Adjuvant Formulation for Companion Animals Vaccines

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Abstract

Companion animals are sensitive species able to strongly react to vaccine. Compared to farm animals, owner’s sensibility to vaccine safety is exacerbated due to emotional links between animal and owner. Adjuvant selection during vaccine development is a key parameter driving vaccine safety and efficacy profile. Our studies demonstrated the ability to use Montanide\textsuperscript{TM} Pet Gel A (polymeric adjuvant manufactured under GMP rules) in cat, dog and horse vaccines. Adjuvants performances were highlighted by local and general safety parameters but also through vaccine efficacy to trigger a protective immune response against the pathogen. Three trials were performed to validate Montanide\textsuperscript{TM} Pet Gel A compatibility with cats, dogs and horses vaccine models. Experimental vaccines were formulated using different antigens according to the animal: inactivated \textit{Rhodococcus equi} (horse), purified ovalbumin (cat) \textit{Leptospira icterohaemorrhagiae} (dogs). In all trials, safety was followed through behavior and temperature measurement. Furthermore, in dog and cat models, histology studies were performed to assess the local reaction in the injection site. A kinetic of blood sampling was performed in all trials. Antigen specific ELISA was used to assess the immune response induced. In cat and dog trials, aluminium based formulation were used as benchmark for Montanide\textsuperscript{TM} formulation while in horse we compare Montanide\textsuperscript{TM} Pet Gel A based vaccine to an already published internal reference. Safety performances of Montanide\textsuperscript{TM} Pet Gel A were superior to aluminium based vaccines in dogs and cats. Transient oedemas were observed in horse vaccine model after each vaccine injection, nevertheless, no impact on the animal behavior was observed. The antibodies production induced by Montanide\textsuperscript{TM} Pet Gel A based vaccines was higher than aluminium-based vaccines or internal reference. Montanide\textsuperscript{TM} Pet Gel A can be used associated with a wide range of antigenic media and recommended to be used as adjuvant for sensitive animal's vaccines.

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1. Introduction

Adjuvants technologies dedicated to veterinary sensitive species are mainly aluminium salts [2, 3]. The other main technologies used in veterinary vaccines are emulsified oil based formulations. Those types of formulations are poorly compatible with the safety expectation of pet’s models. Indeed, added to the animal sensitivity to vaccine injections [4], the emotional links between the animals and it’s owner lead to a perfect safety profile expectations: no local or general reactions would be tolerated in companion animals. Aluminium salts are sometimes considered as a reference in term of safety and are used in most of the more sensitive species because of their safety profile [11]. Nevertheless, aluminium salts are known to induce a TH2 profile and poor long lasting immune response [1]. New adjuvant generation is therefore needed, presenting an equivalent safety profile, compared to aluminium salts adjuvant but inducing a better immune stimulation. To answer this question a new adjuvant based on a dispersion of a high molecular weight polycrylic polymer was created. Produced under GMP like conditions, this sterile adjuvant is a dispersion of highly stable calibrated spheric micronic gel particles of sodium polycrylate in water [15]. This polymeric technology, Montanide™ Gel, has already been used in several vaccine models, including pet’s vaccines, with a promising safety and efficacy profile [6, 12, 13]. Our findings highlight the safety and efficacy profile of this polymer based adjuvants dedicated to species where vaccine safety is sometimes of higher importance than efficacy. We present data collected in pets models (dog and cat) as well as horse model.

2. Material and methods

2.1 Dog model

Dogs were 6 months old male and female cross breed animals. Ten animals were randomly introduced in each group. Montanide™ PET GEL A was used in this field trial at a final concentration of 5%. As a positive control a commercial aluminium based vaccine was used. All vaccines were containing the same amount of antigenic media. The bacterial antigen was kindly provided by ROMVAC Company (Romania, 2007). It was an inactivated culture of Leptospira Icterohaemorrhagiae. Vaccine safety was recorded during the trial by the palpation of the injection site. Temperature of all animals was recorded during 3 days after each injection. Animal behaviour (social, food intake) was also followed during the same period. At the end of the trial on day 120, a subcutaneous biopsy was performed at the injection site on each animal. All biopsy were performed under anaesthesia and analyzed through Hemalun Eosin Safran coloration (HES). This work was performed by IDEXX Alfort laboratories (Alfortville, France). This specific coloration of samples allows the identification in the injection site of all cell populations present as well as necrosis, fibrosis and vaccine remnants. Blood samples were collected every week and sera extracted from blood submitted to antigen specific ELISA.

2.2 Cat model

Fifteen European healthy cats, from 1 to 4 years old, untreated with any medication from 2 month before the day of first vaccination, were randomly separated in three groups of five animals. During all the trial, animals were kept in collective cage and with water adlibitum. Food was individually distributed and weighed to assess the impact of vaccination on appetite of the animals. Table 1 hereunder presents the vaccines tested in this trial.

Table 1: Cat trial vaccine composition per dose of 1ml.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adjuvant %</th>
<th>Antigen μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montanide™ Pet Gel A</td>
<td>7,5</td>
<td>100 μg / dose</td>
</tr>
<tr>
<td>Aluminium salt</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>No adjuvant</td>
<td></td>
</tr>
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</table>
Vaccines were injected twice 21 days apart. Vaccines were injected subcutaneously in the neck; a volume of 1ml was delivered at each injection. The safety of the tested vaccines was assessed by observation during all the trial of the animal behaviour, food consumption and body temperature. The local safety was assessed by observation and handling of the injection site 24, 48, 72, 96 and 120h and a biopsy of the subcutaneous tissues on day 44 post first injection. Efficacies of the vaccines were assessed through a kinetic of blood sampling (D-1 then day 7, 20, 29, 36 and 44). Antigen specific ELISA was performed to detect the OVA specific IgG induced by vaccination.

2.3 Horse model

Female horses aged from one to seven years old were randomly inserted in two groups (twenty animals for adjuvanted vaccine and ten for control group). Antigen was kindly provided by ANSES (Dozulé, France). This antigen was composed of proteins from *Rhodococcus equi*. Vaccine administration was done by intramuscular injection of 1 ml of vaccine in the neck at day 0 and day 28. Table 2 hereunder describes the vaccine composition. Following each injection the animals were observed during three days. Vaccine safety was recorded by palpation of the injection site and rectal temperature of all animals was also recorded. Animal behaviour (social, food intake) was followed during the same period.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adjuvant %</th>
<th>Antigen mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montanide™</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pet Gel A</td>
<td></td>
<td>0.5mg</td>
</tr>
<tr>
<td>Control</td>
<td>No adjuvant</td>
<td></td>
</tr>
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</table>

2.4 Antigen specific ELISA detection of IgG subclasses

ELISA method was used in all trials to assess the level of immunoglobulins induced in the vaccinated animal sera. The protocol was similar for all animals; Only the detection antibody was selected to have species specificity. Detection antibodies used in our trials are Sheep anti-dog IgG:HRP (AAI32P) for dog IgG detection, Mouse anti-cat IgG (MCA2651) for cat IgG detection. OVA (cat model), Leptospira (dog model) was added in ELISA plate (Nunc Maxisorp, ref 055133) and incubated at 37°C for 2 hours. The plates were then washed with PBS / Montanox™ 20 (Polysorbate20, SEPPIC) 0.05%. After three washings, the plates were incubated with 200μl of blocking solution (5% swine gelatin, Prolabo), 0.05% Montanox™ 20 in PBS) for 30 min at 37°C. A mouse serum containing an IgG1 antibody high titre and diluted 1/1000 in blocking solution and was added (100μl). Plates were incubated for 1h at 37°C and washed 3 times. Species specific detection antibody was diluted 1/6000 in blocking solution was added (100μl) and the plates were incubated for 1h at 37°C. The peroxidase activity was visualized with TMB (100μl) (ZYMED), stopped with 50μl of H2SO4 (12.5%). The optical density (OD) was read at 450nm. OD quantifies the amount of IgG subclasses presents in the animal’s sera.

3. Results

No modification of the animal behaviour was detected (social, food intake...) whatever the model.

3.1 Dog model
No impact of the vaccine delivery was observed on the body temperature of dogs whatever the vaccine group. Mild and transient oedemas were observed during the trial, mainly after the booster injections. All reactions observed were of the same type: small oedemas and swelling of the injected site (less than 2 cm²). The duration of the local reactions at the injection site was dependant of the adjuvant. All reactions were observed only 48 hours after injection for the first vaccine delivery (data not shown). But for the booster injection the reactions appeared 24 hours after injection, for both aluminium and Montanide™ PET GEL A based formulation, but lasted until 72 hours for Montanide™ PET GEL A and more than 144 hours for aluminium based vaccine. None of the dog receiving the antigen in saline presented reactions at the injection site. HES coloration performed on biopsy sampled on day 120 gave a higher rate of local reactions for the Montanide™ based vaccine compared to the aluminium based formula. Six out of the ten animal’s injected presented reactions for the Montanide™ PET Gel A based vaccine while only two out of ten for the aluminium formula. Pictures 1 and 2 presents local reactions from Montanide™ injected dogs. Fibrosis as well as a strong infiltration of monocytes can be observed in the injected site. No vaccine remnants and no necrotic tissues were observed in the samples from that vaccine groups. On the opposite, only two animals (Pictures 3 and 4) presented reactions for the aluminium based vaccine but both of those reactions included necrotic tissues and large granulomas. Those two kinds of reaction were also associated with fibrosis as well as a strong infiltration of monocytes.

![Image 1](image1.png) ![Image 2](image2.png) ![Image 3](image3.png) ![Image 4](image4.png)

*Picture 1, 2, 3, 4: a: necrotic tissues, b: granulomas, c: fibrosis, d: normal tissue. Local reaction observed in subcutaneous tissue biopsies performed in dog 120 days after first injection of several vaccines. Pictures 1 and 2 presents reactions examples induced by Montanide™ GEL PETA based formula while pictures 3 and 4 presents reactions observed with vaccines based on aluminium.*
Figure 1 presents the seroconversion rates observed all along the trial. All titers detected were above the protective threshold (data not shown).

![Seroconversion rates graph](image)

**Figure 1:** seropositive dogs to Leptospira antigens at each sampling date.

As presented in Figure 1, only the Montanide™ PET GEL A could induce 100% of dogs to be seroconverted at D84 and up to the end of the trial. Vaccines containing aluminium salts as adjuvant induced only 60% of seroconversion at D84 and presented a decreased percentage at D120.

### 3.2 Cat model

Vaccine tested had no significant impact on the rectal temperature of animals. Histological data collected in our cat model confirmed the reduced inflammatory reaction induced by the Montanide™ adjuvant compared to the aluminium formula. Indeed the booster injection induced 100% of necrosis in the subcutaneous tissue with alum while only 40% of the animals presented such reaction with the Montanide™ Pet Gel A based formulation. Nevertheless, the reactions observed are limited in size and have no impact on the local macroscopic safety: no irritation of the skin could be observed with any of the tested vaccine.

![Histological images](image)

**Pictures 5 to 13:** Local reactions observed in subcutaneous tissue biopsies performed in cats after injection of vaccines based on aluminium salts or Montanide™ PETGEL A. Reactions are observed at three different magnitude: X4 to X40.

As observed in the picture 7, 10 and 13, no reactions could be observed following injection of antigen in saline buffer for the control group. The picture shows a normal subcutaneous tissue section for all animals. Pictures performed on colored cuts from biopsies of animals injected with the adjuvanted vaccines shows a different profile as a strong inflammatory process can be observed. At different
magnification we observe an intense infiltration of the injection site by monocytes and macrophages for the both adjuvanted formulation. Fibrosis can be observed only for Montanide™ based formulation indicating a faster onset of healing activity with this experimental vaccine. Numerous lymphocytes were indentified in the samples of animals injected with Montanide™ but we were not able to compare if that infiltration was significantly higher compared to the aluminium salts induced lymphocyte attraction. Some adjuvant remnants can still be observed in the tissue sections for aluminium salts and Montanide™ Pet Gel A based formulations as biopsies were performed only 23 days after the booster injection.

Figure 2 presents antibody titres in cat sera induced after injection of various cat experimental formulations. Results are means of individuals ELISA measurements.

The last sampling of the trial was performed on day 43 in order to reduce the animal stress (biopsies were performed on day 44). No significant differences were observed between the alum based formulation and the Montanide™ Pet Gel A one. Nevertheless, the Montanide™ based vaccine induced a stronger immune response in all except one animal from that group. Both of the adjuvanted vaccines induced a significantly stronger response compared to the control group.

3.3 Horse model

After the first injection horse’s body temperature increased of 1°C in both groups while at the booster injection only the adjuvanted formula induced an increased temperature of 1°C. Nevertheless this raise of temperature had no impact on the animal behaviour. Local reactions observed were oedemas: those reactions were transient but last one week on some individual. The adjuvant presence in the vaccine formulation increased the size of the reactions in all animals, but not the duration Table 3 hereunder present the size in centimetre square of the local reactions observed in the injection site area (cumulated scores of 20 animals). None of the reactions observed moved from oedemas to sterile or infectious abscesses and no medication was needed.

Table 3 : Cumulative scoring of local reactions observed after booster injection of vaccines (Montanide group: n=20; control group n=10). Individual scoring was defined according to the reaction size in cm²: less than 10cm²=1 ; 10 to 20 cm²=2 ; more than 20cm²=3.

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montanide™</td>
<td>0</td>
<td>14</td>
<td>24</td>
<td>24</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>Antigen</td>
<td>0</td>
<td>3</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
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Differences observed were significant, nevertheless all reactions observed were transient and not related to modification of animal behaviour. We considered the animal welfare or economical performance as unmodified by the vaccines tested.
4. Discussion

Three trials performed in target animal as well as the pre-clinical studies performed in laboratory animal model [7, 8] illustrate the properties of the Montanide™ Pet Gel A in various models. Three different kinds of antigenic media are tested: purified protein as ovalbumin, inactivated virus and antigen potentially more reactive like inactivated bacteria. The bacterial valence used in the dog trial is composed of inactivated *L. Icterohaemorrhagiae*. This bacteria (*Leptospira sp.*) is a gram negative genius. This type of microorganism is lipopolysaccharide (LPS) containing and can be considered as a reactive antigen [14]. There are no or very little reactions observed after injection of the antigenic media without adjuvant: 1 dog out of 9 at the first injection and none of the cats. There is no impact of the vaccine injection upon the general behaviour of any animal during the trials, whatever the adjuvant percentage tested. No increase of the body temperature could be detected in the horse and dog models while during the cat trial we observed a transient increase in body temperature induced by the Montanide™ based vaccine. This increase was nevertheless not significant compared to both alum and control groups. The general safety profile of the adjuvant used in all our trials is equivalent or improved compared to aluminium salts, up to now considered as the golden standard in terms of adjuvant safety [9, 10]. The safety profile observed in the cat and dog was worsened by the subcutaneous injection pathway: the local oedemas induced are easily palpable and an intramuscular injection would reduce the negative impact of local reaction. In the dog trial Montanide™ PET GEL A at 5% was presenting an equivalent macroscopic safety profile compared to Alum based vaccine. In pets market, the macroscopic lesion or reactions linked to the injection are unacceptable. Furthermore, the cat trial performed at a higher percentage of adjuvant also indicated an improved safety profile of the Montanide™ based formulation compared to aluminium based one [5]. The only differences observed were at the primo injection in the dog model: a faster reaction to injection with Montanide™ PET GEL A at 5% compared to Alum based vaccine could be observed. The local safety profile of those adjuvants based vaccines would be improved with vaccine based only on viral antigens or by modification of the injection pathway (IM instead of SC). At last, no evolution of the local reaction observed in all trials performed showed matter production or sterile abscesses created in the injection site. Safety at the injection site followed by histology performed upon muscle or subcutaneous tissues biopsies (from the injection site) showed no significant differences between the inflammatory properties of Montanide™ based formula and aluminium based one. At last, no evidence of larger adjuvant or vaccine remnants compared to aluminium based vaccine was found with the gel based formulas. Dog and cats data collected are consistent upon the adjuvant metabolism: they demonstrate a discrete persistence of the adjuvant in the injected area at 43 to 120 days after vaccine delivery. Nevertheless, the induced inflammatory response is lower for the Montanide™ Pet Gel A based formulation and more important: no chronic inflammatory process with Montanide™ PET GEL A could be observed in any animal like it was the case for aluminium based formula. At last, the healing process observed with Montanide™ based formula indicates a short term strong inflammatory linked to an intense immune response as numerous cells from the innate immune system (macrophages, monocytes) and adaptive immune system (lymphocytes) can be identified in the inflammatory process infiltration of the injected muscle. The choice of gram negative bacteria has certainly worsened the lesion observed at the injection site during dog trial. The lipopolysaccharides from the cell wall of such genus are highly reactogenic inducing strong response even without adjuvant as we observed in the dog biopsies. Safer antigenic media will lead to lowering of necrosis and inflammatory process observed possibly allowing increasing the ratio of adjuvant in the vaccine up to 7% as we did in the cat model. The antigen used for the cat model presents weak inflammatory properties as being 95% purified protein. The safety profile obtain during this trial being almost identical to the profile observed in the dog model, this finding illustrates the balance between the antigenic inflammatory properties and the adjuvants ratio of use. Each formulation will need to be assessed as a single case in terms of safety profile with a Montanide™ Pet Gel A content from 5 to 7 %. Nevertheless, injection site safety profile observed is correlated to the quantity of adjuvant used. Therefore, a specific care should be taken when calculating the optimal amount of adjuvant in the vaccines balancing the safety profile with the efficacy. Works illustrating the cellular set of response induced by both aluminium salts and Montanide™ Pet Gel A in a relevant model would bring some more information upon the immunoproperties and the differences between those adjuvants.
The immune response directed to the bacterial valence of the dog and horse vaccine allowed to discriminate the tested formula. The best results were obtained with Montanide™ PET GEL A at 5% with 100% of seroconversion and a sufficient level to insure protection after 2 injections. The reference adjuvant (aluminium) reached only 60% of seroconversion. The cat model illustrated a higher antibody response induced by the Montanide™ Pet Gel A based formulation, but the differences observed were not significant. A larger number of individuals per groups will allow collecting more consistent data: the results of the Montanide™ based formulation are deeply impacted by one of the animal presenting a very weak response. Efficacy of the vaccines in the horse model is still to collect. Nevertheless preliminary data demonstrated a strong adjuvant effect after booster injection.

The high antibody titres observed in all models with the adjuvanted formulation will allow playing with the antigen content in the vaccine, regulating the efficacy / safety vaccine’s balance. The formulation with adjuvant can be designed with amount of antigen while having a maintained efficacy. Therefore the inflammatory response in the injection site will be reduced. We need to collect long term immunological data in order to observe any booster from natural infection occurring and correlates those titers with the farmer and veterinarian observations of horse health status. None of our study presents long term data collection. Due to trial management we could not go longer than 150 days in horse test. This type of long term study, remain to be started.

5. Conclusion

A synthetic pharmaceutical polymer has been tested in three different companion animals’ experimental vaccines. In two out of these three experiments aluminium was used as the reference already used in registered vaccines. In all cases, adjuvanted formulation gave a stronger and better response than antigen alone, as well as more visible and acceptable reactions at the injection sites. Nevertheless, antibody titres and expected protection are tremendously improved by the use of Montanide™ PET GEL A. The design of modern vaccines, containing different antigens obtained by conventional biotechnologies, but also recombinant or live, can be efficiently made by using robust and flexible synthetic GMP adjuvant.

References


