Available online at www.sciencedirect.com

ScienceDirect

Procedia in Vaccinology 1 (2009) 140–147

Procedia in Vaccinology

www.elsevier.com/locate/ procedia

2nd Vaccine Global Congress, Boston 2008

Adjuvant formulation for veterinary vaccines: Montanide™ Gel safety profile

R. Parker[a], S. Deville[b*], L. Dupuis[b], F. Bertrand[b] and J. Aucouturier[b]

[a]*SEPPIC Inc 30, Two Bridges Road, suite 210 Fairfield, New Jersey 07004-1530.*
[b]*SEPPIC Tour Kupka C, 7 Boulevard Franck Kupka 92039 Paris La Défense Cedex France.*

Abstract

Selecting the adjuvant is one of the key for the success of the vaccine in the field. Selecting a flexible adjuvant that will fit with several vaccines dedicated to one or more animal species is a source of economical efficiency. Frequently the safety or efficacy obtained with one model is different from another: there are few adjuvants fitting with the expectation of more than one animal species. Montanide™ Gel an innovative polymeric adjuvant have been tested in several animals. Our studies demonstrated the ability to use this adjuvant in dogs, cattle and pig vaccines. Three trials were performed to validate Montanide™ Gel ability to be used in cattle, pigs and dogs. Respectively, vaccines were formulated with ovalbumin in cattle, *Pasteurella Multocida* anatoxin and *Bordetella bronchiseptica* cell walls for pig and finally with parvovirus associated to two *leptospira* valence for dog model. All antigenic media used in the three trials were inactivated. In all trial, safety was followed through behaviour and temperature measurement as well as histology studies.
Montanide™ Gel adjuvant can be used associated with a wide range of antigenic media. Nevertheless, the uses of such adjuvant need validation in avian and fish vaccines.

* corresponding author : sebastien.deville@airliquide.com

Keywords: Adjuvant ; Montanide™ gel ; Polymer ; Safety ; Veterinary vaccine ; *Pasteurella multocida* ; *Bordetella bronchiseptica*

1877-282X © 2009 Published by Elsevier Ltd. Open access under CC BY-NC-ND license.
doi:10.1016/j.provac.2009.07.026

1. Introduction

Adjuvants technologies added to vaccines have undergone a large evolution from the first formulation such as Freund's adjuvant. Modern vaccines based on purified antigens from biotechnologies have improved the vaccines safety, but lack of efficacy to induce a protective immune response. Yet, numerous vaccines are still based on inactivated entire microorganism. They present strong immunogenic properties but also a risk to induce local and general side effect if associated to a strong adjuvant formulation [1]. The selection of the adjuvant during the vaccine design is crucial for the vaccine properties. Selecting a flexible formulation fitting with the expectation of various vaccines dedicated to several species is a source of economical benefit for vaccine producers. Numerous adjuvants technologies are available for veterinary vaccines [2, 3], yet very few can fit with multiple expectations as safety and efficacy in various species. Two main technologies for veterinary adjuvant in the field are aluminium salts and emulsions. Aluminium based formulation are used in all vaccinated animal species. These type formulations are described as crystalline particles of amorphous aluminium hydroxyphosphate [4]. Expected mechanisms of action are mainly three: a depot effect, an arrangement of the antigen in a particulate form around the aluminium particles and pro-inflammatory properties [5, 6, 7]. This type of adjuvant has been used for decades in vaccines against Foot and Mouth disease [8]. Nevertheless, histological evidence of adverse effect related to alum in sensitive animals as pets has also been described [9, 10]. The emulsion based formulations are known to induce a long lasting immune response [11]. The properties of oily adjuvant, either inflammatory and immunostimulatory will also deeply be impacted by the composition of the adjuvant on both safety and efficacy profile [12]. The chemical structure of oils used impact the efficacy and safety profile of the vaccine formulated: mineral oils are strong immune stimulators but can induce small to severe side effect at the injection site [13]. Metabolisable oils have lower inflammatory and immunostimulatory properties than mineral ones, conducting usually to improve the safety profile of vaccine formulated [14]. Nevertheless, this type of adjuvant is not able to fit to sensitive species vaccines safety expectations, notably in case of reactogenic antigenic media.

In this study, we tested a novel adjuvant based on a polymer technology already applied in the adjuvant for veterinary vaccine field. This new formulation has been tested for its safety in several models: pig, cattle and dog. These models cover the full range of vaccine expectation with sensitive animals, such as dogs and pigs, and more robust ones as cattle. Furthermore, the antigenic models used (bacterial, viral and purified protein) also covers the various antigens properties encountered in the field. Our finding demonstrates the ability of Montanide™ Gel adjuvant to be safely used in three species, even when associated to crude bacterial antigenic media.

2. Material and Methods

2.1 Trials protocol

Table 1 summarizes all protocols performed during the Montanide™ Gel safety study.

Animal Trial	Injection pathway	Injection site	Injection number	Injection volume (ml)	Duration of the trial (days)
Pig	IM	Loin	2 injections D0, D28	2	110
Cattle	SC	Shoulder			150
Dog	SC	Neck		1	120

Table 1: Summary of vaccine protocols in each species. IM: intramuscular; SC: subcutaneous.

2.2 Pig trial

2.2.1 Animals

Pigs cross breed Large-white and Landrace fattening male pigs from a commercial herd in Brittany were introduced in the protocol. All animals were castrated at birth. Pigs were 6 weeks of age and weighting 10 to 15 kilograms at day 0, and were at day 110 around 100 kilograms when slaughtered. Seven animals were introduced in each vaccine group.

2.2.2 Adjuvants

As a negative control we used the antigen injected in saline buffer. Montanide™ gel was used at 10% of adjuvant in the injected formula. All experimental vaccine formulated contained the same amount of antigenic media.

2.2.3 Antigen

The antigen was kindly provided by Dr Joan Plana from FORT DODGE (Spain).
The same antigen mix was used for all vaccine tested in the study. This antigen was composed of purified *Pasteurella multocida* anatoxins (PMT) and whole bacterial cell wall of *Bordetella bronchiseptica*. Each dose of vaccine was containing 50µg of anatoxin antigen and 1.10^{10} unit forming colonies of inactivated *Bordetella bronchiseptica*.

2.2.4 Data collected

Vaccine safety was assessed through two criteria: the local and general safety was observed during the trial by palpating the injected loin, and by dissecting the injection site after pig euthanasia. The general safety was defined as the absence of pyrogenic reactions following vaccine delivery, as well as the absence of behaviour modification. Therefore, rectal temperature of each animal was taken at 4, 24 and 48 hours after each vaccination. Animal behaviour was also followed during this three days period: various criteria were recorded: reluctance to move, food intake, tendency to lie down, oedema, vomiting and diarrhoea. The local reactions were assessed at the slaughterhouse. Every loin was sampled after pig slaughter and dissected for the research of local reaction in situ.

2.3 Cattle trial
2.3.1 Animals

Thirty Holstein breed calves were used in this trial. Ten animals were inserted in each group. Animals were weighting 45 to 50 kilograms at day 0.

2.3.2 Adjuvants

As a negative control we used the antigen injected in saline buffer. Montanide™ Gel was used in two different groups respectively formulated at 10 and 20% of adjuvant in the injected formula. All experimental vaccine formulated contained the same amount of antigenic media.

2.3.3 Antigen

Ovalbumin (OVA from SIGMA) at a concentration of 2mg per dose was used as a model antigen.

2.3.4 Data collected

The local and general safety was observed during the trial, by palpation of the injection site, and by dissecting the injection site after calve euthanasia. The general safety was defined as the absence of pyrogenic reactions following vaccine delivery, as well as the absence of behaviour modification. Rectal temperature of each animal was then taken at 4, 24 and 48 hours after each vaccination. Animal behaviour was also followed during this three days period: various criteria were recorded: reluctance to move, food intake, tendency to lie down, and oedema. The local reactions were assessed at the slaughterhouse during carcass process.

2.4 Dog trial
2.4.1 Animals

Dogs were 6 months old male and female cross breed animals. Ten animals were randomly introduced in each group.

2.4.2 Adjuvants

For sensitive species such as dog, a specific grade of Montanide™ Gel was designed: Montanide™ PET GEL A. This adjuvant 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.

2.4.3 Antigen

Antigens were kindly provided by ROMVAC Company. Divalent vaccines were formulated with a viral and a bacterial antigen. The viral valence was composed of inactivated Canine Parvovirus (CPV). The bacterial antigen was an inactivated culture of *Leptosipra caniloca* and *Leptosipra icterohemorragiae.*

2.4.4 Data collected

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 population present as well as necrosis, fibrosis and vaccine remnants.

3. Results

3.1 Pig trial

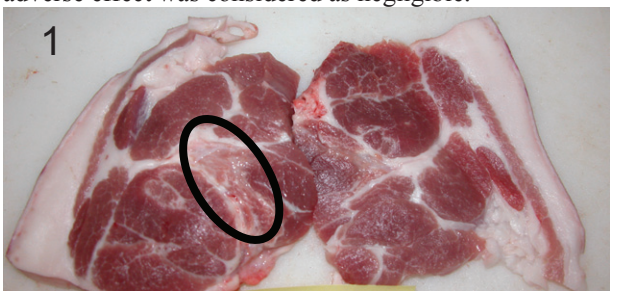
No modification of the animal behaviour was observed in the trial whatever the vaccine. After the first injection, no local reaction could be detected during the three days survey. After the booster injection, oedemas could be detected in two out of seven pigs of the group receiving vaccine based on Montanide™ Gel adjuvant. No local reactions could be observed in the control group. Temperatures of the animals after each injection are presented in table 2.

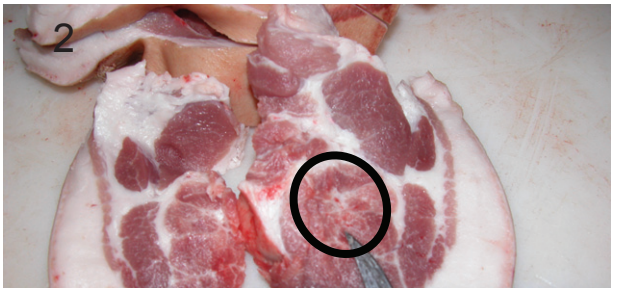
first injection	T0	T4H	T24H	T48H
Montanide Gel 10%, Mean (+/- SD)	39,2 (+/-0,5)	40,3 (+/-0,4)	39,7 (+/-0,3)	39,4 (+/-0,2)
Delta calculated Vs T0	/	+1,1	+0,5	+0,2
Control, Mean (+/- SD)	38,7 (+/-0,4)	40,2 (+/-0,4)	39,5 (+/-0,3)	39,7 (+/-0,3)
Delta calculated Vs T0	/	+1,5	+0,8	+1
booster injection				
Montanide Gel 10%, Mean (+/- SD)	39,9 (+/-0,6)	40,3 (+/-0,6)	39,9 (+/-0,5)	40,1 (+/-0,7)
Delta calculated Vs T0	/	+0,4	+0	+0,2
Control, Mean (+/- SD)	39,9 (+/-0,6)	40,4 (+/-0,3)	39,8 (+/-0,4)	40 (+/-0,7)
Delta calculated Vs T0	/	+0,5	-0,1	+0,1

Table 2: Body temperature survey after intramuscular injection of 2 ml of vaccine or controls in pigs. Animals were observed for three days. Means and standard deviation (SD) from individual measures were calculated.

Increases observed at the first injection were higher than those observed after booster vaccination. This result was observed at 4 and 24 hours after injection while at 48 hours post injection, no reaction could be observed any more. The control group, pig receiving injection of antigen without adjuvant, induced a higher increase of body temperature compared to the adjuvanted vaccine. No differences were observed during the trial between control and adjuvanted vaccines.

At the slaughterhouse, loins were dissected in order to look for vaccine remnants or vaccine related lesions in the meat linked to the vaccine injection. No local reaction in the meat could be observed except in two of the seven animals receiving Montanide™ gel based vaccine. Only discrete fibrosis was observed in those two animals, indicating a healing activity at the injection site. No vaccine or adjuvant residues could be detected in the loin meat. Picture 1 and 2 presents the local reaction detected. The local reactions were observed only in the loins receiving the booster injection. According to the type and size of the reactions observed, as well as the low occurrence, this adverse effect was considered as negligible.





Picture 1 and 2 presents fibrosis areas observed after dissections of loins on day 110. Those reactions were induced by injection of 2ml of vaccine intramuscularly at day 28.

3.2 Cattle trial

No local reactions were observed either during the trial or at the slaughterhouse. The animal behaviour and body temperature remain unchanged after first and second vaccine delivery. No influence of the presence of Montanide™ Gel in the vaccines could be detected.

3.3 Dog trial

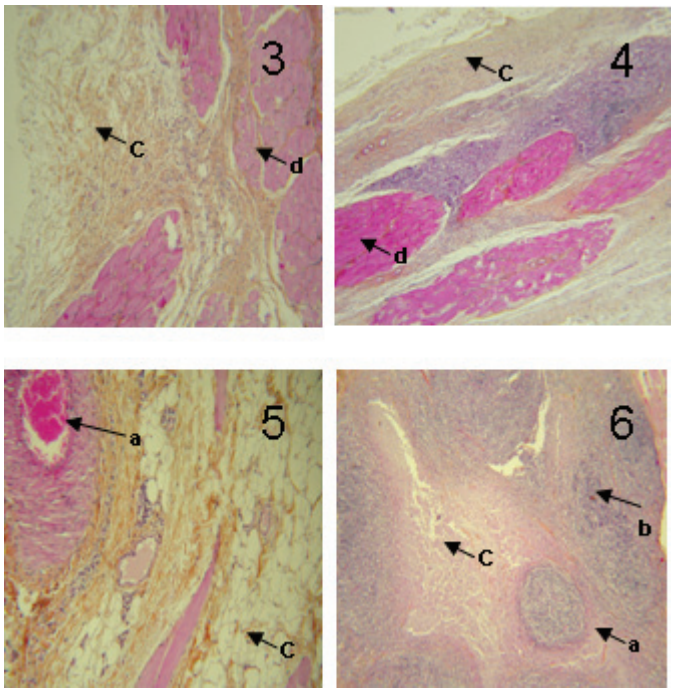
During the trial, no modification of the social behaviour was detected either after first or second injection. Feeding habits of the animals were unchanged. No impact of the vaccine delivery was observed on the body temperature of dogs whatever the vaccine group.
Transient oedemas were observed during the trial, mainly after the booster injections. All reactions observed were of the same type: oedemas and swelling of the injected site. Table 3 presents the percentage of animals with oedema and the size of the reaction observed after the second vaccine injection. The lasting of the local reaction 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.

Alum	**4h**	**24h**	**48h**	**72h**	**144h**
% of dog presennting local reaction	0	11	44,4	22,2	33
Size (mean +/- SD) cm²	0	0,1 (±0,2)	0,4 (±0,5)	0,2 (±0,4)	0,3 (±0,5)
Montanide™ PET GEL A					
% of dog presennting local reaction	0	12,5	37,5	37,5	0
Size (mean +/- SD) cm²	0	0,3 (±0,8)	0,3 (±0,4)	0,1 (±0,3)	0

Table 3: Local reactions induced after the second injection by alum or Montanide™ adjuvanted vaccines. Vaccination was done subcutaneously under 1ml of volume in the neck.

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 reaction for the Gel based vaccine while only two out of ten for the aluminium formula. Pictures 3 and 4 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 5 and 6, 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.



Picture 3, 4, 5, 6: 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 3 and 4 presents reactions examples induced by Montanide™ GEL PET A based formula while pictures 5 and 6 presents reactions observed with aluminium containing vaccines.

4. Discussion

The trials we performed in cattle and pigs demonstrated a good safety profile for Montanide™ Gel based vaccines in both species. Results obtained from the cattle trial can be considered as the adjuvant own safety profile as the highly (95%) purified protein we used as antigen, ovalbumin, do not present any safety risk. However each vaccine, even directed against common pathogen, is unique as the process of antigen production and inactivation impact the safety properties of the antigen itself. Therefore, association of any adjuvant to reactogenic media should be done very carefully as all properties of the antigen (both immunologic and inflammatory) will be enhanced. In the pig trial, Montanide™ Gel was associated to *Pasteurella* or *Bordetella* antigens. Those bacteria are gram negative and containing pro inflammatory lipopolysaccharides (LPS) [16] able to induce strong local and general side effect. Nevertheless, no general side effects were observed and local reactions found while dissecting the injection site were negligible. Montanide™ Gel has also been tested formulated with *Actinobacillus pleuropneumoniae* crude antigen [15] and transient pyrogenic reactions were observed. These finding highlight the crucial role of the antigen upon the safety profile in a vaccine design: *Actinobacillus* like *Pasteurella* or *Bordetella* is a gram negative LPS containing genius. But our results demonstrate vaccine pyrogenic reactions only when Montanide™ Gel was formulated with *Actinobacillus pleuropneumoniae*. Furthermore, when associated with highly purified influenza antigen [17] in a pig trial, no local or general reaction could be observed for the Montanide™ Gel based vaccine while the aluminium based formulation induced a light increase of temperature. In farm animals, Montanide™ Gel safety was found to be better or at least equivalent to the reference adjuvant in terms of safety: aluminium salts. In sensitive species to vaccine, such as dogs, observation performed during three days after each injection showed the

two adjuvants tested (Montanide™ GEL PET A and aluminium) to induce equivalent reactions at the injection site. These reactions were very small and did not interfere with animal welfare. The reactions were also small enough to avoid animal's owners to be worried by post vaccine reactions. Histology results demonstrated a chronic inflammatory reaction induced by aluminium salts in 20% of the dogs leading to necrosis of tissues at the injection site which confirms the long lasting inflammatory properties of alum. This type of reaction has already been observed associated to cytoplasmic macrophages storage of aluminium salts [18] and associated to severe post vaccinal pathologies as sarcomas [9] . None of those intense adverse reactions could be observed with the use of Montanide™ GEL PET A. Nevertheless, moderate inflammatory response was identified in a larger number of dogs (60%). The moderate reactions observed with the polymeric formulation were not associated to presence of adjuvant remnants either in the injection site or cytoplasm of phagocyting cells. Based on these results, no chronic inflammatory response is expected from the use of Montanide™ PET GEL A.
Montanide™ Gel range has been demonstrated to be safe in several species, including pets, and even associated to highly reactogenic bacterial media. Even though, this safety profile must be balanced with the vaccine efficacy in all those models. The Montanide™ Gel based vaccine efficacy has been studied in pigs [IPVS] but is still to challenge in dogs and cattle models.

5. Conclusion

Choosing an adjuvant is crucial in a vaccine design. The choice of vaccine components will drive the vaccine safety and efficacy in the field but also production cost. Antigen media production is done through biotechnologies bringing to vaccine component reliability and consistency. The adjuvant selected must be able to give the same properties to the vaccine production from batch to batch. Selecting a unique adjuvant formulation for several vaccines dedicated to the same species and even to several species allows raising the level of profitability and quality control upon the vaccines production. In this study we demonstrated that Montanide™ Gel range can be safely used associated with various antigenic media and animal species with, at least, an equivalent safety profile than the aluminium salts. We are now challenging Montanide™ Gel range in the field for its ability to induced protective immune response.

6. References

[1] Murphy D, Corner LA, Gormley E. Adverse reactions to Mycobacterium bovis bacille Calmette-Guérin (BCG) vaccination against tuberculosis in humans, veterinary animals and wildlife species.Tuberculosis (Edinb). 2008 Jul;88(4):344-57. Epub 2008 Jan 28. Review.

[2] Cox J.C., Coulter A.R. - Adjuvants : a classification and review of their mode of action. Vaccine 15 (1997), p. 248-256.

[3] Audibert A.C., Lise L.D. - Adjuvants : current status, clinical perspectives and future prospects. Immunol Today Jun;14(6) (1993) p. 281-4.Review.

[4] Lindblad E.B. Aluminium adjuvants--in retrospect and prospect. -Vaccine. 2004 Sep 9;22(27-28):3658-68. Review.

[5] HogenEsch H. -Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 2002;20(Suppl. 3):S34–9.

[6] Ulanova M., Tarkowski A., Hahn-Zoric M., Hanson L.A. -The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect Immun 2001;69(2):1151–9.

[7] Rimaniol A.C., Gras G., Verdier F., Capel F., Grigoriev V.B., Porcheray F., et al. -Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type. Vaccine 2004; 22(23/24):3127–35.

[8] Cloete M, Dungu B, Van Staden LI, Ismail-Cassim N, Vosloo W. Evaluation of different adjuvants for foot-and-mouth disease vaccine containing all the SAT serotypes. Onderstepoort J Vet Res. 2008 Mar;75(1):17-31.

[9] Hendrick MJ, Goldschmidt MH, Shofer FS, Wang Y-Y, Somlyo AP. Postvaccinal sarcomas in the cat: epidemiology and electron probe microanalytical identification of aluminum. Cancer Res 52:5391–5394, 1992.

[10] Verdier F, Burnett R, Michelet-Habchi C, Moretto P, Fievet-Groyne F, Sauzeat E. Aluminium assay and evaluation of the local reaction at several time points after intramuscular administration of aluminium containing vaccines in the Cynomolgus monkey. Vaccine. 2005 Feb 3;23(11):1359-67.

[11] Bahnemann H.G., Mesquita J.A. - Oil adjuvant vaccine against foot-and---mouth disease. Bol. Centr. Panam. Fiebre Aftosa, 53 : 25-30, 1987

[12] Jansen T, Hofmans MP, Theelen MJ, Schijns VE. Structure-activity relations of water-in-oil vaccine formulations and induced antigen-specific antibody responses. Vaccine. 2005 Jan 11;23(8):1053-60.

[13] Oda K., Tsukahara F., Kubota S., Kida K., Kitajima T., Hashimoto S.: Emulsifier content and side effect of oil-based adjuvant vaccine in swine. Research in Veterinary Science 81, 2006 51-57.

[14] Aucouturier J., Deville S., Perret C., Vallée I., Boireau P. - Assessment of efficacy and safety of various adjuvant formulations with a total soluble extract of Trichinella spiralis. Parasite. 2001 Jun;8(2 Suppl):S126-32.

[15] Dupuis L, Deville S, Bertrand F, Laval A, Aucouturier J. Adjuvant formulation for multivalent pig vaccines : Pasteurella multocida anatoxins and inactivated Bordetella bronchiseptica, Montanide™ Gel safety study. Proceedings of the International pig veterinary society 2008, Durban.

[16] Van Amersfoot E.S., Van Berkel T.J.C., Kuiper J. - Receptors, Mediators, and mechanism involved in bacterial sepssis and spetic shock. Clin. Microbiol. Rev. July 2003, 379-414.

[17] Deville S, Parker R, Laval A. Adjuvant formulation for Influenza H1N1 and H3N2 pig vaccines: Montanide™ Gel safety and efficacy study. Proceedings of the Conference of Research of Workers in Animal Diseases 2008, Chicago.

[18] Day MJ, Schoon HA, Magnol JP, Saik J, Devauchelle P, Truyen U, Gruffydd-Jones TJ, Cozette V, Jas D, Poulet H, Pollmeier M, Thibault JC. A kinetic study of histopathological changes in the subcutis of cats injected with non-adjuvanted and adjuvanted multi-component vaccines. Vaccine. 2007 May 16;25(20):4073-84.