EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

4 April 2011
EMA/296057/2010
Veterinary Medicines and Product Data Management

Scientific discussion

This module reflects the initial scientific discussion for the approval of CaniLeish (as published in April 2011). For information on changes after this date please refer to module 8.

1. Summary of the dossier

CaniLeish is a lyophilisate and solvent for suspension for injection, intended for the active immunisation of Leishmania negative dogs from 6 months of age to reduce the risk to develop an active infection and clinical disease after contact with *Leishmania infantum*.

The active substance of CaniLeish is *Leishmania infantum* excreted secreted proteins (ESP).

CaniLeish was eligible for the submission of a dossier for granting of a Community marketing authorisation via the centralised procedure under Article 3 (2) (a) of Regulation (EC) No 726/2004 which refers to medicinal products intended for use in animals containing a new active substance which was not authorised in the Community. The Committee also confirmed that the requirements for veterinary products intended for Minor Use or Minor Markets (MUMS) were met and therefore the provisions of the relevant guideline were applicable for this application.

No specific inspection was considered necessary with regard to CaniLeish. The presented pharmacovigilance system was considered satisfactory.

The benefits of CaniLeish are the stimulation of active immunity in Leishmania negative dogs from 6 months of age to reduce the risk to develop an active infection and clinical disease after contact with *Leishmania infantum*. The onset of immunity is 4 weeks after the primary vaccination course and the duration of immunity is 1 year after the last (re-)vaccination.

The most common side effects are moderate and transient local reactions that may occur after injection such as swelling, nodule, pain on palpation or erythema. These reactions resolve spontaneously within 2 to 15 days. Other transient signs commonly seen following vaccination may be observed such as hyperthermia, apathy and digestive disorders lasting 1 to 6 days. Allergic-type reactions are uncommon and appropriate symptomatic treatment should then be administered.

Canine leishmaniosis is a widespread infectious disease in endemic areas of the Mediterranean basin, Asia and America. This is a zoonosis considered as a serious veterinary problem with an increasing impact on public health. The disease is due to the development and multiplication in the macrophages and mononuclear cells of a protozoan parasite - *Leishmania infantum*. The infected dogs constitute the main domestic reservoir and play a central role in the accidental transmission of parasites to humans. The parasite is transmitted from an infected dog to a non-infected dog by the bites of sandflies of the

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genus *Phlebotomus*. The outcome of the infection is highly variable. Infected dogs may develop symptomatic infection resulting in death if not treated or develop only one or many mild symptoms but a high percentage of infected animals remain asymptomatic.

List of main abbreviations used frequently through the scientific discussion

BSA: Bovine Serum Albumin

Cv: coefficient pf variation (validation of methods)

CMLA: canine macrophage leishmanicidal activity

Con A: Concanavalin A

DTH: Delayed Type Hypersensitivity

ESP: Excreted Secreted Proteins

IFA: indirect fluorescent antibody test

IgG: Immunoglobulin G

LIP: *Leishmania infantum* promastigotes

IFN-γ: Interferon gamma

IL: Interleukin

LLT: Lymphoblastic Transformation test

NNN medium: Novy - Nicolle – Mac Neal medium

Ph. Eur: European Pharmacopeia

PSA: Promastigote Surface Antigens

SC: Subcutaneous

Sd: Standard deviation (validation of methods)

SLA: Soluble leishmania antigens

SPC: Summary of Product Characteristics

Th1: Type 1 T helper cell (lymphocyte)

TSE: Transmissible spongiform encephalopathy

2. Quality assessment

Qualitative and quantitative particulars of the constituents

Each dose of 1 ml of vaccine contains the following:

Freeze-dried fraction:

	Substances	**Quantity per dose**	**Function /Specification reference**
Active ingredient	Excreted-Secreted proteins (ESP)	Not less than 100 µg	Active substance – Virbac specification
Adjuvant	*Quillaja saponaria* purified extract		Adjuvant – Virbac specification
Excipients	mannitol sucrose Trometamol		Bulking agent – Ph. Eur 0559 Lyoprotectant – Ph. Eur 0204 Buffering agent – Ph. Eur 1053

Liquid fraction: for 1 ml of solvent

	Substances	**Quantity per dose**	**Function / reference**
Excipients	Sodium chloride Water for injection	9 mg Qs 1 ml	Stabilizer – Ph. Eur 0193 Diluents – Ph. Eur 0169

The active substance Excreted-Secreted Proteins are constituted of parasitic proteins that are characterised by a defined protein pattern. The quantitative formulation of the vaccine relies on a quantification of the total protein content by a non-specific test. Among these proteins, some antigens have a major role for induction of immunity.

In addition to the quantitative test, other tests (based on proteomic analyses) were developed to appreciate the quality of the parasitic proteins. These tests confirmed the representativeness of the protein patterns obtained at the end of the production process.

Containers

Freeze-dried fraction: A 3 ml insulin type vial made of neutral borosilicate type I glass is used (Ph. Eur. 3.2.1.) and sealed with a buthyl elastomer rubber lyophilisation stopper and an aluminium cap.

Liquid fraction (solvent): A 3 ml insulin type vial made of neutral borosilicate type I glass is used (Ph. Eur 3.2.1.) and sealed with a buthyl elastomer rubber stopper and an aluminium cap.

Treatment:

Vials: dry heat sterilization for sterilization and depyrogenation is implemented (according to Ph. Eur. 5.1.1)

Stoppers: autoclaving takes place.

Filling and stopping are conducted under a class A environment.

The certificates of controls conducted were provided and were acceptable.

Development Pharmaceutics

Canine leishmaniosis is a widespread infectious disease in endemic areas of the Mediterranean basin, Asia and America and is a zoonosis considered as a serious veterinary problem with an increasing impact on public health. The disease is due to a protozoan parasite - *Leishmania infantum* (= *Leishmania chagasi* in South America). The infected dogs constitute the main domestic reservoir and play a central role in the transmission of parasites to humans. The parasite is transmitted from an infected dog to a non-infected dog by the bites of sandflies of the genus *Phlebotomus*.

In the sandfly (vector), the parasites exist as multiplicative procyclic promastigotes and infective metacyclic promastigotes (after differentiation in the digestive tract). After transmission to the mammalian host, through the bite of infected sandflies, the parasites enter into macrophages. They persist as intracellular amastigotes living predominantly in the phagolysosome of macrophages. After initial infection, amastigotes may replicate some time before triggering an inflammatory and adaptative immune response.

Preliminary research on the vaccine – concept and its validity

Research initiated by the Institut de Recherche pour le Développement (IRD) – Montpellier – France focused on Excreted Secreted Proteins (ESP). These proteins have been identified in several protozoan parasites and have been characterised by critical functions during the parasite cycle (implication in host cell interaction, intracellular parasitic development, modulation of the host immune response).

A serum-free medium was patented. This well defined medium allows growth and maintenance of *L. infantum* being free of cells, serum, macromolecules (proteins, nucleic acids) and peptides. From this medium, the culture supernatant containing *L. infantum* Excreted Secreted Proteins (ESP), released by the parasite during its growth, is isolated. The only proteins contained in the medium are parasitic and are found with their native conformation because they are naturally excreted-secreted by Leishmania parasites.

All the development of this vaccine and the production process rely on the cultivation medium for *Leishmania infantum* that is well defined and is free from cells, macromolecules and peptides. Successive steps during the process will allow from cultures of *Leishmania infantum* to specifically recover the proteins that are excreted and secreted by *Leishmania infantum* during the culture stage and to remove parasites. These excreted secreted proteins are the active ingredient.

The active substance, composed of parasitic proteins, is submitted to different testings that will guarantee their presence, their identity and their amount (total amount and also relative amount of the different proteins).

Active ingredient:

Data were provided from initial studies performed to validate the concept of vaccination and use of ESP; they are also described under the safety and efficacy parts of the report and investigated the following:

- protection against experimental visceral Leishmaniasis in dogs immunized with purified excreted secreted antigens of *Leishmania infantum* promastigotes
- long-lasting protection against canine visceral Leishmaniasis using a *Leishmania infantum* Excreted Secreted Proteins – Muramyldipeptide vaccine in endemic areas of France : Double-blind randomized efficacy trial

A field study which included a high number of animals demonstrated better a significant protective effect of the vaccination and data indicated an association between cellular immunity, Th1 response and protection whereas disease is linked with high levels of total IgG antibodies.

Vaccine formulation

Adjuvant – nature and quantity

The *Quillaja saponaria* purified extract was selected as adjuvant as it is known to have immuno stimulant properties and as it induces a strong type-1 immune cellular responses especially mediated by the cytokines interferon-gamma and, interleukin 2, and cytotoxic T-lymphocyte responses. These responses were considered relevant for the development of an effective vaccine against leishmaniosis. The dose of the adjuvant in the vaccine was adjusted according to preliminary safety and efficacy data that were presented in a number of studies.

Pharmaceutical form

A lyophilized formulation was chosen to avoid chemical and physical stresses on proteins and undesirable degradations. Appropriate buffer and lyophilisation conditions were defined.

Analytical methods development

All these methods were described in detail and validations were provided.

Quantification of ESP - active substance bulk and finished product

An adapted colorimetric method is used.

Purity of ESP - active substance bulk and finished product

An adapted electrophoresis method is used.

Identification of ESP - active substance and finished product

An adapted Western blotting technique is used.

Potency test – finished product

A validated potency test based on demonstration of a specific and adapted immune response is used.

It was considered relevant as it measures an immune response that could be correlated with the one obtained in dogs and it is as well appropriate to determine if a new vaccine batch is able to induce an immune cellular response on dogs.

Therefore several tests have been developed and validated to characterise and control the active ingredient of this vaccine:

- one test quantifies the total proteins amount (non specific protein assay). This test specifically quantifies the ESP as they are the sole proteins recovered from the production process,
- one test validates the purity of the proteins (typical pattern – qualitative and semi-quantitative evaluation)
- and one test specifically identifies the active ingredient (revelation of the presence of PSA, a major antigen of ESP).

In the finished product, an *in vivo* potency test has been developed to evaluate the activity of the vaccine and in particular its ability to induce a typical immune response.

Composition of the vaccine batches used for safety and efficacy studies

The formulation of the vaccine based on a quantification of the total amount of proteins relies on a fixed target dose. For development studies of the vaccine, some vaccines were formulated above the target dose for safety assessment purposes and some vaccines were formulated below the target dose for efficacy assessment purposes. The above were acceptable.

Method of manufacture

Flow charts of the production processes and steps were provided.

Lyophilisate

In the first phase the ESP active substance is produced after culture of the *Leishmania infantum*. In the second phase the final product is formulated / manufactured. The formulation is based on fixed antigen content per dose and a fixed amount of adjuvant. Constituents of the excipients and adjuvant are weighed, dissolved in water and sterilised. The *L. infantum* ESP active substance is added under stirring and sterile conditions. The pH of the excipient and adjuvant fraction is adjusted if necessary. After formulation, the product is filled, freeze-dried and packaged. The sealed vials are stored at 5+/-3 °C in a cold room until controls and release.

Liquid fraction (solvent)

This phase starts with the preparation of the vials and stoppers for liquid preparation, the production of the diluent, then sodium chloride powder is weighed, dissolved in water for injection, and sterilised. Then diluents bottles are filled, stopped and sealed and the sealed vials are autoclaved.

Validations of the production process

The key stage in the production of the active substance is the elimination of the biomass. The quantity of antigen is clearly targeted and defined. The addition of the adjuvant is ensured by a manufacturing process under GMP conditions and by reviewing batch records of vaccines. The freeze-drying process is pre-set and automatically triggered. All the parameters are checked and recorded. A number of validation studies were provided and confirmed the consistency of production.

The validation of the whole production of the active substance ESP was supported by the results of two consecutive batches produced using the described process. Data demonstrated the reproducibility of the vaccine production.

Control of starting materials

Starting materials listed in a pharmacopoeia

Dimethyl sulfoxide, hydrochloric acid concentrated mannitol, sodium chloride, sodium hydrogen carbonate, sodium hydroxide, sucrose, trometamol, highly purified water and water for injection. For all the above materials certificate of analyses were provided and found acceptable.

Starting materials not listed in a pharmacopoeia

Starting materials of biological origin

Description of starting materials of biological origin

***Active substance: Leishmania infantum* – origin and history**

Origin: isolated from a man in Morocco in 1967.

History: the strain was adapted to aseric medium and parasites were selected and cloned using defined media. The resulting parasite strain thus originates from the *Leishmania infantum* reference strain and was used for the construction of the seed lot system.

Master seed

Three amplifications were performed on defined and aseric medium. A cryopreservant was added on the last harvest before storage in liquid nitrogen.

Working seed

The working seed comprises the master seed undergone a few passages. The amplifications were performed on defined and aseric medium. A cryopreservant was added on the last harvest before storage in liquid nitrogen.

Controls

The following controls are preformed on the master and working seed: identity, purity, stability after passages and research for extraneous agents.

The absence of extraneous viruses was investigated in the seeds (Master seeds and working seeds) based on the Ph. Eur. monograph 062. All tests produced satisfactory results.

Hemin chloride

Use: growth factor in the culture media

Source/origin: porcine origin – animals from the Netherlands subject to ante and post-mortem examination.

Controls were considered adequate taking into account the suppliers documentation on origin/source, the gamma irradiation certificate, the tests of sterility, growing capacity, physical and chemical characteristics, assay. In order to further guarantee the absence of risk of transmission of extraneous agents through the use of porcin hemin on the vaccine production process, the applicant:

- implemented a systematic control of extraneous viruses in this raw material before irradiation treatment
- provided a validation of the irradiation method to inactivate viruses
- provided a validation of the viral clearance efficacy of the dissolution of hemin chloride in sodium hydroxide 1M for storage before its use in the vaccine production.

Based on the above data, the applicant conducted a risk assessment, which was acceptable and justified that the risk of transmission of extraneous agents through the use of hemin is close to nil.

Purified extract of *Quillaja saponaria*

Use: adjuvant

Source/origin: vegetal origin

Controls were considered adequate taking into account the supplier's documentation and identification by liquid chromatography. Amongst the tests performed on adjuvant by the supplier, there is testing of the haemolytic activity and HPLC profile.

Viral risk assessment

A detailed risk assessment was presented in compliance with Ph. Eur. 5.2.5. and Ph. Eur. 5.1.7 requirements.

Considering:

- the extraneous agent testing performed on the seed lot, manufacturing process including dilutions in aseric and defined culture media,
- the use of only one starting material of biological origin (hemin chloride from porcine origin) which is carefully sourced and undergoes drastic production process as well as irradiation
- absence of use of cells or substrates that could propagate hypothetical viral infectivity,

It can be concluded that the risk of transmitting extraneous agents through the use of this vaccine is close to nil.

Starting materials of non biological origin

Starting material purchased from defined suppliers

These are powder media used as components of the *Leishmania infantum* culture medium. Detailed composition was provided. These media are composed of aminoacids, vitamins and other components (salts, sugars, nucleotides) all from vegetable, mineral, yeast or chemical origin.

In-house media

- *Leishmania infantum* parasites (LIP) culture medium
- Freeze-drying excipient: Composition: sucrose , mannitol, trometamol, water for injection

Specific measures concerning the prevention of the transmission of animal spongiform encephalopathy

A detailed risk assessment was presented in accordance with Ph. Eur. monograph 1483 and existing guidance documents.

Considering:

- that seed materials are prepared in aseric and axenic media and knowing that TSE infectivity is recognised to be established and maintained in vitro with high difficulty and only with cells of neural origin,
- the absence of use of serum and starting material of TSE susceptible species for the manufacture of the vaccine,
- the indication of the vaccine for dogs, which are not susceptible to TSE by subcutaneous route

It can be concluded that the risk of transmitting TSE agents through the use of this vaccine is nil.

On the basis of the above the CVMP concluded that the starting materials of animal origin used in the production of the final product comply with the current regulatory texts related to the TSE Note for Guidance (EMEA/410/01-Rev.2) and Commission Directive 1999/104/EEC.

Control tests during the manufacturing process

Control tests during the manufacturing process of the freeze-dried fraction

- Controls are performed on cultures of *Leishmania infantum*: Aspect of the culture, mobility, purity, numeration
- Controls performed on the ESP fraction of *L. Infantum:* Identification, sterility - absence of bacteria and fungi, purity protein content

Validation of the methods of control of the ESP concentrated fraction

Identification method - Western blot analysis

A validation study was presented for the identification method by Western blot analysis. The method was validated in view of its specificity i.e. univocal identification of the ESP of *L. infantum* when associated to other components and differentiation from components of close structure.

Quantification of the active ingredient - Activity protein content using Bradford test

This method allows a non specific quantification of all proteins contained in the test samples.

A validation study regarding the protein assay method for the active substance "ESP fraction" was presented. The study investigated the specificity, linearity, accuracy, precision and range of the method. Data confirmed the validation of the method with an observed variability acceptable for this kind of method.

Purity control – Protein profile of the ESP by fluorescent staining

Method: Proteins contained in the ESP bulk or finished product are separated by electrophoresis on under reducing conditions according to their molecular weight expressed. A study was provided regarding the validation of the purity control.

Sterility testing of ESP concentrated fraction
Method: Ph. Eur. 2.6.1. membrane filtration method.

A validation study of the sterility test on active substance "ESP fraction" was provided.

Controls during production of bulk vaccine:

Water intake, pH of excipient, osmolarity of excipient, filters integrity, stirring, pH of the bulk.

Controls during filling, freeze-drying, packaging:

Controls of washing and sterilisation of the vials, controls of the sterilisation of stoppers, control of the volume, control of the freeze-drying process.

Control tests performed during the manufacturing process of the liquid fraction (solvent):

Controls of washing and sterilisation of the vials, controls of the sterilisation of stoppers, filter integrity, control of the filling volume, control of the stopper sterilisation cycle, control of the sterilisation cycle of filled vials.

Control tests on the finished product

Freeze-dried fraction

General characteristics of the finished product:
Appearance, pH, solubility.

Identification of active substance(s):
Identification of the ESP by Western blot analysis.

The validation of the identification method was presented. Data demonstrated that the method is appropriate to identify unequivocally the ESP of *L. infantum* contained in the ESP fraction as well as in CaniLeish vaccine without any interaction with the other components (excipient and adjuvant) or with components of close structure. This was considered acceptable.

Batch titre or potency

a) Protein assay: The protein content in the finished product is determined using an adapted protein assay method. A validation of the protein assay method for the vaccine was presented and investigated the specificity, linearity and precision of the method. The method used to formulate the vaccine is also validated to control the protein content, i.e. the antigenic content of the finished product. It is established that the only proteins titrated with the assay are active substances as no components from culture medium or excipient or adjuvant interact with this assay.

Result of this test are regarded together with the results of the identification method that confirms the identity of active substance and also the purity assay that confirms the presence of all the proteins with comparison to reference and their relative amounts. All these 3 tests allow the characterisation of the active substance and confirmation of its conformity.

b) Potency Test: The potency test validates the immune activity of the vaccine. A validation of the potency test was presented. This test allows observation of the activity of the vaccine in term of induction of immune response and confirms the adequate formulation, in particular in the presence of the adjuvant for which there is no specific test available for its quantification in the finished product.

Identification and assay of the adjuvants:

No test is performed for the identification of the adjuvants.

The vaccine is formulated with a fixed amount of highly purified extract of *Quillaja saponaria*. The manufacturing process performed under GMP conditions and the review of batch records ensure the presence of a standardized and well controlled amount of purified extract of *Quillaja saponaria* in the vaccine. Data of the potency test showed that the test would be able to detect a vaccine without adjuvant (within the limit of precision of the assay).

Identification and assay of excipients components:

Not applicable.

Safety tests:

Target animal batch safety test: In accordance with Ph. Eur. 5.2.9. requirements.

Sterility and purity tests:

a) Purity: The electrophoretic profile of the ESP is tested. The method and its validation were presented.

b) Sterility: The bacterial and fungal sterility is tested by membrane filtration. The method is in accordance with the Ph Eur. 2.6.1. membrane filtration method. A validation report was presented for the method. The method provides evidence that the reconstituted vaccine does not show any antimicrobial activity under the conditions of the test.

Residual humidity:

Residual humidity testing the loss on drying method (in accordance with Ph. Eur. 2.2.32).

Inactivation:

Not relevant.

Liquid fraction (Solvent)

General characteristics of the finished product:

Appearance, volume, osmolarity.

Identification of active substance(s):

Not applicable.

Batch titre or potency:

Not applicable.

Identification and assay of the adjuvants:

Not relevant.

Identification and assay of excipients components:

In line with the physico-chemical characteristics (appearance, volume, osmolarity).

Safety tests:

Similar as the described safety tests on the freeze-dried fraction. Safety of the vaccine is tested after reconstitution of the freeze-dried fraction in the diluent.

Sterility and purity tests:

a) Sterility: Sterility is tested using bacterial and fungal sterility controls by membrane filtration in order to identify absence of bacteria and fungus,

b) Endotoxins: Kinetic colorimetric method in accordance with Ph. Eur. 2.6.14.

Residual humidity:

Not applicable.

Inactivation:

Not relevant.

Batch-to-batch consistency

Certificates of analysis for 2 consecutive batches of the freeze-dried fraction were presented as well as certificates for 2 batches of diluents. As the vaccine is classified as a MUMS product, the supplying of data for only 2 production batches was acceptable.

Stability studies

Stability of antigen

ESP fraction

Data were provided to support the storage of the antigen during 24 months and 15 months at -70 °C or -35 °C respectively. The data were satisfactory to validate the storage of the antigen during 24 months at -70 °C. All tests performed on the fraction (identity, electrophoresis and protein content) allow adequate characterisation of the active substance and confirm its stability.

Up to now, results demonstrated the stability of the ESP fraction stored at T ≤-35 °C for 18 months. Complete results after 27 months of storage will be provided three months following the end of the study.

Stability of the vaccine

Freeze-dried fraction

Stability has been studied on an experimental batch of the vaccine formulated with an active substance close to the expiry date and followed during 27 months with storage at 5+/-3 °C. Results for an additional batch will be provided when available.

Investigated parameters included: appearance, pH, solubility, residual humidity, purity, identity, sterility, protein concentration, potency and safety.

Overall the data supported a claim of 2 years stability period for the vaccine stored at +5 °C.

According to guideline on MUMS, results from 1 batch are sufficient for assessing stability. Results for another additional batch will have to be provided after authorisation or as soon as they are available.

Liquid fraction (Solvent)

The stability of the liquid fraction was studied on 3 batches followed for 36 months with storage at 5+/-3 °C. Investigated parameters included appearance, volume, osmolarity, bacterial endotoxins, sterility. Results showed that the physico-chemical parameters (appearance, volume and osmolarity), the sterility and the content in endotoxin remained stable and within the norms established in the specification form. Therefore storage at +5 °C during 36 months was supported.

Stability of the reconstituted product

The quality of the active substance and potency of the vaccine were tested immediately after the reconstitution of the freeze-dried vaccine with 1 ml of the diluent, and 2 hours after storage at 25 °C.

Tests included identity, purity, activity (protein concentration and potency)

Results indicated that after 2 hours at +25 °C there is no change in terms of identity, purity, protein content and potency. The reconstituted vaccine was considered stable for 2 hours after rehydratation with the diluents. The SPC recommends that the reconstituted product should be used immediately.

Conclusions on stability

Data provided were satisfactory to validate the stability of active substance and the stability of the potency of the vaccine after reconstitution.

Overall conclusion on quality

The quality part was adequately documented. The production process is relatively simple and relies on the culture of *Leishmania infantum* in aseric and defined media which allows the removing of Leishmania through adequate processing steps and the recovering of Excreted Secreted Proteins that constitute the active substance of the vaccine.

An overview of the production process and the controls performed during the production of the freeze-drying fraction containing the active substance and of the liquid fraction was presented. The nature of the raw materials, manufacturing process, controls and treatments applied enable to ensure sterility of the vaccine and absence of introduction of any extraneous agent, and to ensure consistency and homogeneity of the production. This is ensured by the controls performed on raw materials and vaccine products as well as process parameters investigated and recorded during the manufacture.

Many tests have been developed by the applicant which enable to:

- Specifically identify the active substance and thus specific recognition of a major protein by specific antibodies after its migration by electrophoresis is achieved allowing confirmation of the presence of this major antigen and its integrity.
- Quantify the active substance: ESP are the only proteins present in the vaccine. A non-specific protein assay allows quantification of the total amount of proteins. This quantification is used to formulate the vaccine on a fixed target. As no protein is added in the finished product, this amount can be controlled by a newly developed test performed on the final vaccine.
- Validate the purity of the active substance: An electrophoresis in defined conditions ensures the conformity of the protein pattern with the expected profile. Validation demonstrated that this kind of test allows detection of the presence of an extra-protein or the over-expression on one particular protein.

On the final product, in addition a potency test is performed which allows to test the activity of the vaccine and the ability to induce an immune response.

These tests provide clear specifications for the active substance and ensure the consistency and homogeneity of the vaccine production and hence the safety and the efficacy of the released batches.

3. Safety assessment

Introduction

CaniLeish is a freeze-dried vaccine containing Excreted Secreted Proteins of the *Leishmania infantum* parasite in the promastigote form, adjuvanted with a purified extract of *Quillaja saponaria.*

One vaccine dose is formulated with a fixed target of protein – 110 µg of ESP – adjuvanted with 60 µg of purified extract of *Quillaja saponaria* and reconstituted with one dose of diluent before use. The vaccine is intended for the immunisation of healthy dogs against *Leishmania infantum* infection. The regimen of vaccination recommends three subcutaneous injections of one dose of vaccine at 3 weeks intervals in dogs from 6 months of age onwards (primary vaccination). An annual booster immunisation with one dose of vaccine is recommended (re-vaccination scheme).

The adjuvant of the vaccine (purified extract of *Quillaja saponaria*) belongs to saponins and derivatives which are known to have haemolytic activity. This lytic action on erythrocytes membrane depends on the structure of the saponin itself and on the physicochemical properties of the cells. For this reason,

the applicant tested the effect of CaniLeish on dog erythrocytes and haemolytical analysis including red blood cell counts were performed during safety studies.

Vaccine batches used in safety studies

One vaccine batch was used for the safety studies and it was produced according to the manufacturing process described in the quality part of the application but was especially formulated with an overdose of antigen – 120 µg of ESP instead of 110 µg. The batch protocol and certificates of analysis were provided. The batch was an experimental batch produced in 2007 containing an overdose of antigen. The batch was retested in 2009 after vaccine storage with the test methods described in the analytical part. All results were within the current described specifications. Considering the nature and purpose of all these tests it was concluded that the antigen quality and the representativeness of the vaccine batch used in safety studies were appropriate.

Laboratory tests

Safety of the administration of one dose and of the repeated administration of one dose

Safety of the repeated administrations of one dose of CaniLeish in dogs

Animals: The vaccinated and control group consisted of dogs of 4 months old (16-17 weeks). All dogs were seronegative at the start of the trial

Vaccine: CaniLeish, pilot batch formulated to contain 120 µg of ESP per ml

Vaccine scheme and administration route: Subcutaneous route, 4 vaccine doses 21 days apart (D0, D21, D42 and D63).

Follow-up:

- Clinical examination: general and local examinations 4 hours after each vaccination and daily during the next 14 days.
- Measurement of the rectal temperature (D-1, D0, D21, D42, D63 and during each clinical examination). Hyperthermia was defined as a rectal temperature superior to 39.6 °C.
- Weighing once a week from D-7 to the end of the follow-up period (D77).
- Collection of blood samples on D-8, D-3, D0 and once a week to the end of the follow-up period (D77) for haematological analyses (white blood cells, red blood cells, thrombocyte counts, haematocrit, haemoglobin concentration, blood formula).
- Serological analysis for Leishmania on D0 (Fluoleish kit, immunofluorescence method).

Results

Only local slight and transient signs were observed in vaccinated dogs (swelling and in some dogs transient nodules), appearing within the first 24 hours and disappearing within the 12 days of observation.

After vaccination, 2 out of 10 showed a transient and slight hyperthermia recorded. No statistical differences between groups considering bodyweight and blood parameters were observed. For all animals the Leishmania serology was negative (serum title < 80).

Conclusions

This study investigated the safety of a repeated vaccination with CaniLeish (4 vaccinations compared to the 3 administrations recommended in the vaccine scheme as primo vaccination and one booster dose), using a vaccine batch containing an overdose of antigen. It was performed in 4 months old seronegative conventional Beagle dogs (younger than the minimal age recommended for vaccination). The study demonstrated that the vaccination does not lead to serious adverse reactions and that some common post-vaccinal reactions may be observed such as transient hyperthermia or local reactions. These reactions are described in the SPC.

Safety of the administration of an overdose

Safety of the repeated administration of one dose

Safety of the administration of an overdose of CaniLeish in dogs

Animals: The vaccinated and control group consisted of dogs at 4 months old (16-17 weeks). All dogs were seronegative at the start of the trial

Vaccine: CaniLeish, pilot batch formulated to contain 120 µg of ESP per ml.

Vaccine scheme and administration route: vaccination by the subcutaneous route with 2 vaccine doses (2 ml) on D0

Follow-up:

- General and local examinations 4 hours after vaccination and daily during the next 14 days. Measurement of the rectal temperature (D-1, 4h and during each clinical examination). Hyperthermia was defined as rectal temperature higher than 39.6 °C.
- Weighing on D-4, D0, D7 and D14.
- Collection of blood samples on D-8, D-4, D0, D7 and D14 for haematological analyses (white blood cells, red blood cells and thrombocyte counts, haematocrit, haemoglobin concentration, blood formula).
- Serological analysis on D0 for Leishmania (Fluoleish kit, immunofluorescence method).

Results

General observations: No abnormal findings.

Local observations: Swelling was observed in 70% of vaccinated dogs, appearing between 4 hours after treatment until D2, measuring 2 to 7 cm diameter and disappearing within 5 days. Neither nodule nor pain was noted.

Temperature: Thirty percent (30%) of vaccinated dogs had a weak hyperthermia on D1 for 1 day. These same animals presented other transient and weak hyperthermia before the end of the study, probably due to animal excitement. Other animals presented normal temperatures. No statistical differences were observed between groups regarding rectal temperature.

Body weight: A regular increase of body weight was noted in each animal but no statistical differences between groups for bodyweight before and after vaccination was shown.

Blood parameters: Blood parameters are within normal ranges – no statistical differences between groups was seen.

Serology: All animals were seronegative (serum titre <80).

Conclusions

This study assessed the safety of the administration of an overdose (2 doses) of vaccine in 4 months old seronegative conventional Beagle dogs (younger than the minimal age recommended for vaccination). Seventy percent of the animals showed mild local reactions (swelling / 2 to 7 cm) between D0 and D2 that disappeared within 5 days. Post-vaccinal reactions are common reactions including transient hyperthermia and local reaction at the injection site. These reactions are mild and disappear quickly within the days following injection.

Examination of reproductive performance

As the vaccine is not intended for use in pregnant animals, no study on reproductive performance was conducted. A specific contra-indication is included within the SPC.

Examination of immunological functions

In order to verify that the vaccine does not affect the immunological functions of the dogs, haematological analyses including white blood cell counts were performed during the laboratory safety studies after the vaccination with a single, a repeated or an overdose. Control animals were included in these trials.

Analysis showed that blood parameters including white blood cells were similar between the vaccinates and the controls during the whole follow-up period meaning that vaccinations did not induce any leucopenia. This was considered an acceptable approach. The vaccine was developed to induce an immune response as investigated in efficacy studies. The adjuvant is known for years and is used in many vaccines present on the market.

Safety studies included an assessment of haematological parameters and confirmed the absence of adverse effects of vaccination.

Special requirements for live vaccines

Not relevant for this vaccine.

User safety

The user safety was assessed according to the guideline EMEA/CVMP/TWP/54533/2006 "Guideline on user safety for immunological veterinary medicinal products".

Hazard identification and characterisation

The manufacturing process of the vaccine and associated control steps guarantee the absence of live agents in the final product. CaniLeish is thus considered as an inactivated vaccine for user safety and environmental risk analysis. Considering the nature of the vaccine, only possible side effects of the *Quillaja saponaria* adjuvant on humans after an accidental self-injection need to be considered.

Exposure assessment

There is no possible exposure of animal owners or caretakers after vaccination. The vaccine is prepared by reconstitution of the freeze-dried fraction containing the ESP and the adjuvant with the liquid diluents. It is considered that the probability of accidental self-injection by the person administering the vaccine is very low.

Risk characterisation

The laboratory safety studies carried out in dogs showed that only moderate and transient general and local reactions occurred after vaccination, even with an overdose of the vaccine. Saponins are used in cosmetics and pharmaceutical products. Saponin-based adjuvants are currently in development for potential use in human vaccines (HIV, malaria) but this use is limited because of their instability in aqueous phase and the induced local reactions i.e. pain and swelling. Therefore, in the case of a hypothetical accidental self-injection, no more reactions than those described in the literature for saponins and those observed during safety studies on dogs are expected in humans, meaning only slight and transient local reactions and not systemic reactions.

Risk management

The only identified risk is the exposure of professional user by accidental self-injection, which is a rare event and can be addressed by the appropriate safety instructions.

Risk communication

An appropriate warning is included in section 4.5 of the SPC: *"In case of accidental self-injection, seek medical advice immediately and show the package insert or the label to the physician".*

Interactions

No information is available on the safety and efficacy from the concurrent use of this vaccine with any other. A standard claim has been retained in the relevant section of the SPC.

Field studies

According to the guideline on the data requirements for Immunological products intended for minor use or minor species/limited markets (EMEA/CVMP/IWP/123243), if laboratory studies sufficiently show no safety risk, field studies are not required. The results of laboratory studies showed the safety of a single, a repeated and an overdose administration of CaniLeish on dogs younger than the minimal recommended age and vaccinated with a vaccine containing more antigen than the target concentration.

The applicant presented results from a field study where CaniLeish was used in dogs from 4 months of age and safety was investigated:

Field study of CaniLeish in dogs from 4 months of age/ Safety investigation

Animals: Dogs of various breeds (51 breeds), aged of at least 4 months on D0, in 28 locations in France were used. Adult dogs were included in the simple follow-up design and puppies in the complete one (see below)

Vaccine: CaniLeish, formulated at 110 µg of ESP per ml.

Vaccination scheme: 3 vaccinations at 3 weeks interval D0, D21, D42

Safety investigation: At inclusion: haematology and serology for anti-Leishmania antibody titres by IFA test. Local and general clinical examinations (including rectal temperature) were carried out by the veterinarian on D0 (before the first vaccination), D0+4h, D2, D7, D14, D21, D21+4h, D23, D28, D35, D42, D42+4h, D44, D49 and D56. Body weights were recorded on D0, D21, D42 and D56.
Further clinical examinations were carried out by the investigator on D1, D3, D4, D22, D24, D25, D43, D45 and D46 for some of the puppies aged from 16 to 24 weeks (i.e. aged from 4 to 6 months).
The owners recorded any reactions observed after vaccination. All dogs were negative for anti-leishmania IgG antibody titres at inclusion. All the puppies presented progressive weight gains

throughout the study, except a single puppy which presented no weight gain with any other general clinical sign. The mean body weight of the adults remained stable throughout the study.

Results

The vaccine did not induce in dogs of various breeds any specific adverse reactions other than those observed in previous safety studies carried out in beagles: local reaction as swelling, nodule, pain on palpation or erythema, hyperthermia, apathy and digestive disturbance.

Conclusions

In the field study described above a total of approximately 15% dogs were Leishmania-positive at the time of the first injection. Most of them did not present adverse effect after any of the three injections. For the remaining dogs no vaccine-related systemic reactions were observed. Three dogs displayed a reaction at the injection site, with pain and swelling 24 hours after the second and third injections. These local reactions are common and were not different from those observed in the Leishmania-negative population, neither in terms of reaction type nor of intensity. Consequently, the tolerance of the vaccine was good in the dogs that had been in contact with the parasite before the first injection. The following sentence can be thus claimed in paragraph 4.5 "Special precautions for use":

"During the trials, injection of the vaccine to dogs already infected by Leishmania infantum did not show any specific adverse reactions other than those described in section 4.6"

Moreover during efficacy studies, safety follow-ups were carried out after the vaccine injections. In particular, the key efficacy study which was a field study included a detailed safety follow-up with examinations of general and local reactions. Reactions that may be observed did not differ from those obtained in the safety studies and remain within an acceptable range for a canine vaccine.

Environmental risk assessment

An environmental risk assessment statement was prepared according to the CVMP note for guidance EMEA/CVMP/074/95: environmental risk assessment for immunological veterinary medicinal products.

Hazard identification

The vaccine is composed of 2 vials:

- one freeze-dried vial containing the active substances formulated at 110 µg of excreted secreted proteins for the *Leishmania infantum* adjuvanted with 60 µg of purified extract of Quillaja saponaria,
- one liquid vial containing a sodium chloride 0.9% solution as diluent.

Active substances

The product contains no live agents and is administered to the dog via the subcutaneous route. It will then be catabolised by the normal cellular process and will not be excreted into the environment.

Other components

The vaccine does not contain any preservative and its content in residual antibiotic is extremely low.

The purified extract of *Quillaja saponaria* is a widely used adjuvant from vegetal origin and this substance is catabolised by the organism.

The components of the product do not constitute a hazard for the environment.

Packaging/Disposal

The vaccine is the reconstitution of the freeze-dried fraction with the liquid diluent. The entire content of the reconstitution of these 2 vials should be inoculated. The administration will be performed by a qualified veterinary surgeon and safe disposal of used containers, syringes, etc is therefore assured.

Assessment of likelihood

The possibility of exposure and the likelihood of hazards occurring following the use of this vaccine can be classified as negligible.

Assessment of level of risk

Considering the composition of the vaccine and the fact that the likelihood of a release of the vaccine in the environment is very low, the risk for the environment posed by this product is negligible.

Study of residues

CaniLeish is an immunological product and is not intended for immunisation of food producing animals therefore no investigation regarding the residues needs to be undertaken.

Overall conclusion on safety assessment

The safety of the vaccination with CaniLeish was investigated primarily in Leishmania free Beagle dogs receiving 3 standard doses (as described in the SPC) or repeated administrations or an overdose (2 doses) of a vaccine formulated with an overage of antigen. Studies demonstrated that mild local reactions such as swellings associated or not with redness, pain or scabs and a weak hyperthermia may be observed after vaccination that will resolve spontaneously within few days. These reactions have been described in the SPC and are regarded as acceptable post-vaccination reactions for a canine vaccine.

The tolerance of the vaccine was good in the dogs that had been in contact with the parasite before the first injection in one of the conducted field studies and therefore considered a statement in section 4.5 "Special precautions for use" was included:

"Injection of the vaccine to dogs already infected by Leishmania infantum did not show any specific adverse reactions other than those described in section 4.6".

For the user there is a risk of self injection, which is however very low. In addition, appropriate warnings and advice on the SPC have been included. For the environment there is negligible risk that the vaccine components may cause unexpected effects to the environment. As the target species is dogs there was no requirement for residue studies.

4. Efficacy assessment

Introduction

The following information on the epidemiology, cycle of transmission, disease and immunity related to *Leishmania infantum* (dossier + bibliography) are considered important for better understanding the rationale followed in the laboratory and field trials presented. Therefore some important information on the disease is presented below.

Leishmaniosis

Epidemiology

Canine leishmaniosis is a widespread infectious disease in endemic areas of the Mediterranean basin, Asia and America. This is a zoonosis considered as a serious veterinary problem with an increasing impact on public health.

The disease is due to the development and multiplication in the macrophages and mononuclear cells of a protozoan parasite - *Leishmania infantum*. The infected dogs constitute the main domestic reservoir and play a central role in the accidental transmission of parasites to humans. The parasite is transmitted from an infected dog to a non-infected dog by the bites of sandflies of the genus Phlebotomus.

It is presumed that the majority of dogs in highly endemic areas are exposed. It has been estimated that at least 2.5 millions of dogs are infected in South-Western Europe alone. In some regions of several countries bordering the Mediterranean sea like Italy, France, Greece or Spain, the sero-prevalences of tested dogs have been found 1.4 to 30% with a supposed underestimation linked to the method.

Cycle of transmission

The vector is the sandfly where Leishmania exists as multiplicative procyclic promastigotes and infective metacyclic promastigotes. They are transmitted to dogs through the bites of infected sandflies. In the mammalian host, Leishmania would maintain as intracellular amastigote living predominantly in the phagolysosome of macrophage. After initial infection, amastigote may replicate some time before triggering an inflammatory and adaptative immune response which could be at the origin of clinical signs. Infected dogs are infective to the sandflies.

Disease

The outcome of the infection is highly variable. Infected dogs may develop symptomatic infection resulting in death if not treated (1/3 of seropositive dogs), develop only one or many mild symptoms (oligosymptomatic) but a high percentage of infected animals remain asymptomatic.

In the absence of an effective protective immune response, the parasites disseminate from the skin and spread in mononuclear phagocytes to the bone marrow, spleen, liver to cause a chronic, possible fatal disease. Replication of the Leishmania in macrophages and nuclear cells is at the origin of inflammation lesions in all organs, which explain the polymorphic clinical signs of the disease that can be observed.

The clinical signs upon physical examination include cutaneous lesions (skins lesions are the most common manifestation in dogs admitted for treatment of the disease), ocular lesions, renal disease, deterioration of the general state, lymphadenopathy, lameness, exercise intolerance, muscular atrophy, splenomegaly, hepatomegaly.

The clinicopathological parameters are the increase of total serum proteins, hyperglobulinaemia, decrease albumin/globulin ratio, high antibody titre, auto-antibodies (antinuclear antibodies, antibodies against myofibres, platelets and red blood cell), presence of the parasites in lymph nodes, bone marrow, spleen, liver, kidneys, lungs and gastrointestinal tract.

Immunity

The protection of dogs is a major point for controlling the parasite transmission and the incidence of leishmaniosis caused by *L. infantum*.

Few data are currently available on the pathogenicity of the disease (and in particular on the mechanisms of the parasites to escape the immune system) and on the immunity of dogs.

The protective response in dogs has been associated with the lack of clinical symptoms, low levels of anti-leishmania antibodies and parasite load, the presence of a patent in vitro lymphoproliferative response or a positive delayed type hypersensitive response to leishmanial antigens in the skin. Cellular response is associated with an activation of Th1 cells producing IFN-gamma, IL2 and Tumour Necrosis Factor- alpha (TNF-alpha).

The active disease is characterised by a marked humoral response, a specific immunosuppression against the parasite: absence of response to Delayed Type Hypersensitivity Test (DTH), decreased T cell numbers in peripheral blood, and absence of interferon gamma and IL2 production by Peripheral blood mononuclear cells *in vitro*. Total immunoglobulin levels are considered as a prognostic marker for the evolution of the disease.

A vaccine against canine leishmaniosis should induce strong and long-lasting cell-mediated immunity. The immune response requires migration of dermal dendritic cells to draining lymph nodes and the presentation of antigens derived from Leishmania to both CD4 and CD8 cells. These then accumulated in the developing inflammatory lesions and promote parasite destruction by producing cytokines able to activate macrophage defences. Vaccination may promote these responses if vaccine antigens are delivered in an appropriate way to trigger both T cell subsets.

The orientation of the immune response has been largely implicated in the evolution of the animal to be infected or resistant after contact with the parasite.

Resistance to the disease seems to be associated with a Th1 or Th1/Th2 mixed type immune response with the predominance of the Th1 cytokines (IFN-γ, TNF, IL-2, IL-3, IL-12, IgG2). Active disease is characterized by a marked humoral response.

The vaccination relies on the administration of proteins which are at the origin of a Lymphocyte T (LT) dependant response. The adjuvant was selected as it is known to participate to the orientation of the immune response.

The applicant chose to focus on the following parameters of the immune response: IgG1 and IgG2, total IgG and cellular response. The starting postulate for development and efficacy investigation of the vaccine is that a mixed immune response (Th1/Th2) should be induced to achieve protection.

Diagnosis approach

During many years, Leishmaniosis cases were only classified based on physical examinations. As many dogs infected with Leishmania do not develop clinical signs or alteration of biochemical and urinary parameters, infected dogs are now defined based on the presence of the parasite (serological investigation, molecular investigation and parasitological examination).

As the disease is extremely variable its manifestation in each animal the categorisation of dogs is not easy.
In this dossier, the demonstration of efficacy of the vaccine was based on:

- a pivotal trial in field conditions involving dogs followed during nearly 2 years, with evaluation of the protective effect of vaccination on the development of an active infection and disease,
- the protective effect of vaccination after challenge based on the investigation of the immune response of vaccinated dogs and detection of the parasite after challenge (study on duration of immunity).

In support of this demonstration, the applicant also conducted exploration of the Th1 immune response of the vaccinated dogs and in particularly investigation of the *in vitro* parameters linked to the cell response: leishmanicidal activity of macrophages, lymphocytes proliferation assay and IgG2 levels to ESP.

Challenge model

Leishmaniosis is a parasitic disease with complex pathology and diagnosis. Reproduction of an experimental disease is difficult and the multiplicity of evolution after natural or experimental challenge makes it difficult to define the criteria to be retained to define efficacy and their interpretation. Considering the difficulties to reproduce the disease in laboratory and to conduct valid challenges, the key data for demonstration of efficacy of this vaccine rely on the "field" trial involving dogs maintained in endemic areas (natural challenge conditions) and followed during 2 years. This key study will be presented first in the assessment dossier. The other studies conducted in laboratory should be regarded as complementary supportive studies. In most of the applications for vaccines, the efficacy of the vaccination is demonstrated in laboratory studies involving challenge and field studies are conducted in support of the demonstration.

Vaccine administration schedule

The vaccine administration scheme for CaniLeish vaccine was based on that of a vaccine, which was the only vaccine against Leishmaniasis commercialised only in one country (Brazil) at the time of the CaniLeish development and of the launch/ design of the natural challenge exposure trials. Furthermore, numerous other vaccinal preparations, consisting of either 2 or 3 consecutive injections were tested for years and were described in scientific literature. Although these trials gave inconsistent data, those providing the more promising results used a three-injection protocol. As no specific risk was associated with 3 injections instead of 2, and given that there was a chance to have an increased cellular immunity, in view of the importance of the availability of such a vaccine in Europe, the complexity of the field trial organisation, and in order to develop rapidly a vaccine as efficacious as possible, the three-injection vaccine scheme was retained for the development of CaniLeish vaccine.

CaniLeish – efficacy investigation: methods and validations

Investigation of the vaccination-induced immune response

Several techniques are commonly used to evaluate the impact of the vaccination on the immune system and to evaluate and follow the evolution of the Leishmania infection in animals. Techniques described below have been used in the assays contained in the dossier – preliminary research studies and studies performed for this application. They have been developed and validated by Virbac or delegated to laboratories specialised in these particular techniques.

Measurement of IgG1 (representative of a Th2 response) and IgG2 (representative of a Th1 response)

Principle*:* The levels of antibodies directed against vaccine proteins ESP (ELISA) or PSA (ELISA) are determined. As the evolution of IgG2 and IgG1 antibody levels reflect the Th1 and Th2 type responses respectively, their levels served to appreciate the intensity and the orientation of the immune response to vaccination. A validation of the ELISA test for measuring the levels of canine antibodies against the ESP was provided.

Measurement of the total IgG antibodies against the whole Leishmania parasite (IFA test):

During the efficacy studies, 3 IFA tests were performed using different Leishmania isolates, one Virbac method and 2 heterologous IFA tests. The validation methods were provided.

Follow-up of the cell mediated immunity:

Levels of CD5+ (T lymphocytes), CD5+/CD4+ (T helper lymphocytes), CD5+/CD8+ (T cytotoxic lymphocytes) and CD21+ (B lymphocytes) were measured after labelling by specific antibodies conjugated with fluorochroms and counting by flow cytometry. The analysis was performed by a specialised laboratory.

Lymphoblastic Transformation test (LLT) and IFN-γ ELISpot assay:

These tests assess the capacity of lymphocytes to proliferate in response to an antigen (assessment of the memory T-cells and their capacity of reaction after reactivation).

Canine macrophage leishmanicidal activity (CMLA):

The method determines the capacity of the macrophages (of the vaccinated dogs) to eliminate the parasite.

Delayed type hypersensitivity test:

This test is commonly used to check the presence of an active immunity against an antigen

Investigation of the Leishmania infection

Serological methods:

Acute infection is associated with a strong humoral response. Several serological techniques tests/kits are commercially available to perform the measurement based on the indirect fluorescence antibody test (IFAT), ELISA or rapid immunochromatographic strip tests.

Parasitological methods:

a) Research of the parasite by culture from a bone marrow, splenic puncture or lymph nodes aspirates in biphasic medium.

This test involves isolation from a biological product (bone marrow, splenic puncture) of the Leishmania parasite on a specific biphasic medium, NNN medium (under promastigotes forms).

b) Research of the DNA parasite by molecular method (PCR)

i) Real-time PCR: This test involves the quantification of the parasite in bone marrow by PCR amplification of a 200 base-pair fragment of leishmania kinetoplast DNA. The limit of quantification is 40 parasites/ml (1.61 in $\log_{10}$). A validation of the method was provided.

ii) Nested PCR: This method was used by Italian and Spanish reference laboratories. The nested PCR (nPCR), performed by both reference laboratories, used the same protocol, based on the small unit ribosomal ribonucleic acid gene amplification.

c) Assessment of the oxidative stress

This was a new method with few data available to allow reliable interpretation. It is based on the fact that an important oxidative stress is observed in an infected cell due to the metabolism of the parasite and the host cell response leading to an overproduction of reactive free radicals.

Vaccine batches used in efficacy studies

The vaccines used for the laboratory efficacy studies were produced according to the manufacturing process described in the quality part of the application but were especially formulated with 100 µg of

ESP instead of 110 µg. The vaccine batches used in the large scale efficacy study were standard vaccine batches formulated with the recommended amount of antigen.

The batch protocols and certificates of analysis were provided. Since the analytical techniques have been developed, optimised and validated after the first control of these vaccines, batches have been re-analysed after storage with new techniques. New certificates with these analytical results were presented and results were acceptable.

Field trials

The efficacy of CaniLeish was tested in the field against a natural challenge exposure to *Leishmania infantum* parasite in 3 high endemic areas of the Mediterranean basin: one in Italy (Naples) and 2 in Spain (Barcelona and Ibiza).

The 3 sub studies followed the same design: A) a primary vaccination phase in laboratory controlled and protected conditions, B) a natural exposure phase to *Leishmania infantum* parasite i.e. exposure of the dogs to sandfly bites in high endemic areas during 2 seasons.

Efficacy of CaniLeish against a Leishmania natural infection in Spain and Italy

This was a pivotal efficacy study. The study included 2 phases, one vaccination phase in laboratory where dogs were maintained in conditions to prevent any possible contact with Leishmania and one exposure phase where dogs were kept in open kennels in places with endemic leishmaniosis in order to ensure possible natural infection. This particular design allowed controlled conditions for vaccination, controlled natural infection and guarantees the identical management of the vaccinated and control dogs in comparable conditions.

This was a multi centric study which included conventional Beagle dogs from 3 different kennels and exposed to natural infection in 3 sites chosen for their endemic situation towards circulation of leishmaniosis. Dogs were checked to be free of antibodies against Leishmania and free from the parasite before vaccination. Their age varied between 5 and 9 months compared to 6 months which is the minimal age recommended for vaccination. However this was not considered as a major deviation. There was a random allocation of dogs to vaccine or control groups taking into account the age, the weight and the litter. Homogeneity of the groups before vaccination has been checked.

After vaccination, the immune serological and cellular parameters were investigated in the same way as in the laboratory studies which will be presented later in the report and a comparable post-vaccine response was observed in the vaccinated dogs.

Vaccination phase

Animals: Conventional beagle dogs aged between 5 months and 9 months were enrolled; equal number of animals was allocated in each substudy (Naples, Barcelona, Ibiza). Animals were seronegative for anti-leishmania antibodies and anti-ehrlichia antibodies

Vaccine: Standard batches were used for the primary vaccination and for the annual boosts, formulated at 110 µg of ESP

Vaccination scheme: In each substudy, animals were divided in 23 vaccinated dogs and 22 controls. Vaccination was implemented with 3 injections administered 3 weeks apart (Day 0 (D0), D21 and D42). A vaccinal boost was performed with one dose of vaccine 1 and 2 years after the 3rd injection of the primary vaccination

Vaccinal phase investigation: This phase was performed under barrier to prevent any Leishmania infection. The clinical follow-up included rectal temperature monitoring and weighing determination of

the IgG1 and IgG2 against ESP and PSA (by ELISA on D0, D21, D42 and D56), measurement of the IgG levels against whole parasite (by IFA using homologous and heterologous isolates on D0 and D56).

Results

No vaccine related general reaction was observed after vaccination. In some cases, a local reaction was observed at the injection site (swelling sometimes associated with redness, pain, or scab) which resolved spontaneously within a few days. Dogs were free from antibodies to the vaccinal proteins and to the whole parasite before the first vaccine injection. All the control dogs remained free from these antibodies. The primary vaccination phase induced a similar immune response in the 3 sub studies: a mixed IgG1/IgG2 response against ESP and a predominant response of IgG2 type against PSA. The dogs also developed IgG antibodies against the whole parasite detectable by IFA tests. These IgG titres were low using heterologous *Leishmania infantum* isolates.

Specific cellular response was also observed (lymphoproliferation and γ-IFN production significantly increased in the vaccinated groups).

Natural exposure phase

Natural exposure: One month after the last vaccine injection at Month 0 (M0), the dogs were transferred to infection sites (open kennels in high endemic areas) to be exposed to the local sandfly vectors. All dogs were tested negative for Leishmania by PCR and culture on M0.

Investigation: During 2 years: assessment of the IgG1 and IgG2 against ESP took place (by ELISA on M0, M3, M9, M11, M12, M18, M23, M24), cellular immunity - lymphocyte T activation testing in 2 testing sites was also performed (by LLT and γIFN-ELIspot assay on M0). The degree of exposure to Leishmania parasites was evaluated by:

- research of the parasite by nested PCR and real-time PCR in the bone marrow (on M0, M9, M12, M15, M18, M21, M24, M25) and by culture isolation in the bone marrow or lymph nodes aspirates (on M0, M9, M15, M18, M21, M24),
- clinical examinations to detect symptoms attributable to leishmaniosis,
- homologous and heterologous IFA tests to measure the levels of the total IgG antibodies against the whole parasite (M0, M3, M6, M9, M11, M12, M15, M18, M21, M23, M24),
- haematological and biochemical analyses (platelets, white and red blood cells counts, ratio albumin/globulin and total proteins on M0, M3, M9, M12, M15, M18, M21, M24) to assess the infection.

The calculation of the clinical scores was as follows:

- Score ≤3: maximum of 3 laboratory abnormal data (e.g. leucopenia, anaemia and/or thrombocytopenia, hyperproteinemia and/or hyperglobulinemia) or 2 laboratory abnormal data and one clinical sign attributable to Leishmania.
- Score >3: more than 3 laboratory abnormal data (skin disorders, enlarged peripheric lymph nodes, splenomegaly, eye affections, arthritis)or more than 2 laboratory abnormal data and one clinical sign attributable to Leishmania.

Other data: 9 dogs were lost for purposes unrelated to the vaccination or leishmaniosis. 65 vaccinated and 61 controls were then included for analyses.

Statistical analysis: α = 0.05

Rationale for the choice of tests performed by the applicant:

PCR in bone marrow

The bone marrow is one of the most sensitive and specific organs for the Leishmania diagnosis by PCR (Solano-Gallego L., 2009). It is easy to sample and commonly used in veterinary practice. For these reasons, the bone marrow was chosen for the PCR tests to detect the Leishmania positive dogs (i.e. subpatent, asymptomatic or symptomatic dogs). The applicant did not detect the parasite in spleen, blood, skin mainly for ethical, technical and practical reasons.

Biomarkers

The starting hypothesis for the CaniLeish vaccine research and development was that a mixed Th1/Th2 response with a predominant Th1 type cell-mediated immunity (Carrillo E., 2009) was necessary to obtain a protection.

Accuracy of the biomarkers

The following biomarkers were thus analysed in this efficacy study to assess this Th1/Th2 immune profile after vaccination:

- in vitro proliferation of T cells (LTT),
- γIFN cytokine production,
- macrophage killing activity (CMLA) associated with the NO (nitric oxide) production,
- IgG, IgG1 and IgG2 humoral responses,
- delayed type of hypersensitivity reaction as determined by Leishmanin skin test

Although the assessment of these biomarkers in the CaniLeish studies revealed a constant and reproducible immune profile with T cell and humoral responses in the vaccinated dogs. i.e.:

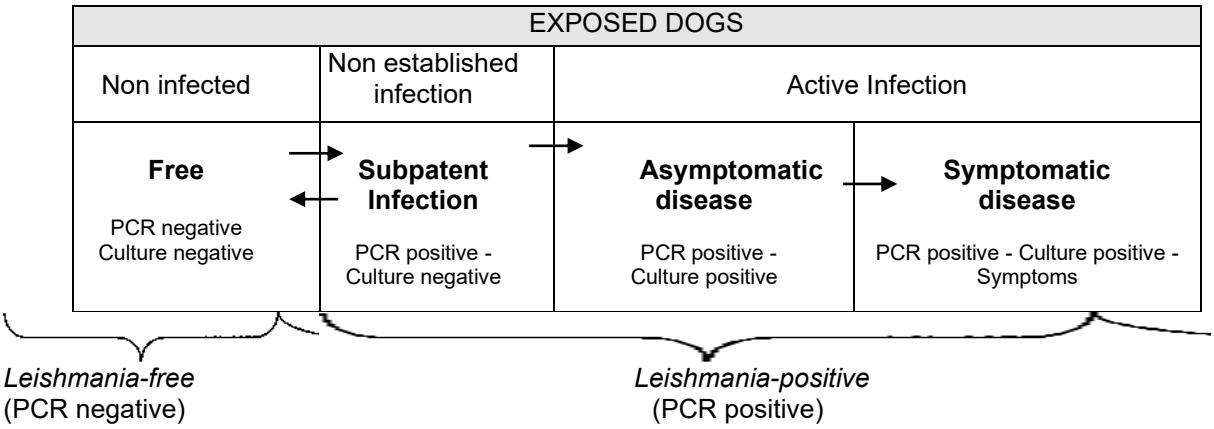
- a preponderant IgG2 response anti-PSA response,
- a mixed anti-ESP IgG1/IgG2 response,
- an increased canine macrophage leishmanicidal activity,
- a specific enhancement of lymphoproliferation and secretion of γIFN.

A different immune profile could not be identified for the vaccinated dogs that developed a persistent infection form that of the vaccinated resistant dogs. As consequence, it was considered inaccurate to assert that this particular immune profile guarantees protection.

Definition of the status of dogs exposed to Leishmania

The definition of the status of the dogs towards leishmaniosis is a major point for interpretation of the efficacy data and assessment of the vaccine efficacy.

The applicant clarified the interpretation of the data and provided an updated definition of the status of dogs exposed to the disease:



The classification proposed by the applicant was considered acceptable as it is also described in literature. All data obtained in the field studies conducted by the applicant confirm that a dog showing a PCR positive result (subpatent infection) can revert to Leishmania-free status whereas as soon as the infection becomes an active one, the immune system of the dog is not capable anymore to go against the active multiplication of the parasite and the status will go from asymptomatic disease for a more or less prolonged period to symptomatic disease.

Exposure rate

The exposure rate was estimated from the percentage of control dogs tested positive at least once for Leishmania.

Naples: 89%; Barcelona: 55%; Ibiza: 14%

Important exposure rates were obtained in Barcelona and Naples. A very low exposure rate was observed in the dog population maintained in Ibiza. This may be linked to several reasons: unfavourable climatic conditions that may reduce the density of the vectors, study timetable (the dogs were transferred to the site in the first season later than forecasted) and use of environmental and topical insecticides and acaricides. This site was considered different and data from Ibiza were excluded from the final analysis of the results.

Results

Taking into account the sites of Naples and Barcelona, the following dogs were included in the analysis of the results:

Approximately 57% of vaccinated and 72% of controls were tested positive for *Leishmania* at least once at different time points of the study. Approximately 43% of the vaccinated and 28% of the controls remained *Leishmania* free during the whole natural exposure phase.

Parasite load follow-up

The evolution of the parasite load measured by RT-PCR was compared. When a dog died, the last value available for parasite load was reported until M24. An increase of the mean parasite load was observed from M9 in both groups until the end of the study. The mean values were significantly higher in the control group than in the vaccinated group all over the period. The evolution of the Leishmaniasis status was presented.

Progression of the number of infected cases: A significant difference between the groups (p=0.0265) with a higher probability to become infected in the control group than in the vaccinated group was observed.

Number of symptomatic cases: A significant difference between the groups (p=0.0466) was observed with a higher probability to become symptomatic in the control group than in the vaccinated. The most severe clinical expression of the Leishmania infection was mainly observed in the control group for which the evolution of the disease led to euthanasia for ethical reasons of some control dogs and the spontaneous death for one while by contrast the health condition of another vaccinated dog required euthanasia.

Significant difference was seen between groups only in relation to the percentage of dogs declared infected at the end of the study M24 with controls having higher levels. Also a significant difference was between groups in relation to the percentage of symptomatic dogs with controls again having higher values.

However it was observed that for vaccinates that became infected and showed clinical manifestation of the disease no benefit could be identified when vaccination continued.

Reduction of active infection

Regarding the progression of the number of infected cases all along the study, as mentioned above a significant difference was observed between the groups (p = 0.0265), with a higher probability to become infected in the control group than in the vaccinated group. Regarding the percentage of dogs declared "Infected" at M24, a significant difference was observed in favour of the vaccinated group (p = 0.0254) which presented fewer cases.

To complete these statistical analyses, the odds-ratio calculation was used. Indeed, the epidemiological studies usually use this method to evaluate the additional probability of exposed population to develop or not a disease.

The odds-ratio is:

$$\frac{\text{(Number of non infected vaccinated dogs) / (number of infected vaccinated dogs)}}{\text{(Number of non infected controls dogs) / (number of infected controls dogs}}$$

The odds-ratio was significantly higher than 1, which meant that vaccinated dog had significantly higher chances to block the active infection than controls. The risk (probability) for vaccinated dogs to develop an active infection was 3.6 times lower than for controls.

Reduction of symptomatic disease

Regarding the progression of the number of symptomatic cases all along the study, a significant difference was observed between the groups (p = 0.0466), with a higher probability to become symptomatic in the control group than in the vaccinated group as mentioned earlier in the results. Regarding the percentage of dogs declared "Symptomatic" at M24, a significant difference was observed in favour of the vaccinated group (p = 0.0455) which presented fewer cases.

Using the odds-ratio calculation meant that vaccinated dogs have 3.8 more chances to prevent the disease than controls, i.e. the risk (probability) for vaccinated dogs to develop a symptomatic disease is quite 4 times lower than for controls.

Conclusions

Overall this was the key study of the application. It showed a benefit of the vaccination according to the recommended vaccine scheme (3 administrations as primo-vaccination and 1 annual booster) when observing the dogs for 24 months in reducing the number of dogs developing an active infection and reducing the probability to develop clinical symptoms in vaccinated dogs. This validates the vaccine scheme and in particular the booster injection after 1 year.

It was observed that the contact with the parasite in Ibiza was low. It was observed that 1 control dog developed a symptomatic disease which lead to euthanasia whereas 1 vaccinated dog developed an asymptomatic disease. As a result no clear conclusion could be made regarding vaccine efficacy in Ibiza due to the specific epidemiological context of this field trial, and therefore the efficacy of the vaccination was more difficult to be observed. This may be linked to the high number of animals needed to demonstrate the benefit in lower prevalence zone.

As a consequence, the section 4.4 of the SPC was updated as follows:

The efficacy of the vaccine has been demonstrated in zones with high infection pressure. The vaccine can be used in dogs living in zones with low or no infection pressure according to the risk-benefit assessment of the veterinarian.

Study of clinical efficacy of the CaniLeish vaccine in dogs in an endemic area

This was an open and multicentric study performed in order to evaluate the efficacy of the CaniLeish vaccine used according to the recommended primary-vaccination protocol: three injections of the vaccine to be given three weeks apart, with the first in dogs aged about six months and older. The study was conducted in an endemic area of canine leishmaniasis.

Animals: Dogs of various breeds were enrolled, aged 5.5 months to 12 years on D0, in 23 locations in France and 12 locations in Italy. Among these dogs, 221 were interpretable for the efficacy analysis.

Vaccine: CaniLeish, formulated at 110 µg of ESP.

Vaccination scheme: 3 vaccinations at 3 weeks interval.

Adverse events: Clinical examination of the dogs at each vaccine visit took place recording any adverse events linked to vaccination.

Efficacy investigation: At inclusion haematology and serology, PCR on bone marrow aspirate; 2 weeks after the last vaccine injection determination of IgG1 and IgG2 against vaccine ESP and vaccine PSA, total IgG against Leishmania.

Interpretation of data: Serological response was assessed according to 2 criteria: the age category of the animals (young i.e. 6 months 26.7% of the dogs/adult 73.3%) and their status against leishmania on D0 (negative 90%/positive 10%).

Results

Adverse events were recorded for 28% of dogs consisting in their majority of injection site reactions (oedema, granuloma, site pain), general signs (lethargy, hyperthermia) or allergy (allergic oedema, anaphylaxis type reaction). Approximately 10% of dogs were Leishmania-positive and 90% were Leishmania-negative. The statistical analysis of serological data showed no significant difference between the groups whatever their L*eishmania* status. The serological response was globally of the same intensity whatever the status *(leishmania* positive or not). Moreover since this study another study performed an explorative analysis of pooled immunological data of CaniLeish vaccine efficacy studies (presented at the end of the efficacy part) and established that serology, whatever the isotype or the antigen (ESP or PSA) was not sufficiently correlated with protection.

Laboratory trials

Onset immunity

Efficacy study of CaniLeish carrying out a DTH test after vaccination

Animals: Conventional beagle dogs were enrolled at 6 months old and free from antibodies against Leishmania

Vaccine: CaniLeish, formulated at 100 µg ESP

Vaccine scheme and administration route: Equal number of dogs received each 3 injections of CaniLeish by the subcutaneous route 3 weeks apart (D0, D21, D42) and equal number of dogs were kept as controls

Follow-up:

- Skin test to assess the presence of a specific T-cell mediated immune response 3 weeks after the last vaccine injection on D63.
- Safety assessment of the vaccination including haematological follow-up and analysis of lymphocytes subset by flow cytometry on D0, D14, D21, D42, D63 and D70.
- Following of the humoral (ELISA IgG1 and IgG2 against ESP and PSA / total IgG against whole parasite) (D0, D21, D42, D56 and D70) and cellular response (canine macrophage leishmanicidal activity – D0, D63 and D70) was implemented.

Results

All animals remained in good health and showed a regular body weight gain. Haematological parameters were within normal or close to normal values with no significant difference between groups. At D70, there was no clear evidence of any effects of the injection of Leishmania antigen on lymphocyte subset counts and haematological counts.

Immunological response investigation

The vaccine induced 3 weeks after the primary vaccination a mixed Th1/Th2 immune response defined by a preponderant anti-PSA IgG2 response against the vaccinal proteins, mixed anti-ESP IgG1/IgG2 response and an increased macrophage leishmanicidal activity, attesting to the capacity of the macrophages to kill the parasite.

A positive DTH reaction against a Leishmania antigen in a majority of vaccinated dogs was observed known to be correlated with the resistance of the animals to infection (Khalil EA 2005).

It was agreed that the applicant conducted all relevant immunity tests according to current literature and knowledge. All data underlined the complexity of the response of the immune system to leishmania infection. Although many parameters were investigated, no specific marker or profile could be defined that correlated with protection or infection as established in the explorative analysis of pooled immunological data (to be presented at the end of the efficacy part).

Conclusions

Onset of Immunity (OOI): The onset of immunity is particularly complex to define due to the slowness of installation of the infection, hence the disease, between the moments of the initial inoculation and the appearance of the disease. Due to the lack of correlation between biomarkers and protection no OOI could be established from this study. Therefore it was that the onset of immunity can be defined on the basis of the key field trial, as the time interval between the 3rd injection and the beginning of

the exposure phase i.e. when dogs were transferred on the infestation sites during the sand fly season, in Naples and Barcelona. As the difference is either 22 or 27 days, the applicant proposed to fix the onset of immunity to 4 weeks which was acceptable.

Efficacy study of CaniLeish carrying out a DTH test after vaccination

Animals: Dogs aged 6 months were enrolled and were divided in 10 vaccinates and 10 controls. They were free from antibodies against vaccinal proteins (ESP and PSA) and the whole leishmania parasite.

Vaccine: The batch used was formulated at 100 µg/ml of *Leishmania infantum* excreted secreted proteins + 60 µg QA-21.

Vaccine scheme and administration route: Primary vaccination was implemented with 3 doses at 3 weeks interval by the SC route – D0, D21 and D42.

Follow-up:

- Clinical follow-up included rectal temperature, weighing.
- Blood collections to follow the humoral (IgG1 and IgG2 against ESP and PSA / D0, D21, D42 and D56 – IFA against whole parasite / D0, D56) and cellular immune response was performed (canine macrophage leishmanicidal activity / D0 and D62, lymphoproliferation assay and γIFN-ELISpot test / D62).

Statistical analysis: α = 0.05

Observations: All animals remained in good health and showed a regular body weight gain. No general reaction or significant hyperthermia was observed after vaccination

Results

Three weeks after the 3rd vaccine injection, T lymphocytes acquired the capacity to proliferate and produce γ-IFN in response to Leishmania antigen, to provoke the destruction of the parasites via the macrophage activation.

Conclusions for the vaccination phase

The vaccine phase of this study and the follow-up of many immune parameters demonstrated a mixed IgG1/IgG2 response against vaccine proteins (ESP) and a preponderant IgG2 response against PSA. This response is correlated with a cellular immune response supported by an increase of CMLA and activation of T lymphocytes (LTT, γIFN ELISpot). These results were comparable and homogeneous with the results of the previous efficacy study which demonstrated that there is a homogeneous response of the dogs after vaccination. However, interpretation of the results from this study phase was difficult as the protective value of these different parameters has not been established.

Challenge phase

Three weeks after the last vaccination (D63/W0), challenge with a virulent *Leishmania infantum* strain via the intravenous route (ITMAP-263 strain at 108 promastigotes per dog) took place.

For 20 months, there was follow-up for the appearance and evolution of the Leishmania infection. The Leishmania parasite detection in the bone marrow included real time PCR and culture isolation (W0, W24, W32, W41, W49, W61, W73 and W86). The clinical and paraclinical follow-up included regular clinical examinations, determination of the anti-leishmania IgG antibody level (IFA / W0, W15, W32, W49, W73 and W88), haematological analysis (W0, W41 and W88) and analysis of 2 biochemical parameters (albumin/globulin ratio, total proteins / W0, W41 and W88) and assessment of the glutathione redox imbalance (W0, W15, W32, W61 and W86).
The immune response follow-up involved the measurement of levels of IgG1 and IgG2 antibodies

against ESP (W0, W15, W32, W49, W73 and W86), canine macrophage leishmanicidal activity test (W0, W32, W61 and W86), lymphoproliferation assay, γIFN-ELISpot test and analysis of the T lymphocytes subpopulations (W0, W32, W49, W61 and W88).

Results

Clinical and paraclinical follow-up: No clinical sign that could be attributed to Leishmaniosis disease was observed.

Biochemical/haematological follow-up: no difference between vaccinated and controls – parameters globally within the standard values.

Infection investigation

The inoculation of the *Leishmania infantum* virulent strain induced a weak and belated infection since the infection was detected (PCR and culture) only in 4/10 dogs in each group from 9 months after the challenge.

The challenge did not allow to induce a disease with clinical expression of the infection.

Immunological investigation

The humoral response against the vaccinal proteins ESP induced by the primary vaccination decreased or even disappeared whereas the specific T cell-mediated immunity persisted. The challenge performed in this study was not virulent enough to assess the protection conferred by the vaccine.

Conclusions – challenge phase

The investigation period of the study was extended beyond one year as the first detection of infection occurred 9 months after inoculation. Despite this long period, the infection rate remained too low to assess the vaccine efficacy. The challenge was not virulent enough to determine the onset of immunity of the vaccine. The difficulty to conduct a challenge in dogs has been reported by several authors (Poot 2005, Leandro 2001). Various methods to conduct such challenges have been described and conflicting results were obtained, underlying the heterogeneity and the lack of reproducibility of the challenge methods. This may be linked to the impossibility to mimic the field conditions as in natural conditions, dogs can receive up to one infectious bite per hour during the night during warm months (frequent administrations of a small parasite quantity). Considering data obtained in field conditions (see related section) and taking into account ethical reasons, the applicant decided not to repeat an experimental challenge to confirm the onset of immunity. The challenge neither allowed to induce a clinical disease, nor to clearly determine the onset of immunity as infection could be detected only 9 months after the experimental infection. Moreover the challenge was a weak challenge as only 4 control dogs were found infected.

Nevertheless, it was observed that in the vaccinated group, some dogs were found infected too and some developed a persistent infection. Vaccination of these dogs did not allow protection against infection although the immune response of these dogs after vaccination was similar to the one of the protected dogs.

It was observed that persistently infected dogs, both controls or vaccinates, present IgG2 against ESP after challenge in contrary to the resistant animals, whereas a starting postulate was that IgG2 was one of the representative parameter of a Th1 protective immune response. While there was some indications of a trend no firm conclusions could be made from this study on the possible correlation between the immunological response and evolution of infection.

Duration of immunity

Duration of immunity with CaniLeish – assessment of the DTH response one year after vaccination

Animals: Conventional Beagle dogs 5 to 7 months of age were enrolled and then allocated to two groups of equal numbers: vaccinates and controls. They were free from antibodies against Leishmania.

Vaccine: CaniLeish formulated at 100 µg/ml.

Vaccine scheme and administration route: 3 injections of one dose at a 3 weeks interval (Week 0 (W0), W3 and W6).

Follow-up:

- Clinical follow-up including weighing and haematological follow-up : blood collections to follow the humoral (IgG1/IgG2 against ESP and PSA by ELISA / W0, W6, W8, W30, W42, W58 and W61: total IgG against parasite by IFA W0,W8,W30,W42,W58 and W61).
- Cellular immune response : T-lymphocyte subpopulation evaluation (W0, W6, W9, W30, W58 and W61), canine macrophage leishmanicidal activity (W0, W9, W58 and W61).

Statistical analysis: $\alpha = 0.05$

Observations: All animals remained in good health and showed a regular body weight gain – haematological parameters remained within or close to normal values with no difference between groups

A DTH test 1 year after vaccination was performed. One year after the last vaccine injection (week 58), both groups were submitted to a Leishmania skin test in order to assess the presence of a specific T-cell mediated immune response

Results

Both groups presented local reactions after the leishmania antigen administration. The DTH response appeared more rapidly in vaccinates and was significantly more intense than those observed in the control group, which was a sign of a specific cellular immunity against the leishmanin antigen in the vaccinates Observation of a boost of the humoral response in the vaccinated dogs indicated the presence of a memory immunity generated by the primary vaccination. Three weeks after leishmanin antigen injection (W61), 70 % of vaccinates presented again anti-ESP IgG1 titres, 90% anti-ESP IgG2, 50% anti-PSA IgG2 and 90% IgG against whole parasite – no boost after leishmanin for anti-PSA IgG1.

Other immunological results

The vaccine induced an onset of humoral and cellular immune responses 3 weeks after vaccination defined by a mixed anti-ESP IgG1/IgG2 antibody response which lasted less than 6 months, an anti-PSA IgG2 response which lasted less than 6 months, an increase of CMLA and the persistence of this activity for 1 year. Finally, the DTH test performed 1 year after the primary vaccination demonstrated the presence of a memory cell-mediated immune response by the observation of a positive delayed type hypersensitivity response and a boost of the humoral response.

Conclusions

This study showed the absence of persistence of the antibodies after vaccination whatever their nature and a boost effect following contact with Leishmania antigen. A persisting cellular immune response was observed in vaccinates. Problems with the interpretation of this study rely on the value of the

investigated parameters and their potential correlation with the efficacy of the vaccination. Data confirmed the presence of a memory immunity revealed by the DTH test in most of the dogs; however no claim for a duration of immunity could be established from this study.

Duration of immunity conferred by CaniLeish after experimental challenge one year after vaccination

Vaccination phase

Animals: Conventional Beagle dogs, 6 months old were enrolled and divided in two groups of vaccinates and controls. They were free from antibodies against Leishmania

Vaccine: CaniLeish formulated to contain 100 µg/ml ESP

Vaccine scheme and administration route: 3 injections of 1 dose at a 3 week-interval by the SC route (W0, W3 and W6) were given.

Follow-up:

- Investigation of the humoral (IgG1 and IgG2 against ESP and PSA – ELISA - total IgG against parasite IFA and
- Cellular immune responses (LLT, CMLA and γIFN-ELISpot assay) over the 12 months following the primary vaccination.

Statistical analysis: $\alpha = 0.05$

Observations: All animals remained in good health.

Challenge phase

One year after the last vaccine injection (newW0) challenge with a virulent *Leishmania infantum* strain via the intravenous route took place.

Follow-up:

- 47 weeks for the appearance and the development of a Leishmania infection: detection of the parasites in bone marrow through real time PCR and culture isolation.
- Clinical and paraclinical parameters: clinical examinations, haematological (W0, W32, W40, W47) and biochemical analysis (W0 W40 and W47) and determination of anti-leishmania IgG antibody levels (IFA / W0, W15, W32, W47).
- Impact of the challenge on the immune system: dosage of the IgG1 and IgG2 antibodies against the vaccine proteins ESP (W0, W15, W32, W47), CMLA test (W0, W32), lymphoproliferation assay and γ-IFN-ELISpot assay (W0, W32, W47) and monitoring of the glutathione antioxidant system (W0, W15, W32, W47).

Results of the challenge phase

There was no difference between groups for haematological parameters.

Cellular immunity:

- CMLA: Results of the controls slightly increased on W32. The mean value slightly increased after challenge in vaccinated dogs and remains significantly higher than the controls.
- LLT and γIFN-ELISpot: The mean γ-IFN production increased 32 weeks after challenge in both groups, this increase being more important in the vaccinated group (not significant at W32, significant at W47).

- A glutathione redox imbalance was registered in most of the control dogs whereas the ratio remained stable for the full study duration in the vaccinated.

Infection status and humoral immunity: see below

Immune response investigation

Dogs controlling or progressively blocking the infection

Parameter	Vaccinated – 70% of dogs	Control – 30% of dogs
Clinical signs	No clinical signs or paraclinical disorders compatible with a leishmaniosis were recorded	No clinical signs or paraclinical disorders compatible with a leishmaniosis. One dog developed a late and asymptomatic infection at the end of the study
Total IgG	Boost of the IgG antibodies response after challenge – titres between 1/200 and 1/1000	The 3 dogs remained negative all along the study
IgG1 against ESP	Challenge induced a boost in 4 dogs – 2 dogs never present any IgG1	No response
IgG2 against ESP	Boost of the response after challenge	No response

Dogs developing persistent infection

Parameter	Vaccinated – 30% of dogs	Control – 70% of dogs
Clinical signs	the clinical and paraclinical disorders noticed in 66% of dogs were too weak to declare a symptomatic infection	several signs characteristics of leishmaniosis were observed in 57% dogs (skin disorders, alterations of haematological-biochemical parameters) but there were not sufficiently numerous and/or developed to diagnose a symptomatic infection
Total IgG	Boost of the IgG antibodies response after challenge – high titres reaching 1/2000 observed in 66% dogs displaying a persistent infection	86% of dogs displaying persistent infection presented IgG titres ranging from 1/200 to 1/1000 32 to 47 weeks after challenge
IgG1 against ESP	Challenge induced a boost in 1 dog – 1 dog never present any IgG1	No response
IgG2 against ESP	Boost of the response after challenge	Response in 71% dogs

At week 47, 20% of controls and 70% of vaccinates were not found to be infected, whereas 80% of controls and 30% of vaccinates presented asymptomatic infection.

Conclusions

Despite the reduction of dogs developing persistent infection in the vaccinated group (300% versus 70% in the control group) this study did not allow to conclude on the efficacy of the vaccine and the duration of immunity as the statistical analysis did not establish statistical differences between groups, probably linked to the number of animals included in the study. However, the DOI is also sustained by the field trial which showed a benefit of vaccination according to the recommended vaccination schedule (3 administrations as primary vaccination and 1 annual booster). The challenge did not induce a clinical disease and the first infection was identified 15 weeks after challenge (in controls as well as in vaccinates). It was nevertheless observed that in the vaccinated group, there was a higher number of dogs controlling the infection (infection never detected) or blocking it (infection detected and progressively blocked) during the 47 weeks of follow-up after the challenge. However the challenge appeared to be more successful in this study in inducing infection on the onset of immunity study.

Considering the parasite load at individual scale (comparison of data of infected dogs – vaccinated or controls), the vaccination did not allow to decrease the parasite load in vaccinated infected dogs compared to control ones. The analysis of individual data showed that the use of serological or cellular immune parameters to describe and predict the future status of the dog towards infection is not clear and evident.

A seroconversion of both humoral responses (anti-parasite IgG and anti-ESP IgG2) was only observed for the permanently infected control dogs, this seroconversion was then correlated with infection for these dogs. On the contrary, the control dogs, which blocked the infection, did not develop any humoral response.

These results again underline the difficulties to define an immune protective profile post-infection.

The PCR and culture to diagnose the infection were performed from bone marrow. A good correlation was observed between the results of these 2 investigation methods. At week 47, 20% of controls and 70% of vaccinates were not found to be infected, whereas 80% of controls and 30% of vaccinates presented asymptomatic infection

Overall even if not definitively conclusive (given the absence of statistical significance), the study supported a beneficial effect of the vaccine 1 year after infection to reduce the development of a persistent infection during the 47 weeks following the challenge, and supports the data obtained in the field trial. As a result a duration of immunity for a year after the last re-vaccination was accepted.

Overall conclusion on the laboratory studies

After vaccination, a constant and homogenous response of the vaccinated dogs was observed in the laboratory studies for the following investigated parameters:

- Humoral response: mixed IgG1/IgG2 response against ESP and predominance of IgG2 against PSA was seen. This humoral response did not persist within time but was boosted after a new contact with the antigen one year after the primo-vaccination.
- Cellular response: an homogenous and persistent cellular response was observed in all vaccinated dogs based on tests such as LLT, CMLA, DTH.

Two challenge studies were performed. The first challenge (onset of immunity) performed 3 weeks after vaccination lead to weak and belated infection in controls as well as in vaccinates and the study did not allow to lead to any conclusion with regard to efficacy of the vaccine. The 2nd challenge (duration of immunity) performed 1 year after vaccination demonstrated a reduction in the number of infected animals in the vaccinated group after experimental infection.

The laboratory data were considered as only supportive data to reinforce the conclusions of the field trial. The observation of a stable and homogenous immune response after vaccination through assays supports the efficacy that may be expected after vaccination.

General studies

Explorative analysis of pooled immunological data of the CaniLeish vaccine efficacy studies

This was an exploratory analysis of the pooled data of all the efficacy studies conducted for the application of CaniLesih, in order to determine if the immune profile induced by the vaccination is correlated with the protection or the resistance to the disease. Therefore, only the results obtained on the vaccinated groups were considered for this analysis. However the CVMP considered that data were nevertheless insufficient to validate any biomarkers.

Assessment of the possible interference of the antibodies generated by the vaccine with different common diagnostics tests

Dogs of 5 to 6 months of age were allocated to two groups: 10 dogs were administered three subcutaneous injections of CaniLeish vaccine at a three-week interval (D0, D21 and D42) and 2 dogs were kept as controls.

Clinical follow-ups were carried out regularly all along the study.

The immunological response towards the vaccinal proteins was checked on through ELISA tests serving to dose the IgG1 and IgG2 antibodies directed against the vaccinal Excreted Secreted Proteins (ESP) and the IgG1 and IgG2 antibodies directed against the Promastigote Surface Antigen (PSA).

Common diagnostics tests were performed in order to evaluate the possible interference of vaccinal response with these tests:

- Three different Indirect Fluorescent Antibody (IFA) Laboratory tests, using as antigen homologous or heterologous Leishmania isolates to the vaccine
- Three rapid commercial tests using purified leishmania antigens.

Results

No vaccine-related general reactions were observed after each injection. All the animals remained in good health throughout the whole study. The serological analyses performed before vaccination confirmed that all the dogs were free of antibodies directed against vaccinal proteins (ESP and PSA) and against the whole Leishmania parasite before the first vaccine injection. All the control dogs remained free of these antibodies throughout the study.

After vaccination, all dogs presented a good immunological response towards the vaccine. The seroconversion rates for the IgG1, IgG2 antibodies against vaccinal ESP and for the IgG1, IgG2 antibodies against the PSA protein were as expected and conformed to established data obtained in previous studies. The humoral response directed against *Leishmania infantum* parasite evaluated through the three IFA tests was different according to the Leishmania isolate used.

Two weeks after the 3rd vaccine injection (D56), the maximum levels of IgG antibodies against the whole Leishmania parasite were obtained:

- 100% of the dogs responded against the homologous isolate used by VIRBAC Laboratory and also against a heterologous isolate. This response rapidly decreased and 4 months after the 3rd vaccine injection the titres were low.
- 70% of the dogs responded against another heterologous isolate. These antibodies rapidly decreased and only 30% of animals were still seropositive 2 months after the 3rd vaccine injection.
- Finally, all the commercial diagnostics tests were negative since no antibodies towards *Leishmania infantum* parasite were detected after vaccination and until the end of the study.

Conclusions

In conclusion, this study demonstrated that the humoral response (IgG antibodies) directed against whole *Leishmania infantum* parasites, obtained after vaccination with the CaniLaish vaccine:

- interferes with IFA tests, performed in reference laboratories using homologous or heterologous *Leishmania infantum* isolates,

- does not interfere with some commercial tests commonly used by the veterinarians as diagnostics test for Leishmaniasis disease.

Consequently, the diagnosis of leishmaniasis should rely on the identification through one of these latter tests as a first step for the veterinarian, before performing other complementary tests as PCR, culture or histology for a definitive diagnosis.

The following special warning was therefore added in section 4.4 "Special warnings" of the SPC:

"Transient antibodies against Leishmania *detected by immunofluorescence antibody test (IFAT) may appear after vaccination. Antibodies due to vaccination can be differentiated from antibodies due to natural infection by using a rapid diagnostic serological test as a first step to a differential diagnosis".*

Protection for public health

Dogs are considered as the most important peridomestic reservoir of *L. infantum* infection to humans (Guerin PJ., 2002). Infectivity of dogs is correlated with the development of the disease (Travi BL., 2001) and symptomatic dogs are more infective to insect vectors than asymptomatic dogs (Molina R., 1994; Moreno J., 2002). Furthermore, after being infected and cured the treated animal continues to harbour the parasite and remains infectious to sand flies (Solano - Gallego L., 2009). Therefore, a vaccine that enables to reduce active infection in dogs would be a useful help to reduce the incidence of human leishmaniasis.

CaniLeish reduces the probability for a dog to become actively infected and to develop a clinical disease. The number of dogs that can act as active reservoir for transmission of leishmaniosis to humans is then reduced following the use of the vaccine. Therefore, a possible impact of the vaccine on public health cannot be excluded. However no conclusion can be reached on the impact of this product in public health with the current data and within the frame of a MA dossier and further investigation needs to be performed.

Consequently, the following sentence has been added in section 4.4 of the SPC under "Special warnings": *"The impact of the vaccine in terms of public health and control of the human infection cannot be estimated from available data."*

Vaccine use in the field according to the different epidemiological situations

The pivotal field trial for this application established the vaccine efficacy in two areas of high prevalence (i.e. mean exposure rate of 72%), and this high prevalence was defined by PCR –molecular diagnosis). In the same trial, no benefit of the vaccination could be demonstrated, on an area with a lower exposure rate. However, due to the complexity of the epidemiological situation only the veterinarian will be able to evaluate the relevance of the vaccine for a dog according to its local living conditions. Therefore, the following sentence is mentioned in section 4.4 of the SPC under "Special warnings": *"In areas of low or no infection pressure a benefit-risk assessment must be undertaken by the veterinarian before deciding to use the vaccine".*

Overall conclusions on efficacy

The vaccine is intended to be used in Leishmania free dogs from 6 months of age onwards to protect against *Leishmania infantum* after 3 vaccine injections as primo-vaccination and an annual single booster vaccination.

Studies presented in this dossier confirmed the difficulty to assess the efficacy of a vaccine against a parasitic disease with heterogeneous evolution and manifestation.

The demonstration of efficacy of the vaccine was based on a key field trial of 2 years duration involving vaccinated and control dogs submitted to natural exposure to infection in zones with high infection pressure. After these 2 years, the vaccine was demonstrated to reduce the number of dogs developing an active infection in the vaccinated group and for a dog to significantly reduce the probability to become infected and to develop a clinical disease. The benefit of the vaccination was therefore estimated in zones with high infection pressure where it may decrease the risk to develop an active infection and a symptomatic disease after contact with the parasite for vaccinated animals. In conditions with weak prevalence of the disease, no clear benefit of the vaccination could be established. This may be linked to the high number of animals needed to demonstrate the benefit in lower prevalence zone.

Laboratory studies provide limited information despite many biological investigations including humoral (IgG1and IgG2, against ESP and PSA, total IgG) and cellular (lymphoblastic transformation test, IFNγ ELISPOT assay, canine macrophage leishmanicidal activity) immunity evaluation. No biomarker or immunological profile correlated with protection or infection could be defined. Nevertheless data on experimental challenges showed that infection can be detected from 4 to 9 months after the experimental infection and allowed a constant and homogenous response to vaccination. However such responses could not be clearly linked to protection and future response of the dogs to infection.

Considering the diversity of evolution of the infection and the variable incubation period that may last for months, it was difficult to define for this vaccine periods such as onset or duration of immunity and protection in a laboratory studies but a duration of immunity lasting a year after the last re-vaccination and an onset of immunity of 4 weeks were supported by the field data.

5. Benefit risk assessment

Introduction

CaniLeish is a vaccine intended to reduce the incidence of asymptomatic and symptomatic forms of Leishmania infection by induction of a specific cell-mediated immunity in vaccinated dogs. It is based on the role of the Excreted Secreted Proteins of *L. infantum* to induce cellular immune response. The vaccine is made of a freeze-dried pellet and a diluent. The adjuvant – purified extract of *Quillaja saponaria* - known to participate to the activation of the cellular immune response is included in the freeze-dried fraction.

The assessment of the application dossier took into account that this vaccine is intended for a limited market and some reductions in requirements according to the guideline on Data requirement for immunological veterinary products for minor use and minor species (EMEA/CVMP/IWP/123243/2006) were implemented.

Benefit assessment

Direct therapeutic benefits

The objective is to induce sufficient immunity to Leishmania free dogs from 6 months of age to reduce the risk to develop an active infection and clinical disease after contact with *Leishmania infantum*.

Field trials demonstrated that the product is capable of reducing the number of Leishmania free dogs developing an active infection and significantly reduce the probability to become infected and to develop a clinical disease after contact with *Leishmania infantum*. The benefit of the vaccination was

estimated in zones with high infection pressure where it may decrease the risk to develop an active infection and a symptomatic disease after contact with the parasite for vaccinated animals.

Additional benefits

CaniLeish is the first vaccine to be authorised for the prophylaxis against *Leishmania infantum* in Europe. The duration of immunity for the vaccine has been shown to be 1 year after the last re-vaccination and the onset of immunity 4 weeks.

Vaccination has been shown to be safe for Leishmania infected animals.

Risk assessment

Main potential risks

a) There is a risk of moderate and transient local reactions may occur such as swelling, nodule, pain on palpation or erythema. These reactions resolve spontaneously within 2 to 15 days. Other transient signs commonly seen following vaccination may be observed such as hyperthermia, apathy and digestive disorders lasting 1 to 6 days. Allergic-type reactions are uncommon and appropriate symptomatic treatment should then be administered

b) For the user there is a very low risk of self injection. Appropriate warnings and advice on the SPC will serve to minimise this risk.

c) For the environment there is negligible risk that the vaccine components may cause unexpected effects to the environment.

Specific potential risks, according to product type and application

a) Efficacy results do not show complete protection of vaccinated dogs. Despite vaccination a percentage of dogs still became infected with *Leishmania infantum* and from those vaccinated dogs that became infected a percentage of animals also developed clinical signs of the disease.

b) The benefit of the vaccination was established in zones with high infection pressure, whereas no clear benefit could be established in areas of low infection pressure.

c) In dogs developing Leishmaniosis (active infection and/or disease) despite vaccination, proceeding with the vaccine injections showed no benefit.

Risk management or mitigation measures

a) Appropriate warnings have been placed in the SPC to warn of the potential risks to the target animal, end user and environment.

b) Appropriate warnings have been placed in the SPC to clarify the limitations of the indication, the limitations of benefit in low infection areas and the lack of benefit in continuing the vaccination in vaccinated dogs that have developed the disease.

Evaluation of the benefit risk balance

Leishmaniosis is an important disease in dogs that is endemic in the Mediterranean countries of Europe, the Middle East and many subtropical areas of the world. In the past decade, an increased incidence of canine leishmaniosis in endemic zones as well as spread of the infection to non-endemic areas of Europe has been observed. Canines are the main reservoir for the parasites and play a

relevant role in transmission to humans. The aetiological agent – *Leishmania infantum* – is transmitted by sandflies of the genus Phlebotomus.

In endemic areas dogs become exposed immediately. Evolution of the infection in dogs is then complex and unpredictable. Some will develop protective immunity, some remain asymptomatic after infection and may relapse later and others develop a clinical disease. It is considered that establishment of infection and development of the disease both depend on the host's immunological response and that once the parasite escapes immunity and is able to multiply, no clearance is possible anymore. Infection may evolve over a period of a few weeks to several months toward disease patterns that can be extremely variable and polymorphic, which makes it difficult to classify dogs within specific categories.

Along with typical clinical signs and history of exposure, the diagnosis is based on microscopic identification of the parasite or PCR testing on bone marrow samples. Serological techniques may reveal active infection.

The management of dogs infected with Leishmania is currently based on sanitary and/or medical prophylaxis but up to now, both showed limited capacity to fulfil eradication, or even control of canine leishmaniosis.

Sanitary measures are based on preventing physical contact of dogs with vector, reducing the microhabitats to sandflies, and employing insecticide (environmental or topical). Despite implementation of all these measures, canine Leishmaniosis could not be reduced efficiently. Moreover culling of seropositive dogs has not proved to be efficient; although this solution was adopted in Brazil it currently has failed to prevent the number of human cases to increase.

If applied medical treatment in diseased dogs consists of symptomatic treatments associated to leishmanicidal molecules (meglumine antimoniate, aminosidine, miltefosine) which reduce or eliminate clinical symptoms but do not achieve parasitological cure. The epidemiological risk persists and dogs that respond to chemotherapy can nevertheless experience clinical relapse after the cessation of treatment or during it. Besides, these medicines have shown to have a number of disadvantages such as price, repeated injections, hepato- and nephrotoxicity, which can make compliance to treatment quite difficult to achieve.

On the basis of the above and although efficacy results have not shown complete protection of vaccinated dogs, it can be concluded that vaccination against Leishmaniosis can become a valuable and/or complementary alternative to the existing tools, despite the limits of the vaccine. Despite the fact that complete protection against Leishmaniosis or eradication of the disease cannot be achieved, this vaccine is able to reduce the risk for developing active infection and disease at the individual scale and to participate to reduction of incidence of the disease at the level of a dog population. Additionally and although the epidemiological impact of the vaccination cannot be estimated from the provided data of this application, it is nevertheless expected that improvement of the situation in dogs with regard to Leishmaniosis will also have a positive impact on human health. Finally, no risk has been linked to the use of this vaccine in dogs (included infected ones with Leishmaniosis).

Hence, the benefit-risk assessment of this vaccine appears favourable for this vaccine, within the limits highlighted in the SPC.

Conclusion on benefit risk balance

The information provided in the dossier and in response to points raised is sufficient to confirm an overall positive benefit risk balance.

Conclusion

Based on the original and complementary data presented the Committee for Medicinal Products for Veterinary Use (CVMP) concluded that the overall benefit-risk balance was considered favourable for authorisation.