to 9. Therefore, we may confirm the worse performance during the fattening stage was for the piglets born to primiparous sows. When analysing the TREATMENT GROUP, we have already mentioned that WEIGHT 3 is the first time in the trial where there is evidence of a significant effect of the vaccine against *M. hyo* with which the piglets had been immunised pre-weaning. Statistically, once the estimation of the marginal means has been performed, it may be affirmed that the group vaccinated against *M. hyo* with the intradermal vaccine (group I) has a final mean weight (WEIGHT 3) that is significantly higher than the piglet group vaccinated with the other intramuscular vaccine (group H). However, no other significant differences are detected, although there are numerical differences with the N group (control group- not vaccinated against *M. hyo*). At this point, it is also important to note that the latter weighing (WEIGHT 3) occurs just before the pigs are sent to the slaughterhouse to preserve as many animals as possible out of those originally identified. However, many of the animals remained for several weeks more in the fattening units until they are sent to the slaughterhouse, and if they were affected at that time by *M. hyo*, the difference in growth between the groups could be greater if one more weighing were to be performed on these animals just before they are sent to the slaughterhouse. Therefore, we could consider likely that, if this last weighing were to be performed a few weeks later, the differences in mean weights could have increased between the different groups due to the effect of the vaccine compared to non-vaccination. Finally, the fact that the differences with the unvaccinated group are only numerical could be based on the mean age of the pigs that belong to that group compared to the other two. We must remember that group N, with 162.3 ± 1.52 (SD) average days of age, is 0.9 days older than group I, and 1.4 days older than group H. At such ages, according to some studies, the growth rate of pigs can approach or even exceed 1Kg per day [23,24].

2. Discussion of the tracheobronchial swab test (TBS)

The analysis of the results of tracheobronchial swabs suggests the hypothesis that the group vaccinated with the intradermal vaccine (group I), enjoys greater protection from *M. hyo*, which would be in line with the results of the weight study. It seems that, according to one study [12], intradermal vaccination offers levels of local immunity (at the level of the lung mucosa), and systemic immunity which is greater compared to other intramuscularly administered bacterin against the same agent (specific IgA antibody levels of *M. hyo* and IL-10 expression levels, responsible for controlling inflammation locally). Continuing with the second part of this study of lung samples, we may confirm, given the results, that not only do the animals vaccinated intradermally show fewer statistically significant positive samples, but also that the value of Ct is greater, which indicates a lower pathogen load, contributing even more to the above hypothesis.

3. Discussion of the assessment at the slaughterhouse:

Some studies have suggested that the greater the lung area affected by lesions the lower the growth rate in the animal [25]. In view of the results of the slaughterhouse visits to assess the lung lesions compatible with both EP and pleuritis, it may be inferred that all groups were infected by *M. hyo*. The least affected group, in relation to the previous results of the tracheobronchial swab tests and growth studies, is group I (Intradermal vaccination). Group I presented data on the prevalence of lesions compatible with EP and pleuritis that are significantly lower than the other two groups, as well as a disease index (DI) and an APPI index numerically lower than those of the other groups, thus offering new evidence of better control of lung involvement, in line with the results obtained in the tracheobronchial swab test as well as in the growth of the animals. No significant differences were detected between the groups in terms of a maximum lesion, perhaps because the number of animals with a maximum lesion is very low in all groups and this affects the power of the statistical test.

It is worth highlighting the role of pleuritis lesions on the C-reactive protein (CRP testing is a blood test marker for inflammation), and this case reveals, once again, a certain association between pleuritis lesions that may have been possibly caused by App and the results of the assessments of EP-compatible lesions at the slaughterhouse [26]. It is clear that the prevalence of pleuritis in group I (intradermal vaccination), with a lower prevalence of EP, is significantly lower than that of the unvaccinated group.

In the lung assessment study, the act of withdrawing the first and last batches from the barns, has enabled us to rule out the potential positive effect of the leader pigs in each batch (normally those that have shown better and more weight growth) and the potential negative effect of the end pigs in the fattening barn from that same batch (on some occasions, the last pigs in a batch at the fattening units stay longer due to marketing and company logistics rather than for their health status).

4. Discussion of the serological study:

The serology and PCR data against the PRRS virus indicates that the virus recirculated in the animals from approximately 6 weeks of age. The negative PCR results for PRRS at 3 weeks of age indicate that the breeders were probably stable [27]. Therefore, at the time of vaccinations against both PCV2 and *M. hyo*, the animals were healthy concerning PRRS, and there was no interference in vaccination due to the immunosuppressive effect of the PRRS virus [28]. However, we can verify that around 6 weeks of age, several of the pools are positive, and as soon as they enter the fattening unit, in the third sampling, all the pools are positive. With this information, we may suggest that the influence of the PRRS virus may have been the same in all the study groups.

Secondly, and referring to another of the agents that play a key role in respiratory pathology, a specific ELISA was performed for the *Actinobacillus pleuropneumoniae* (App) Apx IV toxin to determine only its presence on the farm. Until now, we had only been guided by the slaughterhouse assessments for diagnosis, as well as by lesions found at the level of field necropsies. Given the results, in which almost 100% of the samples were positive (in each of groups H and I, only 1 of 16 samples was a suspect) we may affirm that App could also be involved in the respiratory pathology on this farm and that it could have affected all the study groups equally.

From the serum analysis to detect antibodies against PCV2, we can confirm that, in the first sampling, between 95 and 100% of the animals remain positive in all the treatment groups, due to the colostral immunity received from their mothers. Additionally, this result was seen in the second sampling as a result of the response to vaccination (Porcilis® PCV, MSD Animal Health) [29]. By performing additional quantitative PCR tests in a 4-sample pool, it is clear that in all treatment groups there is no presence of PCV2 viraemia until the last sampling, at a minimum of 21 weeks of age. The viraemia levels found are subclinical in all groups and not related to clinical symptoms of porcine circovirosis [30], which together with having vaccinated all animals, leads us to believe that although the type 2 porcine circovirus was present at the fattening unit, its possible impact has been negligible, and in any case similar in all groups.

Finally, regarding the detection of antibodies against *M. hyo*, we see that group N, unvaccinated, does not show antibodies present against *M. hyo* until the last sampling at the end of the fattening period. We must remember that, at that time, 96.7% of

tracheobronchial swab samples were positive for this agent, as such the animals were facing a recent infection, and hence this fact supports the idea that if we had performed another weighing some weeks after WEIGHT 3, we might have found greater differences than those observed in this trial. On the other hand, the intramuscular treatment group, (group H) seems to be presenting a seroconversion to vaccination with a decrease in antibody levels at around 13-14 weeks of age and a subsequent increase at 17 and 21 weeks of age (when 95% of the animals were ELISA-positive for *M. hyo*). This fact could confirm that, in group H, the recirculation of *M. hyo* occurred earlier than in group N (unvaccinated), and thus perhaps does not show significant differences in growth (although there are numerical differences). Additionally, at 21 weeks of age, the time of the swab, 100% of the samples of group H were PCR-positive for *M. hyo*. Finally, treatment group I, vaccinated intradermally, shows a slight seroconversion to vaccination and it is not until 21 weeks of age, just like group N, when the presence of *M.hyo* is detected again in the serum samples. In addition, and in contrast to groups N and H, only 36.7% of tracheobronchial swab samples are positive, and this fact might be related to greater local protection offered by the vaccine. Moreover, it must be remembered that within the samples of positive lung swabs, the intensity of the positivity or Ct value was significantly lower in the intradermally vaccinated group.

Perhaps the greatest limitation of this trial is its longitudinal character, that is, the development of the trial over 6 weeks, since according to some publications [31] the variability of positive piglets to *M. hyo* at the time of weaning is very high between batches, and such positivity to *M. hyo* at the time of weaning may be a valid predictor for detecting a higher incidence of *M. hyo* problems during the growth phase of the animals. However, other studies make it clear that, although this fact is important, many other factors are also key to the onset of problems related to EP (management, density, concomitant pathologies) [11,26,32]. In Fano's study [31], the importance of primiparous sows as the main responsible agent for the existence of *M. hyo* on farms, due to the horizontal sow-piglet transmission during the lactation phase, is discussed. In this current study, it was taken into account that all batches of animals created week by week had the same number of primiparous sows; therefore, if these sows were the *M. hyo* transmitters to their progeny, the probability would be the same in all treatment groups in terms of the number of possible shedding animals. Future trials may need to include a correction factor by introducing the assessment of tracheobronchial swabs, or nasal swabs to determine the prevalence of *M. hyo* at the time of weaning and thus evaluate the effect of this fact on the results at the end of the trial.

Conclusions

In this study, a better performance was observed for the intradermal vaccine, in a population of vaccinated piglets at 3 weeks of age compared to an unvaccinated control group and an intramuscular vaccine. The claim of better performance is based on the fact that the intradermally vaccinated group showed a greater weight gain at the end of the fattening stage, a lower incidence of *M. hyo*-compatible lung lesions at the slaughterhouse, as well as a lower bacterial load present in the lung tissue at the time of the last weighing.

Abbreviations

M.hyo
Mycoplasma hyopneumoniae; PRDC:porcine respiratory disease complex; EP:enzootic pneumonia; Il-10:interleukine 10; IgA:immunoglobuline A; SD:standard deviation; Ct:cycle threshold; App:*Actinobacillus pleuropneumoniae*; APPI:App Index; PRRS:porcine respiratory reproductive syndrome; PCV2:porcine circovirus type 2; Kg:kilogram; DI:EP disease index; CRP:C-reactive protein; PCR:polymerase chain reaction; TBS:tracheobronchial swab test.

Methods

1. Farm Description

This field study was carried out on a production farm at two sites located in central Spain. One area of the study is a piglet production farm (sites 1 and 2), which houses 1,600 productive sows with self-replacement, and managed in weekly batches of 75 farrows. There are also annexed transition barns where the piglets are kept in large pens that house about 100 animals each,

the piglets are moved here after weaning, from their fourth week of life, until week 8 or 9 (around 20 kg of live weight). It is then when the piglets are loaded onto trucks to take them to the fattening units (site 3), which are located off-farm within the same region. For this specific trial, the animals were in a fattening farm located about 100 km from the piglet production farm, and which has 3 same-sized barns with a capacity for 1,250 piglets each, in pens with a capacity for 13 pigs. All the fattening barns are located on the same farm, built in parallel with a separation of more than 14 meters between them, they have the same source of water, receive feed from the same factory and are managed by the same staff for 8 hours every day.

The fattening unit in this study had a proven history of serious respiratory problems; it is a positive farm for the porcine reproductive and respiratory syndrome (PRRS) virus, porcine circovirus type 2 (PCV2), *Actinobacillus pleuropneumoniae* (App) and *Mycoplasma hyopneumoniae* (*M. hyo*), all of which are involved in the porcine respiratory disease complex [1]. Before the start of the trial, several analyses were performed to determine the disease status concerning some of the pathogens suspected of causing problems in the fattening units. Thus, the presence and recirculation of PRRS (INgezim 2.0, Ingenasa), together with PCV2 (INgezim Circovirus IgM / IgG, Ingenasa) and *M. hyo* (INgezim *M. hyo* Compac, Ingenasa), was found. Additionally, visits were conducted at the slaughterhouse to assess the presence of lung lesions; lesions compatible with catarrhal bronchopneumonia were found, together with pleuritis lesions compatible with an App infection (although said infection was not confirmed by a laboratory diagnosis).

2. Trial design

This study is exempt from the scope of Royal Decree 53/2013, which refers to the basic rules applicable to the protection of animals used in experimentation and other scientific purposes, including teaching, published in: “BOE” no. 34, of February 8th, 2013, Ministry of the Presidency, Reference BOE-A-2013-1337; since it is a veterinary clinical practice not experimental, within the normal farm health program.

At the sow farm, the staff received training in the required work methods related to the trial. The methodology consisted of the veterinarians responsible for the protocol, selecting the animals to be included in the study each week, by visiting the farm on Monday morning. The selection consisted of choosing 22 sows and their corresponding litters from among the 75 sows that were to be weaned on Thursday of that same week. The requirements to be met were as follows:

1. Farrow number: each group included the same number of first-time farrow sows, second to sixth-time sows and sows with more than six farrows. Therefore, 3 population groups were generated: primiparous, farrows 2 to 6, and farrows 7 to 9, with the idea, from an immunological point of view, of homogenising the populations of piglets to be weaned. It is described in some previous studies [15, 16], that piglets born from first-cycle sows have lower levels of antibodies and a higher colonisation rate by *hyo*, and conversely, piglets born from multiparous sows receive greater colostrum immunity from their mothers and have lower levels of colonisation. The aim of such randomisation was to avoid possible interference of this aspect in the results of the trial. As for adoptions, only such litters that were known with certainty to have remained with their mothers since birth, according to the records of the farm, were selected, discarding those with adoptions in their litters.
2. Second, to minimise any possible age spread at the time of weaning, all the piglets were included in one same week, this means that piglets could have a maximum difference of 3 days on their date of birth, as each weekly batch of piglets was weaned on a single day of the week (Thursday).
3. And the third requirement was for the piglets to be healthy. Hence, a group of 220 individually identified piglets (10 piglets for each selected sow) was created each week.

After selecting the litters to be included in the trial, on the afternoon of the same day and at an age between 19 to 21 days of age, piglets were vaccinated according to the corresponding treatment group (TABLE 5), as well as individually identified with a microchip tag, and depending on which treatment group they belonged to, they were identified with another colour tag, different according to the week. The remainder of the piglets belonging to the same week were vaccinated with the same pattern as their siblings and identified with a tag of the same colour for further identification once they were sent to the fattening barns at 8-9 weeks of age.

The fattening unit where the trial was conducted was managed continually by the same staff. Each of the 3 barns had a capacity for approximately 1,250 animals and was filled in two weeks with pigs that had the same pre-weaning vaccine protocol against PCV2 and *M. hyo*. Of the 1,250 animals in each barn, 440 were individually identified and recruited for the trial. Regarding PCV2, all animals were vaccinated with Porcilis® PCV (MSD Animal Health) at 3 weeks of age (preweaning) and regarding *M. hyo*, each of the 3 fattening barns had a different treatment regime.

To rule out the possible influence that housing in a specific barn could have, information was requested from the company that owns the pigs about the productive results of the different barns during the last 5 years. The statistical analysis (data not shown but available), showed that there were no differences between the productive data obtained in the different barns included in the trial (determined as percentage of dead pigs and runts, feed conversion rate (FCR), average daily weight gain, final weight, and duration of the fattening period).

2.1. Field work

I. Treatment groups

The trial was applied to weaned piglets for 6 consecutive weeks. Every 2 consecutive weeks, a different vaccination plan was carried out as shown in TABLE 5. As may be seen in the table, during weeks 1 and 2 of the trial, the piglets did not receive any vaccine against *M. hyo*. At weeks 3 and 4, the piglets received a 0.2 ml dose of a vaccine against *M. hyo* intradermally (Porcilis® M Hyo ID Once, applied with the device IDAL®). Finally, on weeks 5 and 6 of the trial, an intramuscular vaccine against *M. hyo* containing strain 2940 (Hyogen®, CEVA) was applied.

Thus, 3 groups of animals were established (group N, group I, and group H), each with approximately 1,250 piglets identified with a different colored tag, housed in adjacent fattening barns, and within each of those groups there were 440 individually identified piglets.

TABLE 5: Vaccine plan. The duration of each vaccine protocol under study is two weeks.

Week	Treatment	*M. hyo* vaccine	No. of animals in batch	Individually identified animals
1	N	NONE	730	220
2	N	NONE	847	220
3	I	Porcilis® M Hyo ID Once	729	220
4	I	Porcilis® M Hyo ID Once	687	220
5	H	Hyogen®	811	220
6	H	Hyogen®	707	220

II. Weight Study

To monitor the different study groups, different weighings were considered throughout the growth period of the individually identified animals. All animals weaned on a Thursday and with an electronic individual identification tag were weighed individually the following day, Friday, and their weight was recorded (WEIGHT 1). Five weeks later, on a Friday before the piglets were loaded to be sent to the fattening barn, the piglets were again individually weighed, and their weight recorded (WEIGHT 2). All the animals that kept their electronic identification were weighed (unfortunately, a lot of electronic ear tags were lost during the fattening period in all groups). Finally, at the fattening barn, all the pigs that kept their identification were weighed again the week before being sent to the slaughterhouse, thus recording the weight of each individual (WEIGHT 3). The final sample size is shown in TABLE 1.

III. Tracheobronchial swab test

On the same day that WEIGHT 3 was recorded and at the approximate age of 23 weeks, a sample from the lung was taken by a tracheobronchial swab. The technique consists of immobilising the animal by restraining the snout, opening the oral cavity with a mouth opener and introducing a fine cannula, such as those used for post-cervical insemination, through the larynx, deepening into the trachea and large bronchi until causing the animal to cough. The cannula was then removed, the end was cut off and inserted into a tube with 2 ml of physiological saline solution [17,18]. The sample tube was immediately sent to the laboratory for subsequent real-time PCR analysis for *M. hyo* (EXOone *Mycoplasma hyopneumoniae* oneMIX, qPCR kit. Ref. No. MHYP_100, EXOPOL® S.L.U. Spain)

IV. Lung assessments at the slaughterhouse

Once the animals were sent to the slaughterhouse, the assessment of the status of macroscopic lung lesions compatible with *M. hyo* was performed by visual analysis and palpation by the 0-5 method [19], as well as pleuritis lesions [20]. The assessor at the slaughterhouse was a person outside the trial, as such the assessment was blind. From each of the barns that housed the different study groups, the first two and the last two trucks from each barn (the 400 first and last animals) were disregarded from the slaughterhouse assessment, as well as any batches shared between barns that could create confusion between the study groups due to mixing animals from different treatment groups.

The following parameters were assessed from the lungs studied at the slaughterhouse.:

1. The prevalence of *hyo*-compatible lesions, which refers to the number of affected lungs with respect to the total number of lungs assessed.
2. The degree of maximum lesions, that is, lesions of grade 4 and 5 in the method referred above, which refers to the number of affected lungs with grades 4 and 5 with respect to the total number of lungs assessed.
3. The disease index (DI) was calculated for each treatment group based on the total number of lungs inspected as follows: the sum of the result of multiplying each grade of lesion by the number of lesioned lungs of each grade, divided by the number of total lungs assessed [19].
4. The prevalence for pleuritis lesions was calculated considering the number of lungs affected with respect to the number of lungs assessed. The APPI index was calculated for each treatment group, based on the total number of lungs inspected as follows: ratio of lungs with pleuritis lesion with a value> 1 multiplied by the average lung score with a value for pleuritis lesion> 1 [20].

V. Serological study

A regular collection of blood samples from 8 individually identified animals was carried out each week so that, 16 animals from each study group were sampled at 3, 6, 9, 13, 17 and 21 weeks of age. The serum was removed from the blood samples on the same day they were obtained and frozen at -18ºC until the end of the trial, when all these samples were sent to the laboratory at the Center for Diagnostic Solutions (MSD A.H., Boxmeer, The Netherlands), for further joint analysis.

The serum analysis was performed individually for PRRS (PRRS X3 Ab Test, IDEXX®), *M. hyo* (M. hyo Ab, IDEXX®), PCV2 (ALPHALISA PCV-orf2, in-house MSD AH, Boxmeer, The Netherlands), and the 16 serum samples of 21 weeks of age for App (APP-ApxIV Ab, IDEXX®). In addition, in each age group, the samples were divided into 4 pools (viral DNA was extracted from the samples using the DNA/Viral NA SV 1.0 kit (Roche® Diagnostics GmbH)) and each of them was analysed for PRRS and PCV2 as follows: A commercial reverse-transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) kit (Virotype PRRSV RT-PCR, Leipzig, INDICAL BIOSCIENCE GmbH) was used to quantify the PRRSV viral load in the samples. For the quantification of the PCV type 2 viral load the samples were analysed in a PCV2-specific qPCR (Center for Diagnostic Solutions, MSD AH, Boxmeer, The Netherlands). All the analyses were performed at the same time.

2.2. Statistical analysis

For the statistical analysis, only individuals who had all the complete data were studied. For these studies, the SPSS v.22 package (SPSS Inc., Chicago, IL, USA) was used. For the study of WEIGHT 1, a two-way ANOVA was used, considering the sow treatment and cycle as fixed effects. In WEIGHTS 2 and 3, the weights of the previous stages were added as covariates to this model. For the study of the positivity of tracheobronchial swabs, a one-way ANOVA was used (fixed factor: treatment). In the case of qualitative variables (percentage of negatives in tracheobronchial swabs, prevalence and maximum lesions) a Chi-square test was applied. A statistically significant result is considered when $p < 0.05$. For multiple comparisons, the Bonferroni correction was applied.

Declarations

Ethics approval and consent to participate

This study is exempt from the scope of Royal Decree 53/2013, which refers to the basic rules applicable to the protection of animals used in experimentation and other scientific purposes, including teaching, published in: "BOE" no. 34, of February 8th, 2013, Ministry of the Presidency, Reference BOE-A-2013-1337; since it is a veterinary clinical practice not experimental, within the normal farm health program, which will probably not cause pain greater than that caused by the introduction of a needle, always following good veterinary practices.

The owner of the animals signed an informed consent to carry out this field trial.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the trial are available from the corresponding author on request.

Competing interests

Some of the authors of this work are employees at the company MSD Animal Health. The veterinarians responsible for the trial and monitors thereof are personnel completely unrelated to MSD Animal Health. Neither the farmers nor the farm personnel at the fattening unit were aware of the vaccine protocol applied in each treatment group. Although the laboratory personnel that analysed all the samples, both serum and lung samples, were employees of MSD, Boxmeer, the Netherlands, they were unaware of the vaccine protocol applied in each treatment group. The person responsible for the lung assessments at the slaughterhouse was completely unrelated to MSD Animal Health and was unaware of the assessments included in the trial.

Funding

This study was partially funded by MSD Animal Health. Nevertheless, the majority of the field trial was done, without economic interests, by the field veterinarians' team responsible on this farm.

Authors´ contributions

MM and RJ conceived and designed the study. MM drafted the manuscript and coordinated the study execution. RJ coordinated field work at sites 1 and 2. PD coordinated field work at fattening unit (site 3). BJM, RA and PBML, kindly contributed to the sampling and study execution. SH processed all the samples collected. TT performed the statistical analysis. BJM, JM and MR revised the manuscript. All the authors approved the final manuscript.

Acknowledgements

The authors would like to acknowledge the kindly and uninterested cooperation of PROGATECSA company (Valladolid, Spain), as well as the essential and also kindly cooperation of the farmers both in the sow farm and fattening unit.

References

1. Thacker E.L., Minion C. Mycoplasmosis. In: Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J., Stevenson G.W. (Eds.) Diseases of Swine. Wiley-Blackwell. 2012.
2. Straw B.E., Tuovinem V.K., Bigras-Poulin M. Estimation of the cost of pneumonia in swine herds. JAVMA. 1989; Vol195, No.12.
3. Straw B.E., Shin S.J., Yeager A.E. Effect of pneumonia on growth rate and feed efficiency of minimal disease pigs exposed to *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae*. Prev Vet Med. 1990.
4. Maes D., Segales J., Meyns T., Sibila M., Pieters M., Haesebrouck F. Control of *Mycoplasma hyopneumoniae* infections in pigs. Vet. Microbiol. 2008.
5. Nathues et al. Individual risk factors for *Mycoplasma hyopneumoniae* infections in suckling pigs at the age of weaning. Acta Veterinaria Scandinavica. 2013; doi:10.1186/1751-0147-55-44
6. Maes D., Sibila M., Kuhnert P, Segalés J, Haesebrouck F, Pieters M. Update on *Mycoplasma hyopneumoniae* infections in pigs: knowledge gaps for improved disease control. Transbound Emerging Diseases. 2017; doi:10.1111/tbed.12677
7. Del Pozo Sacristán R., Thiry J., Vranckx K., López Rodríguez A., Chiers K., Haesebrouck F., Thomas E., Maes D. Efficacy of florfenicol injection in the treatment of *Mycoplasma hyopneumoniae* induced respiratory disease in pigs. The Veterinary Journal. 2012.
8. Del Pozo Sacristán R., López Rodríguez A., Sierens A., Vranckx K., Boyen F., Dereu A., Haesebrouck F., Maes D. Efficacy of in-feed medication with chlortetracycline in farrow-to-finish herd against a clinical outbreak of respiratory disease in fattening pigs. The Veterinary Record. 2012.
9. Simionatto S., Marchioro S.B., Maes D., Dellagostin O.A. *Mycoplasma hyopneumoniae*: from disease to vaccine development. Vet. Microbiol. 2013.
10. Calsamiglia, M., Pijoan, C. Colonisation state and colostral immunity to *Mycoplasma hyopneumoniae* of different parity sows. Vet. Rec. 2000;146,530-532.
11. Sibila, M., Bernal, R., Torrents, D., Riera, P., Llopart, D., Calsamiglia, M., Segalés, J. Effect of sow vaccination against *Mycoplasma hyopneumoniae* on sow and piglet colonisation and seroconversion, and pig lung lesions at slaughter. Vet. Mic. 2008 ;127,165-170 ; doi:10.1016/j.vetmic.2007.07.027
12. Martelli P., Saleri R., Cavalli V., De Angelis E., Ferrari L., Benetti M., Ferrarini G., Merialdi G., Borghetti P. Systemic and local immune response in pigs intradermally and intramuscularly injected with inactivated *Mycoplasma hyopneumoniae* vaccines. Vet. Microbiol. 2013 ; doi:10.1016/j.vetmic.2013.11.025
13. Tassis P.D., Papatsiros V.G., Nell T., Maes D., Alexopoulos C., Kyriakis S.C., Tzika E.D. Clinical evaluation of intradermal vaccination against porcine enzootic pneumonia (*Mycoplasma hyopneumoniae*). Veterinary Record. 2012; doi:10.1136/vr.100239
14. Beffort L., Weiß C., Fiebig K., Jolie R., Ritzmann M., Eddicks M. Field study on the safety and efficacy of intradermal versus intramuscular vaccination against *Mycoplasma hyopneumoniae*. Veterinary Record. 2017; doi:10.1136/vr.104466
15. Sibila M., Pieters M., Molitor T., Maes D., Haesebrouck F., Segalés J. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae*. The Veterinary Journal. 2009; doi:10.1016/j.tvjl.2008.02.020
16. Garza-Moreno L., Pieters M., López-Soria S., Carmona M., Krejci R., Segalés J., Sibila M. Comparison of vaccination protocols against *Mycoplasma hyopneumoniae* during the gilt acclimation period. Veterinary Microbiology. 2019; doi:10.1016/j.vetmic.2018.12.005
17. Leo A. M. G. van Leengoed, Elbarte M. Kamp. A method for bronchoalveolar lavage in live pigs. The veterinary quarterly. 1989;VOL.II,No.2,APRIL; doi:10.1080/01652176.1989.9694201
18. Vangroenweghe F.A.C.J., Labarque G.G., Piepers S., Strutzberg-Minder K., Maes D. *Mycoplasma hyopneumoniae* infections in peri-weaned and post-weaned pigs in Belgium and The Netherlands: Prevalence and associations with climatic conditions. The Veterinary Journal. 2015; doi:10.1016/j.tvjl.2015.03.028

19. Bollo J.M., Menjón R., Calvo E.: Review of the “0 to 5 scoring method” for enzootic pneumonia slaughterhouse lesions. Proceedings of the 20th IPVS Congress, Durban, South Africa. 2006.
20. Dottori M., Bonilauri P., Merialdi G., Nigrelli A., Martelli P.: Monitoring chronic pleuritis due to *Actinobacillus pleuropneumoniae* at slaughterhouse by newly implemented scoring system. Proceedings of the 20th IPVS Congress, Durban, South Africa. 2008.
21. Knight-Jones TJD, Edmond K, Gubbins S, Paton DJ. Veterinary and human vaccine evaluation methods. Proc. R. Soc. B. 2014; 281:20132839; doi:10.1098/rspb.2013.2839
22. Vidal A. et al. Oral communication. Jornadas de Porcino de la UAB (Universidad Autónoma de Barcelona) y AVPC (Asociación Veterinarios Porcino Cataluña). 2015.
23. Karriker L.A., Ramirez A. Herd evaluation. In: Diseases of Swine, Eleventh Edition. Edited by Jeffrey J. Zimmerman, Locke A. Karriker, Alejandro Ramirez, Kent J. Schwartz, Gregory W. Stevenson, and Jianqiang Zhang. 2019.
24. Lammers P.J., Stender D.R., Honeyman M.S. Feed and growth. Niche Pork Production. Iowa State University. Ames. IPIC NPP340 2007.
25. Pagot E, Pommier P, Keïta A. Relationship between growth during the fattening period and lung lesions at slaughter in swine. Revue Méd Vét. 2007.
26. Marois C., Gottschalk M., Morvan H., Fablet C., Madec F., Kobisch M. Experimental infection of SPF pigs with *Actinobacillus pleuropneumoniae* serotype 9 alone or in association with *Mycoplasma hyopneumoniae*. Vet Microbiol. 2009; doi:10.1016/j.vetmic.2009.09.061
27. Holtkamp D.J., Polson D., Torremorell M., et al. Terminology for classifying swine herds by porcine reproductive and respiratory syndrome virus status. J Swine Health Prod. 2011;19(1):44–56.26.
28. E. Canelli, P. Borghetti, L. Ferrari,E. De Angelis, G. Ferrarini, A. Catella,G. Ogno, P. Martelli P. Immune response to PCV2 vaccination in PRRSV viraemic piglets. Veterinary Record. 2016; doi:10.1136/vr.2016.103637
29. Jiménez M., Menjón R., Marcos M. Respuesta inmunitaria posvacunación frente a PCV2: implicaciones prácticas. SUIS. 2019.
30. Brunborga I.M., Moldalb T., Jonassena C.M. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. Journal of Virological Methods. 2004;122; doi:10.1016/j.jviromet.2004.08.014
31. Fano E., Pijoan C., Dee S. *Mycoplasma hyopneumoniae* prevalence at weaning as a predictor of the group´s subsequent Mycoplasma status. Allen D. Leman Conference. 2005.
32. Sibila M., Nofrarias M., López-Soria S., Segalés J., Riera P., Llopart D., Calsamiglia M. *Mycoplasma hyopneumoniae* infection in suckling pigs: an exploratory field study. 19th IPVS Congress, Copenhagen. 2006.