SCIENTIFIC DISCUSSION

Invented Name:	Nobivac Piro
Active substance / INN:	Soluble parasite antigen (SPA) from *Babesia canis* and *Babesia rossi* cultures
Target species:	Dogs
Therapeutic indication:	For active immunisation of dogs against *Babesia canis*
Withdrawal period:	Not applicable
Pharmaceutical form:	Lyophilisate + solvent for suspension for injection
ATCvet code	QI07AO
Pharmaco-Therapeutic Group	Inactivated parasitic vaccines
Marketing Authorisation Holder	Intervet International BV, Wim de Körverstraat 35, NL - 5831 AN Boxmeer, The Netherlands

1. SUMMARY OF THE DOSSIER

The active substance of Nobivac Piro is soluble parasite antigen (SPA) from *Babesia canis* and *Babesia rossi* cultures.

The benefits of Nobivac Piro are that a vaccine based on SPAs of *Babesia canis* and *Babesia rossi* is the only kind of vaccine having an effect on the clinical signs of babesiosis and reducing the extent of anaemia after a heterologous challenge infection. Protective immunity is associated with a reduction of SPA in plasma after experimental heterologous challenge infection.

The most commonly reported post-vaccinal reactions are a diffuse swelling and/or hardened nodule, accompanied by pain, at the site of vaccination and in general this disappears within 4 days. In rare cases, the reactions after the second dose of vaccine may remain for 14 days. Vaccinated dogs may develop a stiff gait and exhibit a reduction in appetite after vaccination. These reactions should disappear within 2-3 days.

The approved indication is for active immunisation of dogs of 6 months of age or older against *Babesia canis* to reduce the severity of clinical signs associated with acute babesiosis (*Babesia canis*) and anaemia as measured by Packed Cell Volume (PCV).

Onset of immunity: 3 weeks after the basic vaccination course.

Duration of immunity: 6 months after the last (re-)vaccination.

The CVMP, on the basis of quality, safety and efficacy data submitted, considers that there is a favourable benefit to risk balance for Nobivac Piro and therefore recommends the granting of the marketing authorisation.

2. QUALITY ASSESSMENT

Composition
Nobivac Piro is an inactivated vaccine that contains soluble antigen from *Babesia canis A* and *Babesia rossi* with a concentration of 606 (between 301 and 911) Total Antigenic Mass (TAM) units of the active substance per vial. No preservative is included. The lyophilisate is presented with a solvent for reconstitution. The solvent contains saponin as adjuvant and other standard excipients in a 1 ml vial.

The whole application is built on the combination of the soluble parasite antigen (SPA) produced by 5 x 10^7 erythrocytes infected by *Babesia canis A* and the SPA produced by 5 x 10^7 erythrocytes infected by *Babesia rossi*.

Container
Type I glass bottle with a halogenobutyl rubber stopper for both the solvent fraction and freeze-dried fraction, sealed with an aluminium cap.

Development Pharmaceutics
For the antigen selection the following published data were taken into consideration:

Vaccines based on purified parasites from *in vitro* cultures do not confer protective immunity. Soluble parasite antigen (SPA) however directly correlates with protection. Erythrocyte lysate preparation without SPA has no protective effect.

When using vaccines containing SPA of a single *Babesia canis* strain, no protection against heterologous challenge infection was induced. Extensive booster vaccinations (four vaccinations in total) did not improve protection either against heterologous challenge infection. Antigenic diversity between *Babesia* strains limits the spectrum of protective activity. Vaccines needed to be improved to meet efficacy field requirements.

Once dogs had survived a *Babesia rossi* infection, they developed immunity against a subsequent infection with *Babesia canis* parasites. Thus *Babesia rossi* appeared to be an appropriate candidate to stimulate immune responses.

A vaccine based on SPA of *Babesia rossi* alone does not induce immunity against *Babesia canis* infection. However, a vaccine based on SPA produced by equal number of *Babesia canis* and *Babesia rossi* parasites induced protective immunity against heterologous *Babesia canis* challenge infection.

It was concluded that such a bivalent vaccine based on SPA from *Babesia canis* and *Babesia rossi* parasites was worthy of further investigation for the development of a vaccine against *Babesia canis* infection.

Release specification of the antigen component
The formulation combines the SPA produced by 5 x 10^7 erythrocytes infected by *Babesia canis A* and the SPA produced by 5 x 10^7 erythrocytes infected by *Babesia rossi*. Only during the development of the vaccine was the potency measured in an *in vivo* potency test in dogs, that was validated. Repeatability and sensitivity of the *in vivo* potency test were investigated and analysed statistically. No statistically significant difference was seen on both potency titres and proportion of responders. The *in vivo* test is able to detect sub-potent batches. Indeed, the sensitivity test showed that the response obtained after administration of a quarter of dose was significantly different from the response obtained with a single or a double dose. As clinical signs were reduced in dogs vaccinated with only 1/5 of a dose the risk to release a sub-potent batch was considered low enough to be acceptable. On the other hand overpotent

batches cannot be detected, as there is no difference between a standard-dose batch and a batch containing twice the amount of antigen.

Although the above described *in vivo* test is quite effective, the passage to an ELISA quantification of the active ingredients is strongly supported because it leads to an even better standardised product. Indeed, taking into account the ten batches manufactured in the same way (5 x 10^7 erythrocytes infected by *Babesia canis A* and 5 x 10^7 erythrocytes infected by *Babesia rossi*), the coefficient of variation of the *in vivo* potency test is 23%, whereas the coefficient of variation of the *in vitro* potency test is 13%.

With regard to the results obtained in the *in vitro* test compared to the *in vivo* test, the passage to an ELISA quantification of the active ingredients, was proposed, supported and subsequently implemented.

The individual antigenic mass ranges have been defined as *Babesia canis*: 500-1200 ELISA units and *Babesia rossi*: 250-600 ELISA units. Validations of the Babesia antigenic mass ELISA were performed.

Antigen preparation
Fixed amounts of *Babesia canis* and *Babesia rossi* bulk antigen (each produced by 5 x 10^7 infected erythrocytes) are combined. These fixed antigen amounts are quantified by an antigenic mass ELISA.

Choice of the adjuvant
An aqueous adjuvant based on saponin was chosen, based on studies showing that SPA-vaccines were effective when saponin was used as adjuvant, in contrast to vaccines adjuvanted with oil-based compounds, which lacked protective activity.

METHOD OF MANUFACTURE

Lyophilisate
Frozen aliquots of each *Babesia* bulk antigen are thawed. Fixed aliquots of each soluble *Babesia* antigen are combined and stirred, together with culture medium. After filling, the content of the vials is freeze-dried, and closed under vacuum, secured with a coded aluminium cap and stored at 2-8°C.

Solvent
The required weight of saponin is dissolved in a buffer (to a final concentration of 250 µg/ml) and sterilised through filtration (0.22 µm). After filling, the vials are closed and sealed.

CONTROL OF STARTING MATERIALS

The starting materials listed in a pharmacopoeia comply with the requirements of that pharmacopoeia.

Suitable Certificates of analysis were provided. The certificates of analysis show satisfactory results. The vials are sterilised by dry-heat at least 1 minute at 250°C; the stoppers are sterilised by autoclaving at 121°C for at least 15 minutes.

The starting materials of biological origin comply with the requirements of the manufacturer.

The following starting materials were assessed:
- ***Babesia canis A* and *Babesia rossi***

The following details were given:
Controls on the Master Seed Lots
- absence of bacterial and yeast contamination:by a general test (Ph.Eur. test 2.6.1) and by a specific test: search of *Brucella canis*.
- absence of mycoplasmic contamination (Ph.Eur. test 2.6.7).
- absence of extraneous viruses (in various cell cultures)

Controls on the Working Seed Lots:

- absence of bacterial and yeast contamination by a general test (Ph.Eur. test 2.6.1).
- absence of mycoplasmic contamination (Ph.Eur. test 2.6.7).

Suitable Certificates of analysis for each Master Seed were provided. The certificates of analysis show satisfactory results. From these data, it can be concluded that the cells used are sensitive to the viruses to be detected.

Canine red blood cells
For the production of the antigen, red blood cells from SPF beagle dogs are used. SPF and health status of each donor dog are checked. The dogs come from a dog colony tested to be negative for canine adenovirus 2, canine coronavirus, canine distemper virus, canine herpesvirus, canine parvovirus, leptospira spp. and parainfluenza 2 virus. The absence of rabies virus is not checked because rabies does not occur in the country of origin It should be noted that dogs can come from different colonies. In every case, the requirements will be in compliance with the "table of extraneous agents to be tested for" of volume VII, column "SPF herd".

Blood groups do exist in dogs and at least groups DEA_1 to DEA_7, J, K, L, M, N were identified. Amongst them, group DEA_1 is the most important group and about 60% of dogs are A-positive and 40% A-negative. 10% of these A-negative dogs (ie 4%) have natural antibodies against antigen A. If an A-negative dog is transfused with A-positive blood more than once, this might induce safety problems (type-II hypersensitivity). Consequently, the Applicant agreed to use only blood cells from A-negative dogs for the production of the vaccine (working seed production and bulk antigen production).

Blood of different dogs may be pooled. The blood is centrifuged, plasma and buffy coat are removed. Red cells are washed twice with complete RPMI cell culture media.

Saponin (not animal-derived)
Controls performed were for appearance, identification, haemolysis and loss on drying.
A suitable certificate of analysis was provided.

Culture Medium
Satisfactory details were provided on the qualitative and quantitative composition of the culture medium and on the preparation.

As protein source in the culture medium, γ-irradiated canine blood serum from conventional dogs is used. The health status of each donor dog was checked. Information from the supplier on the reduction of virus titres by irradiation was submitted.

The major families of viruses infecting vertebrates are represented. In particular, porcine parvovirus, known to be very resistant, was used.The reduction was always above the requested 6 $\log_{10}$. Consequently, it can be concluded that an irradiation at 25 kGy is sufficient to inactivate any potential viral extraneous agent.

Antibiotics
A mixture of a maximum of 3 of the following antibiotics may be added to the medium:
neomycin sulphate, tylosine tartrate, natamycine and/or gentamycin sulphate. A suitable certificate of analysis was provided for each antibiotic.

Satisfactory details were provided on starting materials of non-biological origin.

SPECIFIC MEASURES CONCERNING THE PREVENTION OF THE TRANSMISSION OF ANIMAL SPONGIFORM ENCEPHALOPATHIES

It is not possible for Intervet to determine which ingredients were used during the passage steps that were performed prior to the establishment of the master seeds (*Babesia canis* A was isolated in France in 1982, passaged through dogs in Utrecht and arrived at Intervet in 1990; *Babesia rossi* was isolated in Germany in 1976, passaged through dogs in Utrecht and arrived at Intervet end of 1989).

For the vaccine production, the TSE risk can be considered as remote, as every effort was made to avoid use of raw materials coming from TSE-sensitive animals. It should also be noted that the vaccine is intended for a species which is, up to now, not recognised to be affected by TSE.

Casein is the only material of ruminant origin involved in the production of Nobivac Piro. This was present in a medium which was used for the preparation of the master seeds and working seeds. It is also a component of the final formulation, as it is used to obtain a constant fill volume of the final vaccine. The milk used to produce casein comes from healthy cows fit for human consumption.

The starting materials of animal origin used in the production of the final product comply with the then current regulatory texts related to the TSE Note for Guidance (EMEA/410/01-Rev.1) and Commission Directive 1999/104/EEC.

CONTROL TESTS DURING PRODUCTION

The following details were given for *Babesia canis A* and *Babesia rossi*:
Origin and history: *Babesia canis A* was isolated from a tick that had fed on a dog that contracted babesiosis in France, and *Babesia rossi* from a dog that contracted babesiosis in South Africa. The parasite was then passaged through 5 dogs for *Babesia canis A* and through 3 dogs for *Babesia rossi* respectively, before the establishment of the Master Seed Lot (MSL).

MSL of both strains: a splenectomised beagle dog was used to establish the MSL. After infection, the dog was sacrificed on the second day. Its blood was collected, aliquoted and centrifuged. After resuspension in culture medium, it was processed for cryopreservation.

WSL of both strains: a splenectomised beagle dog was used again to establish the WSL. After infection with 1 ml of the MSL, the dog was sacrificed upon patency. Its blood was collected, and the *Babesia*-infected erythrocytes are cultured in culture medium. When the culture is stable, it is harvested and processed to be frozen in liquid nitrogen.

Active substance of both strains: the erythrocyte are resuspended in culture medium and the suspension is inoculated with Babesia Working Seed material. The infected erythrocytes are cultured at 37°C. The cultures are refreshed (medium/erythrocytes) and diluted several times during this start-up phase. Once the cultures are growing steadily, they are expanded. The supernatants, containing the SPA antigen, are harvested and stored in aliquots at 2-8°C. The SPA antigen is concentrated using an ultrafiltration system. The SPA bulk antigen is stored in aliquots at ≤ -20°C. The frozen SPA bulk antigen is γ-irradiated at ≥ 25 kGy (validated method) to guarantee absence of live parasites.

Inactivation of Babesia parasites by irradiation was correctly validated. Babesia parasites are killed when subjected to an irradiation of 25 kGy. Although only *Babesia canis A* was used in this test, there was no reason to suspect any other behaviour concerning *Babesia rossi* when γ-irradiated. Despite the fact that the blood is coming from healthy dogs free of any clinical signs of diseases, the Applicant was asked to document inactivation of other extraneous agents by irradiation at 25 kGy.

Control tests during production
Results of the in-process controls were provided for 3 consecutive batches. The release requirements were met.

CONTROL TESTS ON THE FINISHED PRODUCT

Identification and assay of active substance

Antigen preparation
A specific ELISA test for each SPA of the *Babesia canis A* and the *Babesia rossi* strains was used. This method involved specific dog polyclonal antibodies and a reference sample for each strain with predetermined antigenic content. With the 2 results obtained for each *Babesia*, the Total Antigenic Mass calculated using the following formula was: Total Antigenic Mass = (ELISA Units *Babesia canis A* x ELISA Units *Babesia rossi*)$^{1/2}$
Limits of acceptability: Total Antigenic Mass between 301 and 911 units.

Validations of the Babesia antigenic mass ELISA
For the validations of the Babesia antigenic mass ELISA, specificity, linearity, repeatability, reproducibility and robustness were investigated. On request, refined criteria were formulated by the applicant, that further guarantee the robustness and reliability of both assays.
In addition, it was agreed to set the individual antigenic mass ranges to:
Babesia canis A antigen : 500 – 1200 units
Babesia rossi antigen : 250 – 600 units

Safety tests

Reconstituted vaccine: 2 dogs of 2-6 months of age were vaccinated by subcutaneous injection with a double dose of reconstituted vaccine. The dogs were observed for 14 days for local and general reactions. Retesting in 4 dogs was allowed. No abnormal local or systemic reactions are observed. Normal reactions were defined as follows:
Normal local reaction: a slight diffuse transient swelling may occur during the first 3 days of the observation period.
Normal general reaction: dogs may show reduced appetite, malaise/depression and apathy for a maximum of 3 days within a week after vaccination.
If the results are outside the specifications, the batch will be rejected.

Sterility and purity tests

Antigen preparation and solvent: bacterial and fungal sterility, in compliance with Ph.Eur. monograph 2.6.1. No growth is detected.

Residual humidity

Antigen preparation: complies with the Ph.Eur. monograph 2.5.12., with an amount 0.1 - 5% w/w.

Batch to batch consistency

Results of the controls were provided for 3 consecutive batches of both antigen component and solvent. The release requirements are met.

STABILITY

Results were initially available over 17 months for 3 batches of the freeze-dried pellet, and over 15 months for 4 batches of the solvent. Updated the data was provided to include the latest results for both the antigen and the solvent.

Stability of the freeze-dried pellet

Total Antigenic Mass (which should be between 301 and 911 units) and residual moisture (which should be between 0.1 and 5.0 w/w) was established of 4 different batches of finished product up to 27 months and of 1 batch at 84 months of storage at 2-8°C.
The results were within the limits set in the initial dossier and in the responses, except for one value of the *Babesia rossi* antigenic mass in batch IP-008 at 12 months: the results at this time point was of 205 units, whereas the Applicant proposed in his answers to the List of Questions to set the lower limit at 250 units. Nevertheless, taking into account that this threshold was set after the start of the stability testing, and that the lower release limit was arbitrarily set at 250 units (calculation indeed provided a limit of 187 units, which is beneath the result of 205 units found in the stability test), this deviation was considered acceptable.

Stability of the solvent

The pH (which should be 6.5 ± 1.0) and saponin content (which should be 250 µg/ml ± 20%) on 4 batches of solvent were provided over 12 months at -20°C followed by 12 months at 2-8°C and the results were satisfactory.

On provision of these further data, a shelf life of 24 months for the antigen and 24 months for the solvent were supported.

OVERALL CONCLUSION ON QUALITY

The analytical part is well documented. Production of the freeze-dried vaccine and solvent is by conventional production methods. The control tests and specifications for the finished product were suitable to demonstrate that a product of consistent quality could be produced. The TSE risk with this product is condsidered negligilbe and the product is intended for a species which, up to now, is recognised not to be affected by TSE. A shelf life of 2 years for the product was supported by the stability data provided.

3. SAFETY ASSESSMENT

LABORATORY TESTS

Safety of the administration of one dose, of an overdose and of the repeated administration of one dose

Brief description of the trial design
Four- to six-months old Beagles were evenly distributed into 4 groups with regard to the distribution of age, sex and litter. Group A was injected subcutaneously with a double dose of Nobivac Piro once, Group B was injected subcutaneously with single dose of Nobivac Piro 3 times at day 0, 14 and 28, Group C was injected subcutaneously with a double quantity of phosphate buffered saline (PBS) once and Group D was injected subcutaneously with a single quantity of PBS 3 times at day 0, 14 and 28.

Examinations :
All dogs were observed daily for clinical signs (scoring system), during the 14 days after the last injection (up to D14 for groups A and C, and up to D42 for groups B and D). On the day of injection(s), dogs were further observed 4 hours after each injection (D0+4h, D14+4h, D28+4h).

Body temperature was registered 7 days and 1 day before injection, then up to 14 days after the last injection (up to D14 for groups A and C, and up to D42 for groups B and D). Fever was considered as being ≥ 39.0°C.

Local reactions at the injection site were monitored daily (scoring system), at D-7, and from D-1 up to 2 weeks after the last injection (up to D14 for groups A and C, and up to D42 for groups B and D). On the day of injection(s), dogs were further observed 4 hours after each injection (D0+4h, D14+4h, D28+4h).

Haematocrit in groups A and C was determined at D-7, D0, D0+4h, D1, D2, D7 and D14. In groups B and D, it was determined at D-7, D0, D0+4h, D1, D2, D7, D14, D14+4h, D15, D16, D21, D28, D28+4h, D29, D30 and D35.

Statistical analysis
Body temperature and haematocrit: ANOVA and Least Significant Difference.

Results
Clinical signs: prior to vaccination, some dogs showed red eyes and one dog showed a slightly swollen anus. These observations were transient (1 day) and not indicative of disease.

Body temperature: no fever period was registered and no significant difference was found between the mean body temperature of each group on each day, except at day 35 between group B and D (38°C in group B versus 37.4°C in group D). No significant interaction on body temperature between sex and treatment was measured.

Local reactions: all dogs of group A showed transient (2-5 days) diffuse swelling. In one of five dogs, the swelling was painful for 2 days. No local reactions were observed in group C.

All dogs of group B showed transient (3-6 days) diffuse swelling after each injection. In one of five dogs, the swelling was painful after the first and second injection for 1 day, and warm for 2 days after the third injection.

In one of five dogs of group D showed diffuse swellings after the first and second injection that were transient (7 and 2 days respectively).

Haematocrit: no significant difference was found for each mean PCV daily value between the groups. No significant interaction on PCV values between sex and treatment was measured.

Conclusion
Subcutaneous administration of a double dose or repeated administration of single doses are safe.

Examination of reproductive performance
No trial was provided on pregnant or lactating bitches. This was considered satisfactory as the use of this vaccine on pregnant and lactating bitches is contraindicated in the SPC.

Examination of immunological functions
No trial was provided. This was considered satisfactory as there is no reason to suspect any impact of vaccination with Nobivac Piro on immunological functions.

Interactions
No trial was provided. As there is no known interaction, the absence of a trial was considered to be acceptable. Additionally, an appropriate warning is given in the SPC.

FIELD STUDIES

Brief description of the trial design
Dogs of at least 6 months of age and of various breeds, from 5 different sites, were used in this trial. Pregnant bitches were excluded. At each location, dogs were injected either with Nobivac Piro or with a saline solution, twice SC with an interval of 4 weeks between each injection. Treatments were randomly distributed and conducted in a double blind manner.

Examinations
Comparison of both groups was done on the following parameters:
Dogs were observed for local and systemic reactions for a period of 14 days after each injection. Local reactions were appreciated through palpation of the injection site of all dogs. If detectable, size and nature were recorded following a scoring system. Systemic reactions were appreciated through attitude and appetite of all dogs following a scoring system; rectal temperature of 10 pre-selected dogs at each site (or all if less than 10 dogs were present on the site) was recorded, 3 days before each injection, the day of each injection and daily during the four days after each injection.

PCV was determined at 0, 28 and 42 days after the first vaccination on dogs from the placebo group and from the vaccinated group.

Statistical analysis
Incidence, size and nature of local reactions: chi-square test.
Mean age of the groups, rectal temperature, PCV: ANOVA.

Results
There was no difference between the vaccinated and placebo groups and there was no difference seen with regard to breed, gender or age. There was also no difference detected for rectal temperature and packed cell volume. No reaction was seen after the first injection. After the second injection, some vaccinated dogs showed stiff walk and lesser activity and on occasions also less appetite. All these dogs belonged to one single trial site. 17% of vaccinated dogs and 2% of placebo dogs showed reactions after the 1st injection including diffuse and soft swelling, a warm injection site, a hardened nodule, pain and a thickened injection. site. After the 2nd injection, 29% vaccinated dogs showed reactions including diffuse and soft swelling, a warm injection site, a hardened nodule, pain and a thickened injection site.

In all cases, reactions appeared to be moderate and transient, in general disappearing within 4 days. In some cases however the reaction after the 2nd injection remained for 14 days.

Conclusion:
The field trial confirmed that the vaccine is safe.

ECOTOXICITY

Investigations of ecotoxicity in the field was not necessary because

- the vaccine contains no infectious particle or dangerous component.
- the administration is individual and done by subcutaneous injection.
- the SPC recommends any unused or waste material to be disposed of by appropriate channels.

Thus, no hazards can be identified.

OVERALL CONCLUSION ON SAFETY

Two safety studies were conducted in the target species, one in the laboratory and one in field conditions, with vaccine batches formulated with SPA produced by 5 x 10^7 erythrocytes infected by *Babesia canis A* and 5 x 10^7 erythrocytes infected by *Babesia rossi*. Dogs of various ages, breeds and from both sex were involved. Impact of the vaccination on pregnancy was not investigated, but vaccination of pregnant bitches is contraindicated in the SPC. Therefore, it can be concluded that the vaccine is safe when administered as described in the SPC.

4. EFFICACY ASSESSMENT

INTRODUCTION AND GENERAL REQUIREMENTS

Considering the data in on "antigen selection", the following additional data provided by the Applicant needs to be taken into consideration:

Clinical disease in *Babesia canis* infection is associated with an effect on the coagulation system, whereas in *Babesia rossi* infection, clinical disease is associated with the exponentially growing peripheral parasitaemia. The coagulation system plays a role in the sequestration of infected erythrocytes in the microvasculature. *Babesia canis*-infected dogs suffer from severe anaemia, which cannot be explained by destruction due to parasite proliferation. It is mainly due to an immediate hypotensive shock-reaction, which leads to attraction of interstitial fluid to the peripheral blood stream, thus actually diluting the peripheral blood. It is also associated with development of splenomegaly.

The main pathological event is obstruction of capillary blood flow (by sequestration of blood cells in the microvasculature) and increase of plasma volume in the main circulation. A series of clinical parameters was defined to more accurately define clinical disease associated with Babesia infection : behaviour, body temperature, colour of mucosa, capillary refill time, lymph node enlargement and splenomegaly.

Protection is related to an effect on the SPA levels in plasma after challenge infection, and not with an effect on peripheral parasitaemia. When using *monovalent* vaccines, no effect on SPA levels of the challenge strain in plasma was found when animals were challenged with heterologous *Babesia canis* parasites; in contrast significant reductions in SPA levels in plasma were found after homologous challenge.

A *bivalent* vaccine based on SPAs of *Babesia canis* and *Babesia rossi* is the only kind of vaccine having an effect on the clinical babesiosis and reducing the extent of anaemia after a heterologous challenge infection. Protective immunity is associated with a reduction of SPA in plasma after experimental heterologous challenge infection.

Based on these observations, efficacy of Nobivac Piro in the laboratory will rely on following parameters:

- Clinical signs post heterologous challenge
- Haematocrit value (Packed Cell Volume) post heterologous challenge
- Parasitaemia post heterologous challenge
- SPA in plasma post heterologous challenge.
-

The rationale of the Applicant and his choice of the 4 parameters was supported. Indeed, literature provides the following information:

1. Clinical signs post challenge

In summary, Babesiosis is a highly variable clinical disease, whose course can be peracute, acute or chronic. Major features are a shock syndrome and an endotoxaemic-like stage (activation of the kinin system inducing vasodilation, hypotension, and increase of the capillary permeability), hyperthermia (almost constant), anaemia, thrombocytopaenia, hemoglobinuria and splenomegaly. Many atypical forms exist.

The variability observed may be attributable to differences in pathogenicity among strains of Babesia, the degree of parasitaemia, the age of the host (susceptibility decreases with age, puppies being very susceptible to *Babesia canis*), or the immunologic response of the host. Incubation period of babesiosis is 10-21 days. During acute course, death is mainly due to respiratory failure, whereas during chronic course, it is mainly due to circulatory failure. Despite the variability, recording of clinical signs remains, however, an important parameter to demonstrate efficacy of a vaccine against Babesiosis.

2. Haematocrit value (Packed Cell Volume) post challenge

Normocytic, non-regenerative anaemia is frequently found during the early stages of *Babesia canis* infection. Regenerative haemolytic anaemia, associated with haemoglobinuria, is generally found in chronic stages. The PCV is an accurate and practical evaluation of RBC status. In normal circumstances between 43.1% and 52.1% in dogs, PCV values in *Babesia canis* infections are influenced by (i) changes in the plasma volume, (ii) retention of erythrocytes in the spleen, (iii) erythrocyte destruction.

(i) changes in the plasma volume

Babesiosis is associated with a shock syndrome, with activation of the kinin system inducing vasodilation and vascular stasis, hypotension, and increase of the capillary permeability. Some strains of *Babesia canis* not only cause vasodilation but also impede the blood flow even further by a clumping of the erythrocytes to each other and to the endothelium of the capillaries. Together with an endotoxaemic-like stage and the obstruction of capillary blood flow (by sequestration of blood cells in the microvasculature), the shock syndrome induces an increase of plasma volume in the main circulation.

(ii) erythrocyte sequestration/retention

Even if part of the reduction in the number of peripheral erythrocytes appears to be due to a shock-like syndrome, decreased PCV values are mainly due to erythrocyte sequestration and blocking of capillaries with subsequent tissue damage. To reduce interactions with the host immune system, Babesia induce adhesion of infected erythrocytes to the capillary endothelium in the deep vasculature, thus avoiding splenic passage. This mechanism, referred to as “cytoadherence” or “cytoadhesion”, results in the sequestration of infected erythrocytes carrying mature stages of the parasite.

Agglutinates of infected and uninfected erythrocytes are retained because of the open-mesh architecture of the spleen, thus resulting in localised Babesia infections. Blocking of the microcirculation in the spleen appears to be the main cause of splenomegaly with associated anaemia.

Less virulent strains are less likely to be sequestered in internal organs and hence by rapid passaging, the selection pressure results in an enrichment of the less virulent forms in circulating blood. This is in agreement with electron microscopic studies, which demonstrated that red cells parasitized by virulent strains of *B. bovis* had altered external membranes, which attached to capillary walls in the brain and kidney resulting in sequestration. Such changes were not demonstrated in infections with attenuated strains.

(iii) erythrocyte destruction

It is difficult to find evidence of direct parasite-induced damage to erythrocyte membranes. Anaemia is due to immunoglobulin and complement mediated destruction of both parasitised and unparasitised erythrocytes, as well as to erythrophagocytosis of these cells in the spleen and liver. In Babesiosis, immune mediated haemolysis, usually mediated by immunoglobulins (IgG or IgM) and/or complement, is essentially secondary (in which the membrane is altered and recognised as “foreign”).

On the contrary, WBC are of little value: the behaviour of leucocytes during Babesia infection varies greatly and it is extremely difficult to discern a common pattern, even when a particular infection is followed in the same host species. Both leucopenia and leucocytosis have been described in *Babesia canis* infections. Leucopenia is caused by neutropenia, lymphopenia and monocytopenia. Basophil rate seems to remain unchanged.

Therefore, PCV post challenge seems to be an adequate parameter to demonstrate efficacy of a vaccine against Babesiosis.

3. Parasitaemia post challenge

Babesia canis have been shown to proliferate in deep tissues. The various immune responses, the destruction and/or the sequestration of parasitised erythrocytes (including in peripheral organs like the brain, bone marrow and kidneys which seem to be privileged sites for parasitised erythrocytes), make parasitaemia quite fluctuant. Thus, protection against clinical disease cannot always be linked to an effect on the peripheral parasitaemia. Parasitemia remains however an important parameter to demonstrate efficacy of a vaccine against Babesiosis.

4. SPA in plasma post challenge

The importance of SPA, which can be considered as a component inducing an endotoxaemic-like stage, was already discussed in chapter A4. Thus, SPA post challenge seems to be an adequate and important parameter to demonstrate efficacy of a vaccine against Babesiosis.

LABORATORY TRIALS

Brief description of the trial design

Male Beagle dogs of 6 months of age were vaccinated SC twice at an interval of 3 weeks with batches of various amounts of antigens, but always reconstituted in the standard amount of adjuvant. Three weeks after the second injection, the dogs were challenged with heterologous *Babesia canis* strain B administered by intravenous route.

Examinations

They were undertaken during the 12 days post challenge, on the following parameters:

Clinical score value after challenge infection: the dogs were examined daily post challenge for clinical signs, focused on behaviour, spleen size, size of lymph nodes, colour of the mucous membranes of mouth and eye-lid; the clinical scoring included the fact that some animals needed to be treated (with Imidocarb).

Parasitaemia after challenge infection. Two parameters were investigated:

- parasitaemia per day: it is expressed as the log number of red blood cells infected with Babesia parasites per 10^5 erythrocytes in the peripheral blood.
- parasitic load : the daily parasitaemia values were added to establish the cumulative parasitaemia over the observation period.

Haematocrit after challenge infection: it is measured through the maximal decrease of Packed Cell Volume (PCV) and is expressed as a percentage of the 100% value obtained at the day of challenge infection.

Soluble Parasite Antigen (SPA). Three parameters were investigated:

- SPA load before and after challenge infection: concentration of SPA in plasma was detected using a sandwich-ELISA technique and expressed as a value relative to the parasitaemia per erythrocyte (SPA/PE).
- cumulative SPA/PE after challenge infection: daily SPA/PE were added to calculate the cumulative SPA/PE.

Antibodies against SPA in plasma against *Babesia canis A* strain B: they were detected using a sandwich-ELISA technique (using Ig anti-*Babesia canis* because *Babesia canis* was the challenge strain) and read through optical densities. Antibody reactivity against SPA is expressed as the percentage reduction of net OD_{450} signal relative to the 100% value.

Cell mediated immunity: it was investigated through stimulation of leucocytes coming from vaccinated dogs with SPA of *Babesia canis* strain B parasites or phytohaemagglutinin. After addition of [^{3}H]-thymidine solution in the medium and radioactivity was measured after 16 hours.

Statistical analysis

Daily clinical score value, haematocrit, SPA load, parasitemia per day, parasitic load, cumulative clinical score value, maximal PCV decrease values: ANOVA; if there is a difference, Duncan's Multiple Range Test.

Results

Clinical score value:
The mean clinical score per day was statistically significantly lower in vaccinated animals with regard to the control group; a peak at 5 days after challenge infection was seen in all vaccinated groups, after which a similar decrease in clinical scores was registered in all of them; in contrast, the score of the control animals increased further on after day 5 to a peak level 10 days after challenge infection; 1 day after (D11), 2 dogs of one of the control groups needed to be treated to control the infection.
There was no dose-effect relationship between the antigen dose and the mean clinical score value.

Parasitaemia after challenge infection:

- Parasitaemia: it rose exponentially in all the groups the first 4 days. The mean peak of control group was reached 5 days after challenge infection, and resolved quickly during the next 2 days. From day 7 onwards, low numbers of parasites were detected in the subsequent time period. Two of the control animals needed to be treated with chemotherapeutics 9 days after challenge to control the infection. Vaccinated animals showed a pattern similar to the control animals.
- Parasitic load: the increase of the parasitic load over time was reduced in the groups A and C. In the latter group this appeared to be mainly affected by a single dog that showed an extremely low peripheral parasitaemia. When the data of this dog were excluded, the parasite load approached that of group B.

Haematocrit after challenge infection:
PCV of all groups decreased immediately after infection. From day 5/6 after infection onwards, the mean PCV of the vaccinated groups did not decrease any further, but in the control group it dropped further for another 2 to 3 days.
A negative correlation was seen between the antigen dose and the maximal decrease of PCV, the higher the antigen dose the less the decrease of PCV value.

SPA

- SPA load before challenge: there was no dose-effect relationship between the antigen dose and the SPA load.
- SPA load after challenge (relative amount of SPA per infected erythrocyte):
  The amount of SPA in plasma rose exponentially in all groups the first 4 days, but was higher in the control group as compared to the vaccinated groups. Moreover, the amount of SPA in plasma decreased earlier and at a higher rate for the vaccinates. The increase of the SPA load over time was most reduced in groups B and C.
- Cumulative SPA/PE after challenge:
  The cumulative SPA/PE value of group A was higher than that of the groups B and C, but not different from group D. Groups B and C showed a very similar pattern.

Antibodies against SPA of *Babesia canis A* strain B:
During the first 4 days after infection there was a drop in the level of antibodies against SPA of the challenge parasite, which was most pronounced in samples from the control group. From that day onwards antibody titres increased, with a higher rate in the groups B and C.

Lymphocytes stimulation:
There was a dose-effect relationship between the reactivity of the cells and the antigen dose. However, not all dogs within each group reacted similarly and non-responders were seen in each group: 4 dogs of group A responded to SPA from the challenge parasite, 3 dogs from group B, and none from group C. Surprisingly, one animal from the control group did have reactive lymphocytes.

This trial confirmed that a vaccine based on SPA from two different Babesia parasites induces protection against heterologous challenge infection.

On the most relevant parameter for dog owners, i.e. clinical signs, no difference between the 1/5, 1 and 5 times dose level can be observed. In all 3 dosage levels a significant protection against clinical signs was observed.

From the secondary parameters which were looked at, it can be concluded that a clear dose-response effect exist for the lymphocyte stimulation, the antibody response post vaccination and the decrease of PCV. The latter one should be considered as the most important one because it is a direct reflection of the hypotensive shock-reaction: the shock-reaction leads to an attraction of interstitial fluid to the peripheral blood stream, thereby diluting the peripheral blood. This dilution is detected by a decrease in PCV. Thus PCV is the most direct and sensitive parameter to test the protective effect of vaccination against hypotensive shock, which is known to be due to SPA. On this basis, it can be concluded that the level of protection is related to the antigen dose.

Apparently, dogs are able to compensate sufficiently at the beginning of the disease course, with the result that the differences seen in the secondary parameters do not lead to any difference in the primary parameter i.e. clinical signs after infection.

The only secondary parameter, which did not follow this dose-response effect is the SPA produced per infected erythrocyte, but the 1-dose group provides the best results. The animals vaccinated with the highest antigen had significantly reduced parasite load after challenge infection while there was no apparent effect on the amount of SPA produced per infected erythrocyte. In contrast, the group of animals that was vaccinated with only 1/5 dose of antigen showed immediately after challenge infection reduced levels of SPA in plasma while there was no significant reduction of parasite proliferation.

Taking together all the data provided, the group of animals vaccinated with a single dose of antigen takes an intermediate position, combining the best of all responses.

Conclusion
The results show that dogs vaccinated with different doses of Nobivac Piro antigen (within a range of five-fold to one-fifth dose of antigen) are protected against the development of clinical signs upon challenge infection with *Babesia canis* parasites.

Definition of a validated serological marker for Nobivac Piro

Brief description of the trial design
Blood samples were taken from the dogs used in the previous trial, 2 weeks after the first injection and just before challenge (i.e. 3 weeks after the second injection).

Examinations
Antibody titres were determined through ELISA techniques and the overall antibody titre was calculated as follows:
Overall antibody titre = (antibody titre *Babesia canis A* x antibody titre *Babesia rossi*)$^{1/2}$

The following relationships were investigated:

- Relationship between the antigen dose and the maximal decrease of PCV (%) after challenge infection.
- Relationship between the antigen dose and the antibody titres 2 weeks after the first injection or 3 weeks after the second injection.

Results
The extent of anaemia (PCV) is dependent on the antigen dose: the higher the antigen dose, the less the extent of anaemia after challenge infection.

The antibody titre (at 2 weeks) is dependent on the antigen dose: the higher the antigen dose, the higher the antibody titre; such a correlation was not found with antibody titre at 3 weeks after second injection.

Thus, following correlation between antibody titre at 2 weeks and Maximal decrease of PCV (%) could be established, showing a r2 of 0.929: the higher the antibody titre, the less the decrease of PCV value. No correlation could be established between antibody titre just before challenge and maximal decrease of PCV (%).

Immunity to Babesia is mediated by both innate and adaptive immune mechanisms, the later being cell-mediated and humoral. Protection against babesial infections through the humoral component of the immune system is achieved in various manners. Serologically detectable antibodies may not necessarily confer protective immunity, even if antibodies do play an important role in protection. Indeed, literature mentions that:

- protective immunity can be passaged by serum or colostrum collected from immune animals (shown for bovine babesiosis).
- when the infection from a tick first occurs, IgG antibodies can prevent infection by binding and neutralising sporozoites before they succeed in invading their target cells. It had also been demonstrated that antibodies in the serum neutralise babesial sporozoites or merozoites at the extracellular stage. Therefore the protective role of antibodies seems to be restricted to a short window of time between the moment that the parasite gains access to the bloodstream and the time that it invades the target cells.
- infected erythrocytes incorporate Babesia antigens into their membranes. These in turn induce antibodies that opsonize the red cells and lead to their removal by the mononuclear-phagocytic system (mainly macrophages and cytotoxic lymphocytes).
- the protective activity is partially the result of a concerted action of antibody responses that limit parasite proliferation and responses that neutralise the pathological effect of SPA. Indeed:
- control of Babesia infection is due, amongst other mechanisms, to immunoglobulin and complement mediated destruction of both parasitised and unparasitised erythrocytes (IgG2a and IgM were specifically identified).
- in vaccinated animals, recovery from infection is associated with the production of anti-SPA antibodies. The blood level of SPA is not only dependent on the number of parasites actually present in the animal, but also on the level of antibodies against SPA.

It remains however that the humoral component of the immune system is currently considered of limited importance in protection against babesial infections. In some cases, it was also shown that the frequency of recurrence of the disease is linked to the antibody response: recurrence is more frequent in middle or high responders.
The situation we are facing here is very different:

- firstly because the antibodies which are measured are antibodies against SPA, and it was shown that SPA directly correlates with protection.
- secondly because PCV is the most direct and sensitive parameter to demonstrate protection (as already explained). At all three dose-levels a quantitative relationship between the antigen dose and PCV on one hand, and between the antigen dose and the antibody titre on the other hand, was established, and thus also between the antibody titre and protection.

So, despite the fact that literature is very controversial about the protective role of antibodies, it appears that in the present situation, the global antibody titre correlates well with protection, and in a coherent way. An antibody titre of ≥ 1.3 $\log_2$ can thus be considered as protective (to be used in the field trial – see later on).

<u>Conclusion</u>
PCV being correlated to antigen dose and antigen dose being correlated to antibody titre (at 2 weeks), thus PCV (which is a measure for the protective effect of vaccination) is correlated to antibody titre (at 2

weeks): the higher the antibody titre, the higher the protection. The calculated overall antibody titre in serum samples taken two weeks after priming could be used as a validated serological marker of a particular batch of vaccine. An antibody titre of ≥ 1.3 $\log_2$, corresponding to the mean titre obtained with a single dose, can be considered as protective.

Duration of immunity

Brief description of the trial design
Beagle dogs of 6 months of age were split into 4 groups. The first group (A) was vaccinated with Nobivac Piro at day 0, week 6 and was challenged in week 26. The second group (B) was vaccinated with Nobivac Piro at day 0, week 6, week 32 and was challenged in week 58. The third (C) and fourth group (D) were not vaccinated and were challenged in week 26 and 58 respectively. The challenge strain was *Babesia canis* strain B.

Examinations
Examinations were undertaken during the 12 days post challenge, on the following parameters:
Clinical score value after challenge infection: the dogs were examined daily post challenge for clinical signs, focused on behaviour, spleen size, size of lymph nodes, colour of mucous membranes of mouth and eye-lid, capillary refill time. A total clinical score was calculated per day.
Haematocrit after challenge infection: it is measured through the maximal decrease of Packed Cell Volume (PCV) and is expressed as a percentage of the 100% value obtained at the day of challenge infection.
Parasitaemia after challenge infection. Two parameters were investigated:

- Parasitaemia per day: it is expressed as the log number of red blood cells infected with Babesia parasites per 10^5 erythrocytes in the peripheral blood.
- Parasitic load: the daily parasitaemia values were added to establish the cumulative parasitaemia over the observation period.

Soluble Parasite Antigen (SPA). Three parameters were investigated:

- SPA load before and after challenge infection: concentration of SPA in plasma was detected using a sandwich-ELISA technique and expressed as a value relative to the parasitaemia per erythrocyte (SPA/PE).
- Cumulative SPA/PE after challenge infection: daily SPA/PE were added to calculate the cumulative SPA/PE.

Statistical analysis
Score values (total, cumulative, daily), maximal decrease of PCV, cumulative parasitaemia: ANOVA

Results
One dog of group A was excluded from the trial because it developed an atypical hyperacute form of the disease.

Comparison of group A versus group C

- Clinical signs: mean clinical score value started to increase in both groups from day 3 onwards and was similar for both groups during the first 5 days after infection. The clinical score values of group A peaked at days 7-8 after infection, one day earlier and at a lower level than for group C. Days 9-10 after infection, mean value of group A was statistically significantly lower than that of group C. Day 7 after challenge onwards, development of further disease was controlled in group A. Total clinical score was statistically significantly different between both groups at day 12 after infection, in favour of group A.
- Haematocrit: PCV of both groups started to decrease immediately after infection and evolution was similar for both groups during the first 8-9 days after infection. Mean PCV value of group A started to increase from day 9 onwards, 2 days earlier than that of group C. In the period between days 10-14 after challenge infection, mean value of group A was statistically significantly higher

than that of group C. Maximal decrease of PCV value was statistically significantly different between groups, in favour of group A.

- Parasitaemia: it remained very similar between groups. No clear peak could be seen, and parasitaemia decreased in both groups from day 8 after infection onwards. Cumulative parasitaemia at the end of the observation period was not significantly different between both groups.
- SPA: it was detected from day 2 after challenge infection onwards. Dynamics of SPA levels in both groups was similar but mean SPA level of group A was lower than that of group C. Cumulative SPA at the end of the observation period was significantly lower in group A.

Comparison of group B versus group D

- Clinical signs: mean clinical score value started to increase in both groups from day 3 onwards and was similar for both groups during the first 6 days after infection. The clinical score value of group B peaked at days 7-8 after infection, one day earlier and at a lower level than for group D. Maximal clinical score value was statistically significantly lower in group B with regard to group D. Day 9 after infection onwards, mean value of group B was statistically significantly lower than that of group D. Day 7 after challenge onwards, development of further disease was controlled in group B. Total clinical score of both groups was statistically significantly different at day 12 after infection, in favour of group B.
- Haematocrit: PCV of both groups started to decrease immediately after infection and evolution was similar for both groups during the first 3 days after infection. Mean PCV values of group B between days 3 and 8 show that the disease appeared to be controlled, and they started to increase from day 8 onwards. Three dogs of group D were excluded on day 8 because they needed to be treated. PCV values of the 2 remaining dogs of group D started to increase also from day 8 onwards. Maximal decrease of PCV value was statistically significantly different between groups, in favour of group B.
- Parasitaemia: it remained very similar between groups. No clear peak could be seen, and parasitaemia decreased in both groups from day 8 after infection onwards. Cumulative parasitaemia at the end of the observation period was not significantly different between both groups.
- SPA: it was detected from day 2 after challenge infection onwards. Mean SPA level of group B was lower over the entire experimental period compared to that of group D. Cumulative SPA at the end of the observation period was significantly lower in group B.

The vaccination scheme as given by the Applicant (with a revaccination after 6 months) can be accepted: with regard to the main parameters (clinical scoring, SPA and PCV), the results show that dogs of group A were protected 20 weeks after the second injection of the primary vaccination, and that a single injection at revaccination protects dogs of group B for at least 26 weeks. Parasitaemia seems to be under control in the vaccinates, but it appears that there is no complete clearance of the challenge parasite amongst the vaccinates. A carrier status could ensue, with dogs harbouring parasites but resistant to further challenges.

Conclusion

For a Babesia vaccine to be effective, vaccination should provide protection for about 4-5 months, the duration of a seasonal wave of babesiosis. Result show that 6 months after initial vaccination or re-vaccination, dogs effectively limit the level of SPA in plasma upon challenge infection, which is reflected in limited duration and extent of clinical manifestations. It was further shown that the level of immunity of primary vaccinated dogs and that of repeatedly vaccinated dogs was comparable. Thus, it was concluded that vaccination with Nobivac Piro induces protective immunity against clinical babesiosis for a period of 6 months, and that a single dose at revaccination is sufficient.

FIELD TRIALS

Brief description of the trial design :

Dogs of at least 6 months of age and of various breeds, from 5 different sites, were used. Pregnant bitches were excluded from the trial. At each location, dogs were injected either with Nobivac Piro or with a saline solution, twice SC with an interval of 4 weeks between each injection. Treatments were randomly distributed and conducted in a double blind manner.

Examinations
Comparison of both groups was done on following parameter: the proportion of dogs showing seroconversion to *Babesia canis* and *Babesia rossi* at 0 and 2 weeks after the 1st vaccination was determined. Seroconversion was defined as a mean geometric titre ≥ 1.3 $\log_2$.

Statistical analysis
Antibody titres: ANOVA
Proportion of dogs showing seroconversion: Fisher's exact test

Results
Seroconversion (defined as an increase in total antibody titre of ≥ 1.3 $\log_2$) was seen in all but one vaccinated dogs and in none of the placebo dogs.
Antibody titres correlated correctly with protection. The field trial confirmed the results seen in the laboratory trials, as all but one vaccinate seroconverted.

ADDITIONAL INFORMATION

Relevance of the challenge strain

Numerous Babesiosis strains were analysed. Information on immunogenic relationship and protection was submitted. It was concluded that the choice of *Babesia canis* B as a challenge strain is relevant.

OVERALL CONCLUSION ON EFFICACY

Demonstration of efficacy is based on a laboratory model, whose relevance is well demonstrated, and supported by a field trial. Efficacy was shown with vaccine batches always formulated with SPA produced by 5 x 10^7 erythrocytes infected by *Babesia canis A* and 5 x 10^7 erythrocytes infected by *Babesia rossi*. Relevance of the challenge strain is documented. An onset of immunity of 3 weeks is supported by report 97R/0209, which shows that dogs were protected when challenged 3 weeks after the primary injection. Report 99R/0821 validates the duration of immunity of 6 months.

Therefore, it can be concluded that the vaccine is efficacious when administered as described in the SPC.

5. RISK BENEFIT ASSESSMENT

Nobivac Piro is a vaccine intended to protect against canine babesiosis. It is based on original research on Babesia disease in dogs, showing that soluble parasite antigens produced by Babesia parasites may induce protective immunity, but only under special conditions. It is composed of a lyophilisate and a solvent. The lyophilisate contains a soluble parasite antigen from *Babesia canis A* and *Babesia rossi* cultures. The solvent contains saponin as adjuvant.

The analytical part is well documented. The vaccine is produced by conventional methods of manufacture and the process is validated. Appropriate tests are conducted on the finished product to ensure that a product of consistent quality is produced. A shelf life of 24 months for the product is supported by stability data provided.

The production and control of starting materials follows the recommendations of the EU note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMEA/410/01-Rev.1).

Two safety studies were conducted in dogs, a laboratory study and a field study, with vaccine batches always formulated with SPA produced by 5×10^7 erythrocytes infected by *Babesia canis A* and 5×10^7 erythrocytes infected by *Babesia rossi*. Dogs of various ages, breeds and from both sex were involved. Impact of the vaccination on pregnancy was not investigated, but vaccination of pregnant or lactating bitches is contraindicated in the SPC. It is concluded that the vaccine is safe when administered as described in the SPC.

Demonstration of efficacy was based on a laboratory model, whose relevance is well demonstrated, and supported by a field trial. Efficacy was shown with vaccine batches always formulated with SPA produced by 5×10^7 erythrocytes infected by *Babesia canis A* and 5×10^7 erythrocytes infected by *Babesia rossi*. Relevance of the challenge strain is documented. An onset of immunity of 3 weeks was supported, which showed that dogs were protected when challenged 3 weeks after the primary injection. The duration of immunity was proven for 6 months.

It is concluded that the vaccine is efficacious when administered as described in the SPC.

Based on the original and complementary data presented, the Committee for Medicinal Products for Veterinary Use concluded that the quality, safety and efficacy of the product were considered to be in accordance with the requirements of Council Directive 2001/82/EEC.