SCIENTIFIC DISCUSSION

This module reflects the scientific discussion for the approval of Startvac (as published in December 2009). For information on changes after this date please refer to module 8 (Steps taken after authorisation).

1. INTRODUCTION

An application for the granting of a Community marketing authorisation of STARTVAC has been submitted to the EMEA in accordance with Council Regulation (EEC) No. 726/2004 on 27 April 2007 by Laboratorios Hipra, S.A. STARTVAC is presented in packs/containers of 3 ml (1 dose = 2 ml), 10 ml (5 doses) and 50 ml (25 doses). It contains inactivated *Escherichia (E.) coli* J5 and inactivated *Staphylococcus (S.) aureus* (CP8) and is indicated for herd immunisation of healthy cows and heifers, in dairy cattle herds with recurring mastitis problems, to reduce the incidence of sub-clinical mastitis and the incidence and the severity of the clinical signs of clinical mastitis caused by *Staphylococcus aureus*, coliforms and coagulase-negative staphylococci.

The route of administration is intramuscular use.

The target species is cattle (cows and heifers).

2. QUALITY ASSESSMENT

Composition

STARTVAC is an immunological product containing whole cells of heat-inactivated *Escherichia coli J5* strain and whole cells of a formaldehyde-inactivated *Staphylococcus aureus* (CP 8) strain. The vaccine is adjuvanted with liquid paraffin and contains benzyl alcohol as preservative. The quantitative composition has been well defined.

Active substances:

Escherichia coli J5 inactivated..... > 50 RED_{60} *
Staphylococcus aureus (CP8) strain SP 140 inactivated, expressing Slime Associated Antigenic Complex (SAAC) .....…… > 50 RED_{80} **

* RED_{60}: Rabbit effective dose in 60 % of the animals (serology).
** RED_{80}: Rabbit effective dose in 80 % of the animals (serology).

Adjuvant:
Liquid paraffin..... 18.2 mg

Excipients:
Benzyl alcohol……..... 20 mg
Liquid paraffin
Sorbitan monooleate
Polysorbate 80
Sodium alginate
Calcium chloride, dihydrate
Simeticone
Water for injections

Container

The colourless vials (3 ml/1 dose, 10 ml/5 doses and 50 ml/25 doses) are of type I glass according to European Pharmacopoeia *(Ph. Eur.)* 3.2.1. The vials are closed with grey bromobutyl stoppers. These stoppers are classified as Type I rubber stoppers and comply with Section 3.2.9. of the current *Ph. Eur.*.

Development Pharmaceutics

STARTVAC contains inactivated whole cells of two bacterial strains incorporated in an oil-in-water-emulsion in order to stimulate immunity. There is a comprehensive justification regarding the choice of the bacteria, adjuvant and preservative.

Staphylococcus aureus **(***S. aureus)* is recognised as the main contagious pathogen in bovine mastitis. The strain included in the STARTVAC vaccine is based on the presence of the Slime Associated Antigenic Complex (SAAC), which is an exopolysaccharide. This is an important virulence factor implicated in the adhesion of the bacteria to the epithelium of the mammary mucous. The induction of anti-slime antibodies will help the minor colonisation and subsequent multiplication of *S. aureus* in the glandular epithelium.
Escherichia coli (*E. coli*) are wide-spread in the dairy environment and considered as the most important cause of environmental mastitis. The strain *E. coli* J5 lacks the enzyme Uridin Diphosphate Galactose 4-Epimerase, which is responsible for binding the somatic antigen (O-chain polysaccharide) to the LPS molecule of the cell wall. Thus, the core antigen, which is common to many gram negative microorganisms, is better exposed to the outside of the bacterium and, therefore, better recognised by the immune system.
Liquid paraffin is chosen as adjuvant component. In spite of its mineral origin (non-biodegradable), the low percentage (oil-in-water-emulsion) used in this vaccine confers a good safety profile.
Benzyl alcohol is chosen as preservative. The efficacy of antimicrobial preservation is properly evaluated according to *Ph. Eur.* 5.1.3 and the Guideline for the Testing of Veterinary Medicinal Products, 1994: "Inclusion of antimicrobial preservatives in immunological medicinal products" (III/3469/92). The proposed in-use shelf life of 10 hours after first opening of the bottle is considered sufficiently substantiated by appropriate data (microbial safety as well as sterility and potency results, which were provided in a separate study).
Sodium alginate – calcium chloride was added to the vaccine composition to obtain a more viscous and stable emulsion.

Method of manufacture

The manufacturing process corresponds to a classical procedure. Bacteria used in manufacture are handled in a seed-lot system. The strains are propagated in a scale-up system. In case of *S. aureus,* the culture of the fermentor is inactivated and afterwards the antigens are concentrated by centrifugation. The harvest of *E. coli* J5 is washed with PBS and then a concentration (centrifugation) is performed.
The inactivation procedures are adequately validated by appropriate inactivation kinetic studies.
The concentrated antigens are stored at +2 °C - +8 °C for a maximum period of 12 months until they are used for blending purposes. The bulks of active ingredients are blended with other components to an emulsion, filled in defined containers, labelled and packed to obtain the finished product. The maximum blending volume will be 300 litres.
The volume of antigens to be added in order to obtain the target concentration of 1 x 10^{10} microorganisms of each antigen per dose of 2 ml is calculated on the basis of concentration of total bacteria determined after the concentration step which follows the inactivation step. The method is considered properly validated for both antigens *E. coli* J5 and *S. aureus*.
The consistency of the production is demonstrated on three pilot batches and one commercial batch.

Control of starting materials

Active substances

The original strain of *Staphylococcus aureus* CP8 was obtained from the isolate collection from DIAGNOS, the Diagnostic Centre of LABORATORIOS HIPRA, S.A. This strain was characterized as a phenotype producer of Slime (SP) by means of immunoelectrophoresis (IEP) and the Congo Red test. It was also determined by immunoelectrophoresis (IEP) as a strain belonging to Capsular Polysaccharide 8 (CP8).

The strain *Escherichia coli* J5 used in the production of STARTVAC vaccine was isolated from old cultures *E. coli* O111:B4 by selection and subsequent cultivation of colonies "galactose-negative" (characterised by its lack of colour) and sensitive to galactose (characterised by its tendency to disintegrate after prolonged incubation).

Information relating to the vaccine strains *E. coli* J5 and *S. aureus* CP8, their origin, characterisation, passage history, preparation and storage conditions has been provided. Seed lot systems have been followed. Identity and purity of MSB and WSB have been confirmed by morphology, growth characteristic and biochemical analysis. Serotyping will be introduced as a routine control in any new *E. coli* working seed and *S. aureus* working seed.

Excipients

Starting materials listed in a pharmacopoeia are sodium alginate, calcium chloride dehydrate, liquid paraffin, benzyl alcohol, Polysorbate 80, simeticone, sorbitan oleate, sodium hydroxide, glucose monohydrate, formaldehyde solution (35%), sodium chloride, potassium chloride, disodium phosphate dodecahydrate, gelatine (from porcine skin), sucrose, povidone, monosodium glutamate, water purified, water, highly purified and water for injections.
All starting materials are referred to *Ph. Eur*. with the exception of monosodium glutamate for which reference is made to the US Pharmacopoeia (USP). Defined specifications are provided in the Certificates of Analysis (CoA). The Certificates of Analysis comply with the related monograph and results match every specification.

Starting materials of biological origin not listed in a pharmacopoeia are Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB) and yeast extract. The following information about the TSA and TSB is included in the dossier: Certificate of analysis, raw material quality control sheet, copy of the catalogue of the supplier, technical sourced raw materials document, animal origin position statement, letter about the bovine milk component as well as information about their sterilisation. Yeast extract is derived from the soluble part of the yeast cells after autolysis. The corresponding certificate of analysis is provided. It is sterilised together with the culture medium once prepared.

The following in-house media are used: freeze-drying excipient, TSB-G medium, CB120 culture medium, PBS solution, sodium hydroxide solution and antifoam solution. The composition, preparation and sterilisation are adequately described. Sterility control is performed by direct inoculation. Shelf lives and storage conditions are defined.

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

The following 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 Directive 2001/82/EC: *Escherichia coli* J5, *Staphylococcus aureus* CP8, Seed Lots TSA and TSB .

Control tests during production

During manufacture the following *in-process* controls are carried out to assure the quality parameters.

***Staphylococcus aureus* CP8:**
Gram stain, viability/ purity, identity, count of viable bacteria, count of total bacteria, inactivation, pH, sterility and SAAC concentration.

***Escherichia coli* J5:**
Gram stain, viability/ purity, identity, count of viable bacteria, count of total bacteria, inactivation, pH and sterility.

Detailed information of the methods, their frequency, their function and their specifications are included in the dossier. Maximum pre-inactivation specifications (viable count according to the inactivation kinetic studies) are established.

The following methods are adequately validated:
- Concentration of viable bacteria
- Concentration of total bacteria
- SAAC concentration
- Test for complete inactivation.

Control tests on the finished product

The description of the methods used for the control of the finished product (appearance, viscosity, identification and quantification of the preservative, pH, volume control, residual formaldehyde, conditioning, sterility, determination of endotoxins, safety test, potency test: vaccination of rabbits and indirect ELISA for determination of *E. coli* J5 antibodies and *S. aureus* anti-slime antibodies) and the specifications are provided.

The specifications proposed are appropriate to control the quality of the finished product.

The results of the analysis of three consecutive pilot batches and one commercial batch were presented and comply with the required specifications.

The following methods are adequately validated:
- Identification and quantification of the preservative
- Residual formaldehyde
- Sterility test
- Determination of endotoxins
- Batch potency tests.

The handling of OOS results has been satisfactorily addressed. Furthermore, amendments regarding procedures for safety testing and vaccination of rabbits were made in accordance with the list of outstanding issues.

Stability

An antigen stability study has been provided. It has been properly demonstrated that antigen stocks stored for 12 months (2° C – 8° C) before blending are stable over the claimed shelf-life of the vaccine.

Samples (1 dose and 25 doses, respectively) from three consecutive pilot batches filled in glass bottles (colourless, Type I with rubber stoppers, Type I) were included in the stability studies. The parameters evaluated, their specifications and the methods were the same as established for the final product testing with the exception that volume and endotoxin content were not controlled. This was sufficiently justified.

The results support a shelf-life of 18 months post-manufacturing. This shelf-life is substantiated by appropriate data.
It was further demonstrated that batches released with the minimum acceptable value of potency will be stable over the claimed 18 months storage period.

The proposed in-use shelf-life of 10 hours after first opening of the bottle was considered sufficiently substantiated by appropriate data (microbial safety, sterility and potency results).

New real time stability studies with one commercial batch (Batch no. 5Z5Y filled in 25-dose presentations and 1-dose presentations using the newly introduced 3 ml vial) have been initiated. The Applicant has undertaken the commitment to provide the results in regular intervals until the foreseen end of the stability study (Feb. 2010).

Overall conclusion on quality

The product is manufactured in accordance with the principles of Good Manufacturing Practice at a licensed manufacturing site.
Process validation data on the product have been presented in accordance with the relevant European guidelines.
The documentation meets the requirements of Directive 2001/82/EC and the current *Ph. Eur.* monographs as well as the relevant guidelines.

The necessary data on the qualitative and quantitative composition have been provided.

The majority of starting materials used in production comply with pharmacopoeial monographs.
Biological starting materials used are in compliance with the current regulatory texts related to the TSE Note for Guidance (EMEA/410/01-Rev.2) and Directive 2001/82/EC.
The master and working seeds have been produced according to the Seed Lot System.

The tests performed *in-process* are described and the results of 3 consecutive runs, conforming to the specifications, are provided.
The tests performed on the final product are in compliance with the relevant requirements. The tests include validated potency tests (vaccination of rabbits and indirect ELISA for determination of *E. coli* J5 antibodies and *S. aureus* anti-slime antibodies), content of residual formaldehyde, sterility and determination of preservative (benzyl alcohol) as well as endotoxin measurement. Furthermore, appearance, viscosity, safety, extractable volume and pH measurement are established as final product tests.
The controls during production and on the finished product guarantee compliance with the specified quality parameters.

Demonstration of the batch-to-batch consistency is based on the results of 3 batches (pilot) produced according to the method described in the dossier. Other supportive data (commercial batch) provided confirm the consistency of the production process.

Real time stability data on the finished product have been provided, demonstrating the stability of the product throughout its shelf-life (18 months) when stored under the approved conditions.
It has been properly demonstrated that antigen stocks stored for 12 months (2° C – 8° C) before blending are stable over the claimed shelf-life of the vaccine**.**
The in-use shelf-life of the broached vaccine (10 hours) is supported by data (microbial safety, sterility and potency).

3. SAFETY ASSESSMENT

Laboratory tests

Four laboratory studies were performed to assess the safety of the administration of a single, double and repeat single dose using batches of standard antigen content (antigen load is fixed to 1 x 10^{10} microorganisms for *S. aureus* and *E. coli* J5, respectively). The studies have been conducted according to GLP. The animals used were of the appropriate target species cattle and the most sensitive category of the target species for which the vaccine is intended for (heifers = primiparous cows, in the last trimester of pregnancy) has been included in the trials. Only heifers, verified serologically, were included.
One additional laboratory study was performed with two experimental vaccine batches which were not fully in compliance with the composition of the vaccine STARTVAC.

Safety of the administration of one dose

Three studies were performed to investigate the safety of the administration of a single dose of the vaccine STARTVAC to target animals.
Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route 45 and 10 days before the expected parturition date (EPD) with a single dose of either the vaccine STARTVAC or a placebo (without antigens).
The parameters local reactions, general clinical signs, rectal temperature, serological responses and evolution of pregnancy and new born calves were examined.
No general reactions or adverse side effects were observed. In a few animals, a slight local swelling, scored as 1 (nodule < 2cm) was noticed 24 and/or 48 hours after 1st injection. No histopathological lesions were observed in the musculature of the immunised animals.

One additional laboratory study was performed with two experimental vaccine batches to investigate the safety of the administration of a single dose. The composition of these vaccine batches did not comply completely with the composition of the vaccine STARTVAC. The concentration of *S. aureus* was 2 x 10^{10} total bacteria per dose instead of 1 x 10^{10}. Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route 45 and 10 days before the EPD with a single dose of either one of the two vaccine batches or a placebo (without antigens).
The parameters local reactions, general clinical signs, rectal temperature, serological responses and evolution of pregnancy and new born calves were examined.
No local reactions and no general reactions or adverse side effects were observed.

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

One study was performed to investigate the safety of the administration of an overdose and the safety of the repeated administration of one dose of the vaccine STARTVAC in target animals.

Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route as follows: 1st injected with a double dose 45 days before the EPD, 2nd injection 35 days thereafter (corresponding to 10 days before the EPD) with a single dose and 3rd injection 28 days after the 2nd injection (corresponding to 18 days after the EPD).
The parameters local reactions, general signs, rectal temperature, serological responses and evolution of pregnancy and new born calves were examined.
No general clinical signs or adverse side effects in the gestating cows or their progeny were observed after all three injections. Slight to moderate local reactions were noticed after administration of the double dose characterised by swellings (up to 5 cm^2) or nodules and local pain. The reactions disappeared within a few days.

Examination of reproductive performance

In four laboratory studies, the influence on gestation, calving and the progeny was investigated after immunisation of pregnant heifers during the last trimester of pregnancy.

Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route as follows: 45 and 10 days before the EPD with a single dose of either the vaccine STARTVAC or a placebo (without antigens) or 1st injection with a double dose 45 days before the EPD and 2nd injection 35 days thereafter (corresponding to 10 days before the EPD) with a single dose.
The evolution of pregnancy and new born calves was examined.
No negative influence on gestation, calving and the progeny of the heifers was observed after immunisation

Examination of immunological functions

No specific studies have been carried out based on the justification that *E. coli* J15 and *S. aureus* have not the potential to adversely affect the immune system.
STARTVAC is a vaccine containing inactivated bacteria. Replication of vaccine bacteria in any cells involved in the immunised animals immune system is therefore not applicable and subsequently impairment of the immune system is not to be expected.

Special requirements for live vaccines

Not applicable as STARTVAC is an inactivated vaccine.

Study of residues

No specific study on residues was performed. A withdrawal period of zero days was proposed and accepted.

The vaccine is inactivated and therefore the determination of residual organisms at the injection site is not applicable. The adjuvant and other components used for the formulation of STARTVAC are included in the Regulation (EEC) No. 2377/90 and included in Annex II.

Interactions

No specific studies on interactions with other immunologicals or veterinary medicinal products were performed since no interaction is likely to occur.

Field studies

One multicentre field study was carried out in primiparous and multiparous cows using a batch of standard antigen content to assess the safety of the vaccine in dairy cows under real field conditions. The field trial was conducted according to GCP as a randomised double-blind, placebo controlled study.

Cows and heifers at an age of 22 months onwards were immunised in accordance with the proposed immunisation schedule by intramuscular route either with the vaccine STARTVAC or with a placebo: 1st injection 45 days before the expected parturition date, 2nd injection 35 days thereafter (corresponding to 10 days before the expected parturition date) and 3rd injection 62 days after the 2nd injection (corresponding to 52 days after the expected parturition date).

The parameters local reactions, general clinical signs and adverse events, rectal temperature, effects on reproductive parameters and milk production (as secondary safety parameter) were examined.

Only individual animals showed general clinical signs scored as 1 or 2 at one or several observation time points. A significant increase of the rectal temperature in immunised cows was observed 4 hours after each injection. Single immunised animals showed an increase of up to 1.8° C, two immunised cows even more than 2° C. The rectal temperature became normal within the next 24 hours. Slight (< 2 cm) to moderate (2-5 cm) local swellings or nodules were observed after injections which normally disappeared within a few days. Local pain was recorded in single animals. Injections did not have any adverse effects on gestation, calving or the progeny of the immunised cows. Milk production was not affected.

User safety

STARTVAC is an inactivated vaccine.
The raw materials used to prepare active ingredient and vaccine comply with the relevant *Ph. Eur*. monographs (where applicable) and are carefully controlled to prevent contamination with other infectious agents. The adjuvant comprises liquid paraffin. The excipients are sorbitan monooleate, Polysorbate 80, sodium alginate, calcium chloride dehydrate, benzyl alcohol, simeticone and water for injections. All components are included in Regulation (EEC) No. 2377/90/ and included in Annex II.
Liquid paraffin is a mineral oil but its concentration in the vaccine is very low compared to other oily vaccines. It is known that, in case of accidental self-injection, an oily adjuvant might cause local tissue irritation and lesions to the person administering the vaccine. For that reason, the applicant has included in the SPC (section 4.5.) an advice to the user and to the physician in case of accidental injection/self injection.

Benzyl alcohol is used as preservative with a quantity of 20 mg per dose in order to limit risks of product contamination after first use. It is used extensively as an antimicrobial preservative in a wide range of cosmetics and pharmaceutical formulations, including oral and parenteral preparations. Therefore, the preservative is not expected to represent a hazard to the user.
Sorbitan monooleate and Polysorbate 80 (authorised food additive-E433) are emulsifiers which promote the dispersion of watery droplets of antigen throughout the oil. The use of these emulsifiers does not represent a toxicity hazard to the user.

Sodium alginate is a gelification polysaccharide extracted from giant brown seaweed that precipitates in presence of calcium chloride. Simeticone is a commonly used antifoam, water for injections is the dilution vehicle of the vaccine and also commonly used in medicinal products for parenteral administration. Their utilisation does not represent a risk.

The conclusion that no specific risk associated with the use of this vaccine is identified is supported.

Environmental risk assessment

A Phase I environmental risk assessment was conducted, including a hazard identification and assessment of the exposure to the hazard as well as the likelihood that the hazard may occur. As no hazard can occur, the likelihood of hazard is negligible and the consequences of the occurrence of any hazard can be considered as negligible. Therefore, the risk can be considered effectively zero.

Therefore, a Phase II study has not been considered necessary or adequate given the very low environmental risk potential of the vaccine.

Overall conclusion on safety

The studies presented in the safety part were satisfactorily described. The Applicant conducted adequate laboratory studies and one field study to assess the safety of a single, double and repeat single dose after intramuscular administration using batches of standard antigen content in primiparous and multiparous cows during the last trimester of gestation. The vaccine may induce slight to moderate local reactions in the target animal, cows and heifers. These local reactions were characterised by slight (< 2 cm) to moderate (2-5 cm) local swellings or nodules and local pain. The reactions were transient; normally they disappeared within a few days. An increase in rectal temperature could be ob-

served 4 hours after injection. The rectal temperature became normal within the next 24 hours. Other general clinical signs scored as 1 or 2 were only observed in a small number of animals. That means the vaccine will be well tolerated by primiparous and multiparous cows immunised in the last trimester of gestation.

No negative influence on gestation, calving and the progeny of the cows was observed after injection of pregnant cows during the last trimester of pregnancy. An assessment of ecotoxicity risks showed that the overall risk represented by the vaccine to the environment is effectively zero. No specific risk is expected for the user, the consumer and other animals.

4. EFFICACY ASSESSMENT

Introduction and General Requirements

Efficacy studies have been carried out in the target species, heifers (dairy cows in their first pregnancy) and pregnant cows (multiparous cows).

The selected challenge strain of *S. aureus* has been described as virulent strain in several published works and is assayed for virulence in own trials. The challenge strain is a slime producing strain which can even afford cross-protection immunity against coagulase-negative staphylococci (CNS).

The selected challenge strain of *E .coli* was assayed for virulence in own trials. The challenge strain possesses the *E. coli* core oligosaccharide-lipid A complex (common core antigen). It features chemical, structural as well as immunologic homology across species and genera of Gram-negative bacteria, which can even confer cross-protection immunity against coliforms.

Six laboratory studies and one field trial have been conducted:

- Studies for verifying the capacity of the test challenge strains to reproduce mastitis
- Studies assessing efficacy by challenge with *S. aureus* and *E. coli*
- Studies assessing duration and onset of immunity after basic immunisation and re-immunisation
- A field trial.

Laboratory trials

Two intramammary challenge studies were performed to study the pathogenicity of *S. aureus* and *E. coli* strains.
Three laboratory studies were performed to assess the efficacy of the vaccine STARTVAC using batches of standard antigen content (antigen load is fixed to 1 x 10^{10} microorganisms for *S. aureus* and *E. coli* J5, respectively). The studies have been conducted according to GLP. The animals used were of the appropriate target species, cattle, and the most sensitive category of the target species for which the vaccine is intended for (heifers = primiparous cows, in the last trimester of pregnancy) has been included in the trials.
One additional laboratory study was performed with two experimental vaccine batches which were not fully in compliance with the composition of the vaccine STARTVAC.

As requested, the results of a previous study were provided to assess the immunogenicity of the slime-associated antigen of a *S. aureus* bacterin. Heifers were immunised according to the immunisation scheme (first and second injection) with STARTVAC or PBS. Blood samples were taken from all heifers on day 0, 35, 49, 56, 77 and 98 respectively. It was shown that the experimental vaccine, manufactured with *S. aureus* slime antigen, is able to induce a high and persistent humoral immunity in heifers.

Establishment of a Challenge Model

The capability of challenge strains of *S. aureus* and *E. coli* was assessed to reproduce mastitis in cows in their first lactation cycle after inoculation of a suspension of these bacteria by the intramammaryroute to different quarters.

In each case, two *S. aureus* and *E. coli* strains were used as challenge strains, one strain in two inoculation doses.

The parameters examined were general clinical signs including rectal temperature, histopathological analysis of mammary tissue, clinical signs of mastitis (quarter and milk appearance), bacterial count and somatic cell count in milk, milk production, serological evaluation (anti-slime antibodies in serum and milk, determination of anti-*S. aureus* antibodies in milk).

It was shown that the chosen infection method – intramammary route – was adequate for this purpose for both strains.

After intramammary challenge with *S. aureus,* a significant increase of the rectal temperature was detected in all animals 24 hours after challenge, an inflammatory reaction caused in quarters infected with *S. aureus* was observed*,* clinical signs of mastitis manifested in all quarters inoculated with the strains of *S. aureus* after challenge and a multiplication of both strains in the cistern of the mammary gland was observed. The correlation between the bacterial count in milk and clinical signs of mastitis was statistically significant and an increase in the number of somatic cells was detected in all inoculated quarters from 24 hours after challenge onwards. Daily milk production was reduced 1 and 2 days post-challenge, respectively.

The results obtained demonstrated the capacity of the test strains to cause sub-acute clinical mastitis in infected quarters. Although the results were satisfactory for both strains tested, the strain producing slime was chosen.

After intramammary challenge with *E. coli*, clinical signs of mastitis scored 2 and 3 were seen in the form of clots in the milk 24 and 48 hours post-inoculation in two of three quarters and in 3 of 6 quarters. Maximum titres of the *E. coli* challenge strains were reached mainly within the first 24 hours post-challenge in all the infected quarters. An increase in the number of somatic cells in the milk of most of the infected animals was seen.

The results obtained demonstrated the capacity of the test strains to cause sub-acute or sub-clinical mastitis in infected quarters. Because of the score of the clinical signs of mastitis and the results of the count of cfu/ml, the *E. coli* strain was chosen.

Onset of protection

One laboratory study was performed with two experimental vaccine batches to investigate the efficacy of a bacterial strain of *S. aureus* compared to an intramammarian challenge in cows with a heterologous strain of *S. aureus* and to assess the degree of protection that a slime antigen associated with *S. aureus* confers. The composition of these vaccine batches did not fully comply with the composition of the vaccine STARTVAC. The concentration of *S. aureus* was 2 x 10^{10} total bacteria per dose instead of 1 x 10^{10}.

Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route 45 and 10 days before the EPD with a single dose of either one of the two vaccine batches or a placebo (without antigens) and challenged at an average of 23 days post-parturition. Non-immunised, non-challenged animals were also included.

The parameters examined were: general clinical signs including rectal temperature, clinical signs of mastitis (quarter and milk appearance), milk production, bacterial count and somatic cell count in milk, serological evaluation (anti-slime and anti-*E. coli* J5 antibodies in serum and anti-slime, anti-*S. aureus* and anti-*E. coli* J5 antibodies in milk).

Immunisation with the two experimental vaccines induced a serological response of anti-slime antibodies in serum and also of anti-*E. coli* J5 antibodies in serum. On day 0 of the challenge, the average IRPC anti slime value in milk was higher in animals immunised with the higher concentration of SAAC.

After challenge, the maximum severity of clinical symptoms in the form of presence of lumps in milk and/or induration or local inflammation of quarters was seen 48 to 72 hours post-challenge in quarters inoculated with *S. aureus*. A multiplication of the challenge strain in the mammary gland was observed; the number of cfu per ml of milk progressively increased until 24 hours after challenge. Taking the entire post-challenge period as a whole, animals immunised with the higher concentration of SAAC had lower bacterial counts in milk than the others.

Nevertheless, it is difficult to draw a conclusion on the efficacy of these vaccine combinations from the results of the study. The argumentation that there is a connection between the higher value of IRPC anti-slime in serum and a lower bacterial count in milk on the day of challenge and a smaller severity of clinical symptoms of mastitis after challenge can be supported.

Vaccines with a greater amount of slime associated per dose displayed protection against a challenge, from the point of view that the immunised animals had lower bacterial counts than the control group during the post-challenge phase of the study.

Another study was carried out to demonstrate by intramammarian challenge that immunisation with STARTVAC confers protection against virulent *S. aureus* in dairy cows.

Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route 45 and 10 days before the EPD with a single dose of either one of the two vaccine batches or a placebo (without antigens) and challenged at an average of 23 days post-parturition. The parameters examined were: general clinical signs including rectal temperature, clinical signs of mastitis (quarter and milk appearance), milk production, bacterial count and somatic cell count in milk, serological evaluation (anti-slime antibodies in serum and milk).

The count of *S. aureus* in milk showed that temporarily less infected quarters were proven in the immunised animals (9 hours, day 1+2) and generally lower bacteria counts in the infected quarters of the vaccinates were found. The vaccine significantly reduces the multiplication of *S. aureus* in the first 48 hours after challenge. Thus, the conclusion can be supported that the trend to the elimination of the infection is more favourable in the group of cows that received the vaccine.

The number of somatic cells in the immunised group after the first few hours of the challenge increased. The greater increase in somatic cells observed 9 hours post-challenge in the immunised cows can be attributed to the opsonisation of the bacteria by pre-existing specific antibodies (opsonins).

Immunisation induces a significant seroconversion of anti-slime antibodies in blood (humoral immunity) and milk (local immunity) with respect to the not immunised group. Since the humoral defence (formation of opsonins) is closely intertwined with the cellular defence and since both protection mechanisms aim at the elimination of pathogens, it can be assumed that the seroconversion in serum and milk accompanied by the negative correlation in a significant form with the count of *S. aureus* in milk 24 hours, 4, 7 and 21 days after the challenge can be an indicator of the protective effect of the vaccine against *S. aureus*.

In order to establish a minimum value of anti-slime antibodies of *S. aureus* (IRPC anti-slime), indicative of protection, the anti-slime response in serum obtained in the immunised group on the day of challenge, was used to calculate a so-called minimum protective value.

A third study was performed to demonstrate by intramammarian challenge that immunisation with STARTVAC confers protection against virulent *E. coli* in dairy cows.

Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route 45 and 10 days before the EPD with a single dose of either one of the two vaccine batches or a placebo (without antigens) and challenged at an average of 23 days post-parturition. Non-immunised, non-challenged animals were also included.

The parameters examined were: general clinical signs including rectal temperature, clinical signs of mastitis (quarter and milk appearance), milk production, bacterial count and somatic cell count in milk, serological evaluation (anti-*E. coli* J5 antibodies in serum and milk).

The count of *E. coli* of the challenged quarter was lower in the immunised group with respect to the non immunised group until day 8 post challenge, which may mean a reduction of the sub-clinical signs of *E. coli* mastitis. Cell numbers in the milk were increased in both groups after challenge; however, they then decreased more rapidly in the immunised animals. The greater severity of mastitis evaluated by the appearance of milk was observed in the non immunised group which may be due to the fact that the immunisation might reduce the clinical signs of *E. coli* mastitis.

The lower drop of milk production following challenge should be indicative of a reduction of the clinical signs of coliform mastitis in the immunised group. Also, a positive correlation between this reduced drop in milk production and the bacterial count of *E. coli* in milk has been demonstrated. In addition in the vaccinates, the milk quantity achieved 100 % of the pre-challenge level, while in the control group the milk production remained under the pre-challenge level throughout the post-challenge period. These observations demonstrate the reduction of the clinical severity of an important bovine variable such as milk production by means of immunisation.

In the immunised group, a significant anti-*E.coli* J5 seroconversion in serum was observed on day 14 after the first dose. The differences with respect to the non immunised group remained significant until the day of challenge, except for the day of immunisation (day 0) and day 35 post-immunisation (day of administration of second dose).

Although the anti-*E. coli* J5 response in serum in the immunised group was significantly greater than in the non immunised group, no significant differences in the response in milk were detected between the two groups on the day of challenge (59 days post-immunisation)

In order to establish a minimum rate of anti-*Escherichia coli* J5 antibodies (IRPC anti-*E. coli* J5), indicative of protection, the anti-*E. coli* J5 response in serum obtained in the immunised group on the day of challenge, was used to calculate a so-called minimum protective value.

Onset and duration of Immunity

In the case of STARTVAC, both immunisations were considered, i.e. the basic immunisation (1st injection: at 45 days before the EPD; 2nd injection: 35 days thereafter, corresponding to 10 days before the EPD) and the 3rd injection at day 97 (62 days after the 2nd injection, corresponding to 52 days after the expected parturition date) which is considered as a booster injection necessary to maintain the immunity, and being part of the regimen of immunisation. This regimen of immunisation must be carried out in each gestation period.

One study was carried out to investigate the onset and duration of the immunity of STARTVAC.
Heifers at an age of 22 months onwards were immunised by the recommended intramuscular route as follows: 1st injection 45 days before the expected parturition date, 2nd injection 35 days thereafter and 3rd injection 62 days after the 2nd injection.

In order to establish the onset and duration of the immunity of the STARTVAC vaccine, the serological response against *S. aureus* and *E. coli* obtained in the immunised group was compared with the serological response of the non immunised control group at different time intervals. Therefore, the antibody response against slime of *S. aureus* and against *E. coli J5* in serum was determined.

These established minimum protective values were tried to used as a basis for definition of onset and duration of immunity, but it was not accepted by the CVMP. Based on the results, obtained in the field study the following phrase was recommended: The full immunisation scheme induces immunity from approximately day 13 after the first injection until approximately day 78 after the third injection (equivalent to 130 days post-parturition).

Influence of Maternal Antibody on the Efficacy of the Vaccine

Not applicable as heifers and cows in the last trimester of gestation were immunised.

Field trials

One multicentre field study has been carried out in primiparous and multiparous cows using a batch of standard antigen content to determine the efficacy of the vaccine in dairy cows under real field conditions. The field trial was conducted following GCP as a randomised double-blind, placebo controlled study.

Farms with different conditions (type of milking, working procedures, park design, etc.), were included. The type of management employed in these farms (housing conditions, feeding, type of milking, parameterisation...) as well as the genetics of the animals used and the habitual mastitis problems found are common in the dairy farms around Europe.

Cows at an age of 22 months onwards were immunised in accordance with the proposed immunisation schedule by intramuscular route either with the vaccine STARTVAC or with a placebo: 1st injection 45 days before the expected parturition date, 2nd injection 35 days thereafter (corresponding to 10 days before the expected parturition date) and 3rd injection 62 days after the 2nd injection (corresponding to 52 days after the expected parturition date).

The following parameters were examined and evaluated:

- incidence of clinical mastitis (appearance of new cases of mastitis) by means of evaluating the general clinical symptoms and local clinical symptoms;
- incidence of subclinical mastitis by means of the aseptic taking of milk per cow (from the 4 quarters) for microbiological analysis and somatic cell count and individual recording of the daily milk production in the totality of the animals included in the trial;
- severity of the symptoms - somatic cell counts, general clinical signs, clinical signs (milk and quarter appearance), dead cows due to mastitis or severe mastitis, mastitis treatments with pharmacological products;
- spontaneous cure rate (cured cases of mastitis per number of infected animals);
- milk production, mastitis treatments and rate of antibodies in serum and milk samples.

The immunisation program as well as the dosage of 2 ml/animal and the administration route of the vaccine is efficacious in the reduction of the incidence of intramammary infection due to *S. aureus,* coliforms or coagulase-negative staphylococci, with clinical or subclinical manifestations in cows (multiparous) and heifers (primiparous) in the period of maximum incidence, i.e. post parturition. Immunisation also significantly reduces the severity of the symptoms, causes a significant increase in the spontaneous cure rate of the infected cows, significantly reduces the number of cows that need to be treated for mastitis and has positive effects on both the quantity and quality of milk production.

The rate of specific anti-slime antibodies of the STARTVAC group was significantly higher compared to the placebo group until the end of the study (130 days post-parturition) and the rate of specific anti-*E. coli* J5 antibodies of the STARTVAC group was significantly higher compared to the placebo group until approximately 90 days post-parturition.

The correlation between the serological response in serum (humoral) and milk (local) observed in the challenge laboratory trials and the two antigens was confirmed under field conditions.

The results obtained in the field trial demonstrate the efficacy of the STARTVAC vaccine.

Overall conclusion on efficacy

Main findings in the laboratory or pre-clinical trials
The vaccine significantly reduces ($p < 0.05$) the multiplication of *S. aureus* in the first 48 hours after challenge. A significant increase ($p < 0.05$) is also observed in the number of somatic cells in the immunised group after the first few hours of the challenge, which would indicate a greater cellular response that contributes to an increase in phagocytosis and, therefore, to a reduction in infection.
Immunisation significantly reduces ($p < 0.05$) the drop in milk production caused by an intramammarian challenge with a virulent strain of *E. coli*. Also, a positive correlation between this reduced drop in milk production and the bacterial count of *E. coli* in milk has been demonstrated.

Main findings in the field trials
The immunisation program as well as the dosage of 2 ml/animal and the administration route of the vaccine are efficacious in the reduction of the incidence of intramammary infection due to *S. aureus,* coliforms or coagulase-negative staphylococci, with clinical or subclinical manifestations in cows (multiparous) and heifers (primiparous) in the period of maximum incidence, i.e. post-parturition. Immunisation also significantly reduces the severity of the symptoms of clinical or sub-clinical mastitis, causes a significant increase in the spontaneous cure rate in the infected cows, significantly reduces the number of cows that need to be treated for mastitis and has positive effects on both the quantity and quality of milk production.

The indication also includes the protection against the coliforms. The vaccine strain can confer cross-protection immunity against the coliforms attributable to the common core antigen. Also in consideration of the fact that the results of the field trial showed a significant reduction in intramammary infections caused by coliforms in the immunised heifers and cows in comparison to the placebo group, the conclusion that the vaccine also protects against coliforms is acceptable.

The indication also includes the protection against coagulase-negative staphylococci (CNS). The slime characteristic of the vaccine strain can afford cross-protection immunity against CNS species. In consideration of the fact that the results of the field trial showed a significant reduction in the incidence of mastitis attributed to CNS in immunised heifers and cows in comparison to the placebo group, the conclusion that the vaccine also protects against CNS is acceptable.

5. BENEFIT RISK ASSESSMENT

STARTVAC is intended for use in healthy cows and heifers in dairy cattle herds with recurring mastitis problems in order:

- to reduce the incidence of intramammary infection caused by *S. aureus*, coliforms or coagulase-negative staphylococci, with clinical or subclinical manifestations (incidence means new cases of mastitis per number of healthy animals at risk during the observation period)
- to reduce the severity of the symptoms of the intramammary infection caused by *S. aureus*, coliforms or coagulase-negative staphylococci in the immunised group with respect to the control group based on analysis of Somatic Cell Counts (SCC), clinical signs, mastitis treatments and dead cows due to mastitis or severe mastitis.

Immunisation has positive effects on both the quantity and quality of milk production. Furthermore, a significant reduction of the number of mastitis treatments with antibiotics per cow was observed in the immunised group. Vaccination also increases the spontaneous cure in the immunised group (cure rate means the cases cured of mastitis per number of infected animals during the observation period).

The risk assessment is based on the estimated risks to target and non-target animals, users, consumers of animal derived food and to the environment.

STARTVAC is an inactivated vaccine. The inactivation procedures and test on inactivation have been adequately validated. The intramuscular administration route does not allow release of the vaccine into the environment. The inactive antigens will be metabolised in the target animals and are therefore considered irrelevant concerning possible risks through residues. Thus, the proliferation, persistence and excretion of vaccine germs can be excluded. The adjuvant, the preservative and other constituents can be regarded as safe in the concentration used for the product.
Therefore, there is no risk regarding the transmission of live organisms to target and non-target animals, no shedding and no capacity of live product organisms to survive, establish and disseminate, and no pathogenicity to other organisms.

Based on a Phase I environmental risk assessment it can be concluded that the vaccine represents a negligible risk to the environment.

The vaccine does not contain any ingredients that are likely to pose a risk for consumers of milk and meat.

STARTVAC contains liquid paraffin, a mineral oil as adjuvant but the concentration is low. It is known that, in case of accidental self-injection, an oily adjuvant might cause local tissue irritation and lesions to the person administering the vaccine.

In the target animal, slight to moderate transient local reactions may occur after the administration of one dose of vaccine. They would mainly be: swelling (up to 5 cm^2 on average), which disappears within 1 or 2 weeks at most. In some cases, there may also be pain at the inoculation site that spontaneously subsides in a maximum of 4 days. A mean transient increase in body temperature of about 1° C, in some cows up to 2° C, may occur in the first 24 hours after immunisation.
No negative influence on gestation, calving and the progeny of the cows was observed after immunisation of pregnant cows during the last trimester pregnancy.

Based on the data presented immunisation with STARTVAC reduces the incidence of sub-clinical mastitis and the incidence and severity of the clinical signs of clinical mastitis caused by *S. aureus*, coliforms and coagulase-negative staphylococci.

The risk of the use of the vaccine STARTVAC for the immunised animal can be evaluated as minimal. Only slight to moderate transient local reactions and a transient increase in body temperature in the first 24 hours after immunisation may occur.

For the user, special safety precautions (risk management measure) are mentioned in the product information as the vaccine contains mineral oil as adjuvant and it is known that, in case of an accidental self-injection, local tissue irritation and lesions to the person administering the vaccine can occur.

The benefits of the vaccine as stated above have been sufficiently substantiated. The risks identified for the target species, the user and the environment are considered acceptable. Therefore, the overall benefit-risk balance is considered as favourable.

Preventive measures other than immunisation for mastitis control that should be applied, within a good management program, include: (a) a clean, stress-free environment (b) proper maintenance and operation of milking equipment; (c) good milking procedures including teat dipping; (d) a dry cow treatment program and culling chronic cows when necessary; and (e) a program for monitoring the health status of udders.

Based on the original and complementary data presented the Committee for Medicinal Products for Veterinary Use (CVMP) concluded that the quality, safety and efficacy of STARTVAC were considered to be in accordance with the requirements of Directive 2001/82/EC, as amended, and that the benefit-risk balance was favourable.