Control of pig vaccine safety through adjuvant design and vaccination protocol: example of a divalent *Pasteurella multocida* toxin and *Bordetella bronchiseptica* vaccine


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**SUMMARY

Vaccination may induce adverse effects in pigs. These effects may be general and/or local, and may have a negative impact on the herd performance and the meat quality. For optimal safety and efficacy of the vaccine, several parameters should be controlled. This article reports the findings of a study in pigs comparing different adjuvants for optimal vaccine safety. The study also allowed the comparison of vaccine protocols with either one or two intramuscular injections. Two adjuvants were studied, Montanide™ISA 563 VG and Montanide™ IMS 251 C VG and compared to a classical formulation based on Tween/Span W/O emulsion. A commercially available Tween/Span W/O formulation served as positive control, while a non-adjuvanted vaccine was used as a negative control. The immune response to the antigens (*Pasteurella multocida* anatoxin(\(P\)) and bacterial cell wall of *Bordetella bronchiseptica*) was measured by antigen-specific ELISA on days 0, 42 and 113. After each injection, the general status, behaviour and body temperature of the pigs were recorded. The injection site was dissected after slaughter in order to check for local tissue reaction in the muscles. No change of behaviour was detected during the trial. Some animals showed transitory pyrexia, mainly those that had received the water-based vaccines. Strong local reactions were found in the tissues with both Tween/Span formulations. The Montanide™ IMS 251 C VG adjuvant induced the strongest immune response, but was linked to pyrexia. The results indicate that it is possible to modulate the vaccine safety through adjuvant selection. However, the adjuvant should be selected according to the intrinsic reactivity of the antigenic medium.

**Keywords:** Pig vaccine, adjuvant, safety, immune response.

**RÉSUMÉ

La vaccination des porcs peut être source d’effets secondaires néfastes. Ces effets secondaires peuvent être locaux et/ou généraux, réduisant les performances d’élevages ou la qualité de la viande obtenue. Afin de maîtriser l’innocuité et l’efficacité d’un vaccin, plusieurs paramètres doivent être contrôlés. Nous présentons ici les résultats d’une étude vaccinale réalisée sur des porcs ou les adjuvants ont été sélectionnés pour améliorer l’innocuité des vaccins. De plus, le protocole mis en place a également permis d’étudier des protocoles en une ou deux injections intramusculaires. Les adjuvants sélectionnés pour cette étude étaient : Montanide™ ISA 563 VG et le Montanide™ IMS 251 C VG. Deux formules classiques basées sur des émulsions Tween et Span contenant une huile minérale ont également été étudiées. Le témoin négatif était une solution antigénique non adjuvée. La réponse immunitaire induite par les antigènes formulés (anatoxines de *Pasteurella multocida* (PMT) et corps bactérien inactivés de *Bordetella bronchiseptica*) a été suivie par ELISA spécifique d’antigène à J0, J42 puis J113. Après chaque administration de vaccin, le comportement des animaux ainsi que leur température corporelle a été suivi. Après abattage des animaux, les sites d’injections ont été disséqués pour rechercher des réactions locales dans les muscles. Aucune modification comportementale n’a pu être observée pendant l’essai. Quelques réactions pyrogènes transitoires ont été observées pour les formules ayant une phase continue aqueuse. De très grosses réactions locales au site d’injection ont été observées pour les deux formules sur émulsions Tween et Span contenant une huile minérale. La formule contenant le Montanide™ IMS 251 C VG a induit la réponse immunitaire la plus intense mais cette efficacité a été liée à des réactions pyrogènes. Les résultats démontrent qu’il est possible de gérer l’innocuité des formules vaccinale par une sélection des formules adjuvantes et ce en fonction de la purification du milieu antigénique.

**Mots clés**: Vaccin porcs, adjuvant, innocuité, réponse immunitaire.

**Introduction**

The vaccination of pigs with inactivated antigen requires adjuvanted vaccines. In general, two kinds of adjuvant are used to enhance the immunogenic properties of an antigen: aluminium salts or oil-based formulas [1, 9]. Aluminium salts often fail to induce a long-term response or a cellular immune response [7]. Emulsified vaccines are frequently used in pigs as they induce a long-lasting immune response [3]. However, they are also known to induce side effects [5]. General side effects, such as fever, abortion and loss of appetite, or local side effects such as transient lesions (e.g. local oedema) or abscesses can be observed. These adverse effects may result in reduction of the productive performance or carcass quality, causing economic losses. This highlights the need to improve the tolerance, either local or general, of oily
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adjuvants without reducing the efficacy of the vaccines. At least four parameters play a role in vaccine safety: (1) the intrinsic antigen (non) reactogenicity, (2) the vaccination protocol (one or more injections), (3) the adjuvant formulation and (4) animal susceptibility. This paper presents the results obtained in pigs vaccinated with various adjuvant formulations. The impact on vaccine safety of a one-shot vaccination protocol was also studied.

Material and methods

ANIMALS

42 male crossbred Large-white and Landrace fattening pigs from a commercial herd in Brittany were included in the study. All animals had been castrated at birth. On day 0 of the study, pigs were 6 weeks of age and weighed 10 to 15 kilograms, and on day 113 (slaughter) they weighed around 100 kilograms. There were six groups of seven animals each.

ADJUVANTS

Two vaccine adjuvants were studied: the nanoparticle-based Montanide™ IMS 251 C VG (water-based formulation) and the Montanide™ ISA 563 VG adjuvant (W/O emulsion based on non-mineral biodegradable oil), both from the SEPPIC range. They were compared with a widely used Tween/Span mineral oil in water (O/W) emulsion. The water-based formulations ensure good injectability of the vaccines.

A commercially available vaccine with a Tween/Span water in mineral oil (W/O) emulsion was used as a positive reference, while antigen in saline buffer was used as a negative control. Table 1 describes the vaccine formulations used.

ANTIGEN

The antigen was composed of purified Pasteurella multocida anatoxins (PMT) and whole bacterial cell wall of Bordetella bronchiseptica. Each vaccine dose contained 50 µg of anatoxin antigen and 1.10^10 CFU of inactivated Bordetella bronchiseptica. The same antigen mix was used for all vaccines tested in the study.

TRIAL PROTOCOL

All vaccines were administered by intramuscular injection. The vaccination protocol was as follows: 2 ml of vaccine was injected in the left side of the neck at the ear base on day 0 with a further 2 ml injection in the right side of the neck, 42 days after the first injection, according to the vaccination protocol recommended for the commercial reference.

All pigs received a booster injection except group 1, which was one of the two groups inoculated with Montanide™ ISA 563 VG, and received a single injection on day 0.

Blood samples were collected on day 0 (before the primo vaccination), day 42 (before the booster injection) and at day 113 (before slaughter).

The efficacy of the vaccines was evaluated as the ability to induce a strong and long-lasting immune response. The immune response was evaluated by the titration of antigen-specific antibodies. Specific antibodies directed against PMT and bacterial cell walls were measured using an indirect ELISA method.

Vaccine safety was measured by two criteria: local and general safety. Local safety was defined as the absence of a local reaction at the injection site during the trial, by palpation of the injected muscle, and by dissection and inspection of the injection site after slaughter. General safety was defined as the absence of pyrexia following vaccine administration, and an absence of changes in behaviour or the general status. The rectal temperature of each animal was measured at 4, 24 and 48 hours after each vaccination. The behaviour and general status were monitored during this three-day period by recording movement, food intake, time spent lying down, oedema (especially around the eyes), vomiting and diarrhoea.

TITRATION OF ANTIBODIES IN PIG SERA

The titres of the antigen-specific IgG against PMT or Bordetella cell walls were assessed by ELISA. The titre was expressed as the inverse of the last positive dilution in ELISA. The positive threshold was given by the optical density of the negative control ELISA procedure (without serum sample), multiplied by 4.

Plates (96 wells) were coated with 100 µl of either PMT or bacterial cell wall antigen diluted at a ratio of 1/100 in 0.05 sodium carbonate buffer (pH 9.6) per well and incubated for 2 hours at 37°C. Separate antigen solutions (PMT and Bordetella bacterial cell) used for plate coating were from the same batch as the solution used to prepare the antigen mix formulated in the vaccines. The plates were washed with PBS/Montanox™ 20 (Polysorbate20, SEPPIC) 0.05%. After three washings, the plates were incubated with 200 µl of blocking solution (5% swine gelatin, Prolabo), 0.05% Montanox™ 20

<table>
<thead>
<tr>
<th>Adjuvant formulation</th>
<th>Type of final vaccine formula</th>
<th>Nature of the oil</th>
<th>% adjuvant in formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montanide™ ISA 563 VG</td>
<td>W/O (water based)</td>
<td>non mineral oil</td>
<td>50</td>
</tr>
<tr>
<td>Tween/Span</td>
<td>O/W (water based)</td>
<td>mineral oil</td>
<td>50</td>
</tr>
<tr>
<td>Montanide™ IMS 251 C VG</td>
<td>Nanoparticles (water based)</td>
<td>Nanoparticles</td>
<td>20</td>
</tr>
<tr>
<td>Tween/Span</td>
<td>W/O</td>
<td>mineral oil</td>
<td>50</td>
</tr>
</tbody>
</table>

Table I : Vaccine formula compositions.
in PBS) for 30 min at 37°C. The serum samples were diluted at 1/10, 1/100, or 1/1000. Each sample (100 µl) was applied in the first row and serial dilutions of blocking solution were then carried out. According to the initial serum dilution, the final dilution reached 1/1280, 1/12800 or 1/128000. Plates were then incubated for 1 hour at 37°C and washed 3 times. Peroxidase-conjugated rabbit anti-pig IgG (SIGMA) diluted 1/6000 in blocking solution was added (100 µl) and the plates were incubated for 1h at 37°C. The peroxidase activity was visualized with TMB (100 µl) (ZYMED), stopped with 50 µl of H2SO4 (12.5%) and read at 450 nm.

The significance of the difference observed in antibodies titrations was determined using Mann-Whitney test dividing the significance threshold by the number of tests (software statbox 6 STATBOX). Results were therefore considered as significantly different if \( P < 0.008 \). Paired comparison of groups using the Mann-Whitney test required division of the \( P \) value by the number of comparisons in order to keep the significance relevant.

**LOCAL SAFETY**

Tissue lesions of the injection site observed at the slaughterhouse were classified into three kinds of local reactions: granulomas, fibrosis and sterile abscesses of various sizes. For the evaluation of the local reaction, a scale was used ranging from 0 to 5 according to the size of the tissue reaction. The size of the reaction of either abscesses or granulomas at the injection site was measured (cm³) following tissue dissection at the slaughterhouse.

**Results**

**GENERAL TOLERANCE OF THE VACCINE**

During the trial, no change behaviour/ demeanour was observed in the animals. Three formulations induced an increase of temperature: the oil in water (O/W) Tween/Span formulation, the Montanide™ IMS 251 C VG and the W/O commercial reference. Table 2 summarizes the increase of body temperature observed after the first injection. The booster injection induced milder pyrexia (data not shown).

**LOCAL TOLERANCE OF THE VACCINE**

The prevalence of abscesses and granulomatous/fibrotic reactions is summarized in table 3 and results from the sum of local reaction evaluation are presented in table 4.

<table>
<thead>
<tr>
<th>Group</th>
<th>Formulation / protocol</th>
<th>T4</th>
<th>T24</th>
<th>T48</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Montanide™ ISA 563 VG</td>
<td>-0.01</td>
<td>-0.26</td>
<td>-0.27</td>
</tr>
<tr>
<td>2</td>
<td>2 injections</td>
<td>-0.77</td>
<td>-0.53</td>
<td>-0.59</td>
</tr>
<tr>
<td>3</td>
<td>Tween/Span O/W</td>
<td>1.57</td>
<td>0.26</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>Montanide™ IMS 251 C VG</td>
<td>2.00</td>
<td>0.90</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>Commercial vaccine Tween/Span W/O</td>
<td>0.96</td>
<td>0.53</td>
<td>0.36</td>
</tr>
<tr>
<td>6</td>
<td>Antigen in saline</td>
<td>1.29</td>
<td>0.07</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**TABLE II :** Pyrogenic reactions after primo vaccination. Results are expressed in °C. Results were obtained by subtracting the average temperature for each group of pigs from the average group temperature on day 0.

<table>
<thead>
<tr>
<th>Group</th>
<th>Formulation / protocol</th>
<th>% Sterile abscess</th>
<th>% granulomas and fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Montanide™ ISA 563 VG</td>
<td>33.3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2 injections</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Tween/Span O/W</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Montanide™ IMS 251 C VG</td>
<td>0</td>
<td>28.5</td>
</tr>
<tr>
<td>5</td>
<td>Commercial vaccine Tween/Span W/O</td>
<td>50</td>
<td>8.3</td>
</tr>
<tr>
<td>6</td>
<td>Antigen in saline</td>
<td>0</td>
<td>16.6</td>
</tr>
</tbody>
</table>

**TABLE III :** Evaluation of local reactions: prevalence of sterile abscess and granulomas at the injection site observed at the slaughterhouse.

<table>
<thead>
<tr>
<th>Left side neck</th>
<th>Right side neck</th>
<th>Sum of score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montanide™ ISA 563 VG 1 injection</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Montanide™ ISA 563 VG 2 injections</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Tween/Span O/W</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Montanide™ IMS 251 C VG</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Commercial vaccine Tween/Span W/O</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Antigen in saline</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

**TABLE IV :** Evaluation of the local reactions: cumulative scores of individual local reaction score for each injection site. For each lesion, the scale ranged from 0 to 5 according to the size of the local reaction.
The Montanide™ IMS 251 C VG based formula was the best tolerated formulation. It caused a slight fibrosis at the injection site in a restricted number of pigs (28.5%). The poorest result was obtained by the oil in water emulsion, with a cumulated ratio of 70% of the pigs presenting local reaction for the O/W Tween/Span formulation and 80% for the Montanide™ ISA 563 VG based formula. The use of the latter adjuvant in a single vaccine administration greatly reduced the score (33% of pigs with local reactions). This result was equivalent to the commercial vaccine based on W/O Tween/Span. However, only one side of the neck site underwent tissue reaction while the commercial two-shot formula induced a reaction on both sides.

**ANTI BB IMMUNE RESPONSE**

Pigs presented low levels of IgG on day 0 (Figure 1a). There was no significant difference in values between groups.

Results on day 42 (Figure 1b) showed an important increase in IgG levels, representing the early response to vaccination. The response of the two groups that received Montanide™ ISA563 VG differed significantly: the one-shot group (1) had an average IgG level of 13714.28±6841.88, the two-shot group (2) 34742.85±15614.4 (Figure 1b, *P < 0.008*). Group 1 mounted a comparable response to that of the other adjuvant groups after 2 shots except the Montanide™ IMS 251 C VG group which induced significantly higher antibodies levels (40000±13856.40 versus 14857.14±10480.27 in the Montanide™ ISA 563 VG group 1, *P < 0.008*).

Before slaughter at day 113 (Figure 1c), all groups showed a strong response to bacterial cell walls except the negative control. However, the difference between the commercial reference and negative control was not significant at the end of the trial. Montanide™ IMS 251 C VG induced the highest response compared of all adjuvants (40000±13856.40). However, the difference with group 2 (Montanide™ ISA563 VG 2-shot protocol) and group 3 (Tween/Span O/W) was not significant (*P > 0.008*).

**ANTI PMT IMMUNE RESPONSE.**

The immune response to Pasteurella multocida anatoxins (Figure 2) was weaker than the response to bacterial cell walls. The short-term response (Figure 2a) showed a higher response induced by Montanide™ IMS 251 C VG (2264.28±4672.32). There was no significant difference between vaccines due to the lack of within-group homogeneity of the response induced. After the booster injection (Figure 2b), the Montanide™ ISA563 VG two-injection protocol induced an equivalent response to the same adjuvant-based vaccine used in the one-
shot protocol (1188.57±2133.33 and 990.0±1550.15, respectively). Both responses were higher than the commercial reference (617.14±407.17). The O/W Tween/Span formulation induced the strongest immune response (2880±1610.63). At day 113, only the Montanide™ IMS 251 C VG induced a significantly higher response compared to the commercial reference.

Discussion

Oil-based adjuvant formulations associated with bacterial antigens are known to induce adverse local and general reactions [5]. These adverse effects may be reduced by using purified or non mineral oils [2]. Such oils are better metabolized and eliminated [4, 11].

Surfactants used in Tween/Span formulations are based on sorbitan oleate esters and are commonly used in various industrial applications. In this trial, the use of non-injectable grade of surfactants like Tween/Span lead to a high level of local reactions, even for water continuous phase formulations. We expected O/W Tween/Span formulation to be less reactive than the W/O formulation but the level of local and general reactions induced are similar. This could be due to the fact that a high amount of mineral oil is reactogenic whatever the type of emulsion (W/O or O/W).

The Montanide™ adjuvant range is based on injectable quality surfactant from mannitol and purified oleic acid of vegetable origin. The Montanide™ range differs from Tween/Span formulation in the chemical structure of raw material as well as purity. Tween/Span formulations either in O/W or W/O formulation are based on surfactants not specifically dedicated to injections. All Montanide™ range formulations are free of maximum residues limits and registered under annex II of Regulation EEC 2377/90.

We modulated the tolerance of the vaccine by changing the adjuvant composition and the number of injections. The depot effect induced by oil-based vaccines and the slow release of the antigenic media from the emulsion once injected could explain the delayed contacts between the LPS included in the vaccines and the pig immune system.

Vaccines formulated with a water continuous phase may induce pyrexia due to the fast release of the antigenic components.

Antigen purification is a critical point in the vaccine safety improvement as the lipopolysaccharides (LPS) from the bacterial cell wall are highly reactogenic [10] and induce strong pyrogenic reactions. The W/O formulation used in our trial associated with reactive antigens showed the lowest pyrogenic effect. Transient fever may be tolerable for fattening pigs as they disappear within 24 hours after injection. However, they may be critical in pregnant sows due to the risk of abortion. However, W/O formulations are poorly compatible with reactogenic bacterial antigens as they may induce local abscesses. Indeed, the local reactions at the injection sites with that kind of formulation were too strong to be acceptable for fattening pigs.

The modification of the vaccine protocol was combined with a modification of the adjuvant oil phase to reduce the inflammation induced by each injection. There was a risk of losing vaccine efficacy by reducing the number of injections and changing the oil for the same vaccine. Although the adverse effects observed at the slaughterhouse in the two-shot Montanide™ ISA 563 VG-based vaccine group were significantly higher than the local adverse reactions induced by the commercial vaccine, the one-shot protocol induced an equivalent result compared to the reference in term of tolerance. The advantage of a one-shot protocol is that the adverse local reactions can only occur in one site, in while the commercial reference and the two-shot Montanide™ ISA 563
We did not examine the one-shot vaccination protocol for antigens, as only slight pyrogenic reactions were observed. It is especially suitable for fattening pigs when using LPS. Montanide™ is a VG offers the best compromise between safety and efficacy. This emulsion prevents the pyrogenic effect induced by the bacterial cell wall. The modification of the vaccine protocol to a one-shot vaccination allowed inducing a strong immune response with the Montanide™ ISA563 VG, while reducing the local reaction to a level equivalent to a commercial reference.

Low levels of IgG observed at day 0 (before vaccination) were induced by a previous contact with wild bacterial valence of the vaccine or most probably with cross-reacting, non-pathogenic bacteria. Contact with other gram-negative bacteria may also have induced some cross-reactive antibodies. Nevertheless, the differences between the titres observed after vaccine injection clearly demonstrates the activation of the immune system by the vaccines.

The efficacy of the vaccine based on Montanide™ ISA563 VG in a one-shot protocol was higher than the mineral O/W commercial reference. The significant differences observed between the two groups receiving this vaccine formula before the booster injection could not be explained. In absence of a challenge procedure, validating the protection induced by the vaccines, the formulation conferring a level of immune response equivalent to the commercialized formula can be considered as efficient.

Conclusion

Controlling the parameters that influence the vaccine efficacy and safety is difficult as it is antigen, animal and adjuvant-dependent.

Even though the level of local reactions observed with the Montanide™ ISA grade is acceptable, carcass damage may be reduced with a one-shot vaccination protocol. Furthermore, this emulsion prevents the pyrogenic effect induced by the antigen through a slow release of the bacterial component from the vaccine. Nevertheless, the use of a W/O based adjuvant for bacterial LPS containing antigen presents an important risk of developing sterile abscesses at the injection site.

In the case of reagogenic antigens, Montanide™ IMS 251 C VG offers the best compromise between safety and efficacy. It is especially suitable for fattening pigs when using LPS antigens, as only slight pyrogenic reactions were observed. We did not examine the one-shot vaccination protocol for water-based vaccine adjuvants. The use of highly tolerated and efficacious adjuvants like Montanide™ IMS 251 C VG in a one-shot protocol could help improve both animal welfare and pig farm management, as one-shot protocols are less time-consuming. These vaccine formulations are also easier to manufacture and to inject than classical emulsified forms of vaccines.

Two parameters of vaccine efficacy remain to be investigated: the age of the animal at vaccination and the nature of the antigen (viral and/or bacterial). The age of the pig, which may influence the intensity and duration of the immune response, and the nature of the antigen, which may influence the vaccine tolerance of the animals.

Acknowledgements

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