

CIFSRF final technical report: Development of a subunit vaccine for contagious bovine pleuropneumonia in Africa (CIFSRF Phase 2)

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Research Organizations involved in the study:

Kenya Agricultural and Livestock Research Organization (KALRO)

Vaccine and Infectious Disease Organization – International Vaccine Centre
(VIDO-InterVac)

Department of Veterinary Services – Kenya (DVS)

Kenya Veterinary Vaccines Production Institute (KEVEVAPI)

International Livestock Research Institute (ILRI)

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1. Executive Summary

The objective of this project was to develop a safer and more effective vaccine for Contagious Bovine Pleuropneumonia (CBPP) - one of the most economically important diseases in sub-Saharan Africa. We developed a novel subunit vaccine based on a combination of four recombinant proteins, which was 81% effective versus 22% conferred by the existing live attenuated vaccine. The vaccine formulation was patented and licensed to KEVEVAPI for commercial production and marketing.

In Phase 1 we developed and patented three prototype vaccines, which protected animals challenged with the CBPP infectious agent. In Phase II of this project, we evaluated formulations of the experimental vaccine, tested and selected vaccine adjuvants, constructed vectors and strains for industrial production, and compared the new vaccine formulation to the existing vaccine using animal trials. The two most prevalent cattle breeds in East Africa,- Boran and Zebu were used in the animal trials. The project also supported transition to manufacturing of the vaccine at the Kenya Veterinary Vaccine Production Institute. A socio-economic program integrated at the beginning of the project addressed aspects of disease impact and vaccine adoption in support of a product rollout.

The trials for selection of adjuvants took place at the station in Muguga, Kenya. Adjuvants tested included Emulsigen, CpG ODN 2007, host defence peptides, EP3 and Montanide ISA 61 VG. The groups immunized with formulations containing Montanide ISA 61 VG produced the most desirable immune responses and was compatible with existing formulation capabilities at KEVEVAPI.

The final vaccine formulation was tested in a vaccination experiment in Zebu administered twice (vaccination and boost) at an interval of 28 days. It included four *Mycoplasma mycoides* proteins and the adjuvant Montanide ISA 61 VG and was compared to the currently used live attenuated CBPP vaccine, administered once. The results showed that the new recombinant vaccine conferred 81% protection while the live attenuated vaccine had protection of 22% in the same experiment.

In support of transition to manufacturing, the team developed and tested vaccine formulations, aiming at selection of most effective protection, accompanied with reduction of production costs. The efforts continued by the laboratory in Muguga, which developed small scale production protocols. The molecular biology laboratory at the same centre performed the analytical procedures for the animal trials conducted in 2017-2018. These activities established new capacity to produce and evaluate recombinant vaccines, and to support vaccine production development independently. Another step towards manufacturing was the execution of a license

agreement by the three IP holder institutions (KALRO, ILRI and VIDO-InterVac) on one side and the vaccine manufacturer KEVEVAPI on the other side.

The focal point of the socio-economic team was the economic impact of CBPP and the potential of the new vaccine to reduce it. Studies aimed at the impact along the value chain calculated the cost of one outbreak in a single county (out of 14 counties directly affected with outbreaks) to be \$125,000 in loss of production and control measures cost. Examination of factors that may affect adoption and rollout of the vaccine revealed low correlation between willingness to pay and net incomes. The proportion of farmers willing to pay for the benchmark price of KES. 34.6 (0.44CAD\$) was only 59%, while 80% vaccination is required to interrupt transmission. Additionally, we found that a significant proportion (39.9%) of households have negative net incomes even with additional support.

A policy study was carried out to map policies, related to CBPP control and eradication. These studies demonstrated KAP gaps and low vaccination coverage (20-60%) were linked mainly to undesirable post-vaccination reactions, and deficiencies in control resources, benefit-cost justification and policies.

The project term ended just before production of the vaccine for a large field trial in commercial cattle to assess safety, efficacy and duration of immunity of the vaccine.

The results of our work were published in three peer-reviewed journal publications, eleven presentations (five of them invited, and two keynote addresses) at scientific meetings, and in several media reports. Two stakeholder meetings informed key partners among which were Pharmacy and Poisons Board, Department of Veterinary Services, National Biosafety Board, Kenya Veterinary Board, National Council for Science and Technology, Directorate of Veterinary Medicines (DVM), FAO, GALVmed, and Kenya's Veterinary Medicines Directorate.

In January 2018 the Deputy President of Kenya William Ruto honoured the Kenyan CBPP vaccine project team with the Public Service Excellence Award (PSEA), with certificates issued to the team and accepted by Dr. Salome Kairu Wanyoike, (Deputy Director of the Directorate of Veterinary Services and CBPP vaccine project socio economic team leader). This was a unique team composed of project, national and sub-national personnel. During the award, a citation was broadcast which highlighted the achievements of the project as: development of new vaccine to control CBPP in Africa, assistance offered to farmers in Laikipia and Kajiado counties during CBPP outbreaks through investigations and launching of mass screening as well as policy studies. It was recognized that in Laikipia, the highest national and sub-national authorities were involved in communicating the risk of CBPP to the farmers in a high impact awareness creation campaign organized by the project and captured in national media. Gendered project activities in Garissa county in 2014/15 were also cited. The citation included two video clips which summarized the key results of the project. The award also covered other activities of the team towards control of

CBPP, among them preparation of the CBPP control strategy and contingency plan in Kenya as well as national training on CBPP surveillance in collaboration with the AU-IBAR, responsible for the control of CBPP in the African region.

2. The Research Problem

Animal infectious diseases are the main cause of economic losses in the livestock sector worldwide. The effects of an outbreak directly decrease productivity and product quality and are further magnified by the additional costs of treatment, and by the downstream countermeasures like limitations of livestock movement and restrictions of national and international trade. Movement restrictions and culling of infected or exposed animals have historically achieved a degree of success in infectious disease eradication. This approach is limited to historical periods when a given country has both, the funding and the level of control over the livestock industry to carry out quarantines and culling. However, funding and logistics are necessary, but not sufficient as demonstrated by the political backlash, which followed quarantines and culling in the last few years when African swine fever, lumpy skin disease and *peste des petits ruminates* occurred in several EU countries. Therefore, we have suggested vaccination as an evidence-based method of preventing infectious disease and aimed to contribute to prevention of contagious bovine pleuropneumonia (CBPP).

Both AU-IBAR and OIE recognise CBPP as one of the livestock diseases in Africa with the highest economical importance. This disease poses a direct threat to the food security of over 24 million people - all in the low-income bracket in the African countries. The impact on the economies is very difficult to calculate, but has been estimated to be 2 billion dollars per annum at a minimum (1)

Commercially available CBPP vaccines are based on attenuated *Mycoplasma mycoides* strains, the most widely used being T₁/44, T₁-SR, KH₃J, KH₃J-SR, and PG₁. Despite their relatively low protective efficacy, they have been used to control CBPP outbreaks. A serious set of problems for these vaccines includes severe post-vaccination reactions, lung lesions, weak immune reactions, difficulty in establishing master seed stocks, difficulty in differentiating infected from vaccinated animals, variability of dose titers, and variance between passages. Our project aimed at improving food security in Sub-Saharan Africa by developing a novel vaccine for the prevention of CBPP. Our objectives had two focus areas. One was the identification of a CBPP vaccine with high protective efficacy, high safety in both production and application, low cost, easy for production and quality control, with capacity to differentiate vaccinated and infected animals (DIVA) and with a good potential for acceptance by the public (absence of any pathogens in the

formulation, low post-vaccination reactions). All these conditions could be met by exploring recombinant *M. mycoides* antigens for their ability to induce protective immunity, followed by the formulation of a vaccine and scale-up for production. The second focus area was the identification of socio-economic factors, which will enable the use of the new vaccine in the field. For this we aimed at studying the knowledge, attitudes, practices and perceptions of various stakeholders, the willingness of cattle farmers to pay for the vaccine, the policies of CBPP control, and the economic impact of the disease.

The search for a protective combination of recombinant antigens involves very high numbers of proteins, and a corresponding high number of protective efficacy trials. To identify antigens and formulate a vaccine, we have used reverse vaccinology – an approach, which relies on genome analysis, and on computational predictions of protein structure and immune reactions to reduce the inherent biases of traditional methods for antigen selection. The identified proteins were then produced as recombinant antigens and tested for protective efficacy against CBPP in cattle.

The vaccine candidates were patented in “*Mycoplasma Vaccines and Uses Thereof*”, US Patent Application Serial No. 62/195,581 filed on July 22 2015. Laboratory-produced vaccine prototypes were tested for protective efficacy in animal trials, followed by construction of strains for industrial production, and by formulation of the new vaccine. At this stage our attention was focused on potential for protection, but also on factors influencing the production cost of the antigens. We have selected an expression system which is well characterized, has showed high yields and good purity of the expressed products – all characteristics aiming at low cost of a vaccine dose. We also constructed some fusion proteins to further reduce the cost but could not implement fusion antigens in the final formulation because the size of the best protective antigens (plus time and budget restrictions) did not allow us to search for protective epitopes in these large proteins.

Our trials confirmed the main advantages of the new vaccine: easy and safe production, good potential for production scale-up, high vaccine safety, potential for high stability, well defined vaccine formulation, and very good protective efficacy.

The socio-economic program determined that most pastoralists perceive vaccination as the only effective control measure for CBPP. Despite this, vaccination coverage in endemic areas has been shown to be rather low. The underlying problems include vaccine and vaccination related policy issues as well as lack of cost-benefit analyses to justify use of the disposable incomes of livestock keepers and/or public funds to control the disease. Most of the survey participants also rated highly the vaccine attributes which our project is aimed to deliver. In addition, the improved vaccine with preferred attributes is predicted to have a higher demand by pastoralists when compared to the current vaccine. Completed willingness to pay (WTP) studies have shown that livestock keepers are generally willing to pay for CBPP vaccines and vaccination with women

showing a higher WTP than men. However, this could be just an indication of the value attached to vaccination by livestock keepers; which calls for an assessment of the affordability. Although the impact of vaccination has been demonstrated using deterministic models, this may be under or overestimated and requires the use of stochastic models. Besides, CBPP affects many players within and outside the cattle value chain and in a country at large. This necessitates further analysis of impact along the value chain and macro-economic impact analysis.

3. Progress Towards Phase II Milestones

Project level milestones

- a. ***Inception workshop held, report developed and circulated – completed:*** joint project launch meeting with CIFS RF 107848 was in London, UK on January 18 and 19, 2015
- b. ***Memoranda of Understanding or Letters of Agreement signed between VIDO and KALRO, and between KALRO and each of the third party organisations (ILRI, KEVEVAPI and DVS), with clear deliverables, roles and responsibilities – completed:*** MOUs were signed between KALRO, ILRI and KEVEVAPI, a collaborative research contract was executed between KALRO and VIDO-InterVac. KALRO-ILRI signed an agreement for access and use of ILRI BecA Hub
- c. ***Development of key project implementation strategies including: i) impact pathway, ii) project M and E / results framework, iii) communication/scaling up strategy, and iv) gender and socio-economic studies framework. – completed:*** all plans and strategic documents were drafted at the project launch meeting and accepted
- d. ***Equipment ordered – completed***
- e. ***Annual workshop held – completed.*** The joint Annual workshop was held together with CIFS RF project 107849 in Pretoria, South Africa (September 03-05, 2015). Also, members of the project team participated in a scaling up workshop in Cotonou, Benin in October 2015, interacting with other CIFS RF projects.
- f. ***Annual workshop held – completed:*** Saskatoon, SK October 09-11, 2016
- g. ***Annual workshop held – completed:*** Nairobi, July 11-12, 2017
- h. ***Project Final meeting/workshop/conference held to disseminate project results and recommend key strategies for future scaling up – involving project partners, stakeholders and policy makers – completed:*** June 7, 2018 in Nairobi, Kenya.
- i. ***End of project evaluation and impact assessment documenting progress towards the key objectives and research for development outcomes, highlighting progress towards development of recombinant CBPP vaccine – completed:*** see the current document and all project outputs

- I. **Milestones related to Objective I - Define the vaccine formulation, including choice of adjuvant, route of delivery, dose and duration of immunity**
 - a. ***Adjuvant selection trial complete in Canada – completed:*** cancelled in Canada because of delay in obtaining CFIA permits. To keep progress on track, we transferred trial to Kenya and used the time to determine plasmid stability, induction conditions, and growth media (milestones II.f and II.g)
 - b. ***Adjuvant selection trial completed in Kenya – completed:*** Montanide ISA 61 VG selected as adjuvant
 - c. ***Publication on vaccine composition and efficacy submitted – completed***
 - d. ***Vaccine formulation finalized – completed***
 - e. ***Manuscript on final vaccine formulation, safety, dosage and duration of immunity prepared – not completed*** because of delayed manufacturing
 - f. ***Evidence of efficacy, safety and shelf life of vaccine collected – not completed:*** Evidence is collected except for shelf life and field trial data.

- II. **Milestones related to Objective II - Scale up of production of vaccine components from recombinant *E. coli***
 - a. ***Antigen fusion proteins constructed – completed:*** Protein fusion 610-581, containing YP_004400610.1 and YP_4400581.1 proteins and 127-790, containing YP_004400127.1 and YP_00399790.1 proteins were constructed.
 - b. ***Antigen fusion proteins inserted in industrial expression vector – completed:*** Leukotoxin-610-581, containing YP_004400610.1 and YP_4400581.1 proteins; Leukotoxin-127-790, containing YP_004400127.1 and YP_00399790.1 proteins; and Leukotoxin-160, containing MSC_0160 protein were inserted in vector pAA352.
 - c. ***Equipment installed, set-up, and fermentation process established – incomplete:*** All equipment was installed, laboratory scale protein production was established. however, fermentation process was not established
 - d. ***Production in industrial expression vector tested in laboratory conditions – completed:*** SOP and notes sent to KALRO and KEVEVAPI
 - e. ***Seed stocks ready – completed:*** seed stocks developed for KEVEVAPI
 - f. ***Tests completed, and results reported for: a) plasmid stability, b) induction conditions, c) growth media - completed***
 - g. ***Antigen harvesting and isolation/purification optimized – completed*** for laboratory scale processes. Limited large scale process is under development at closure of the

project. Merck has thereafter provided a budget for this activity to go on between KALRO and KEVEVAPI after closure of the project.

- h. **Batch release and potency evaluation methods developed – completed:** methods chosen, and assays developed.
- i. **Antigen purity assays developed – completed:** sterility, LAL endotoxin and SDS-PAGE
- j. **Scale-up for manufacturing completed – not completed,** industrial consultation with Merck ongoing
- k. **Shelf life evaluation in progress – not completed** because of delayed manufacturing
- l. **GALVMED consultation report finalized – completed:** The industrial consultation partner was changed to MCI Sante Animale, Morocco, who finished an audit earlier in the project, followed by Merck, who consulted and assisted the establishment of production.
- m. **Dossier complete – not completed.** Estimated 50% complete and field trial data needed for completion
- n. **Report from PANA-VAC on quality control submitted – not completed** because of delayed manufacturing

III. Milestones related to Objective III - Define the regulatory pathway for approval of the vaccine in Kenya and elsewhere in sub-Saharan Africa

- a. **Consultations on regulatory pathway initiated, contracts signed – completed:** Dr. Jane Wachira (Director KEVEVAPI) started consultations and provided a list of documents for product dossier
- b. **Dossier collection started – completed:** data entered from laboratory tests and animal trials
- c. **Dossier collection 30% completed – completed:** Experimental results were included in the following dossier components: 2. A. Quantitative and Qualitative Particulars; 2. E. Control Tests of the Finished Product; 3. A. Laboratory Tests (Safety); 4. A. Laboratory efficacy.
- d. **Dossier assembly 50% complete – completed**
- e. **Dossier assembly 60% complete – not completed,** estimated 50 % of the dossier is completed because of delayed manufacturing
- f. **Dossier documentation 80% complete – not completed,** estimated 50 % complete because of delayed manufacturing

IV. Milestones related to Objective IV - Test the vaccine under field conditions

- a. ***Permits and approvals for dose, safety and duration of immunity trials submitted – completed:*** all permit and approval materials were prepared
- b. ***Dose, safety and duration of immunity trial ongoing*** – not completed: safety data collected, dose and duration of immunity not completed because the time was taken up by vaccine formulation work and trials that were repeated and needed to be finalized first. Duration of immunity now requires further funding which is not yet secured.
- c. ***Dose, safety and duration of immunity trial in progress*** – not completed: Adjuvant trial and efficacy tests to select optimal vaccine formulation had higher priority
- d. ***Permits and ethic approvals obtained for large field trial*** – not completed because of delayed manufacturing. This delay was due to the length of time required to negotiate the license agreement.
- e. ***Large field trial started*** – not completed during project term, because manufacturing was not established
- f. ***Large field trial completed*** – not completed due to delayed manufacturing.

V. Milestones related to Objective V - Analyze the social and economic factors, including gender constraints that will influence the acceptability and adoption of the vaccine

- a. ***First report on policy study completed*** – completed
- b. ***Report and manuscript ready on cross-sectional study of CBPP epidemiology and risk – completed:*** manuscript submitted.
- c. ***Analytical report and manuscript ready for KAPP on vaccine coverage with the existing vaccine – completed:*** manuscript in submission: “Preferred Attributes of Contagious Bovine Pleuropneumonia Sub-Unit Vaccine among Somali Pastoralists in Kenya” by Mary R. Ndanyi, Salome W. Kairu-Wanyoike, Rose A. Nyikal, Hezron O. Wesonga, and Philip M. Kitale.
- d. ***Second policy study finished, report prepared*** – completed: Data from relevant policy documents collected/collated, interviews in seven counties and NGOs completed.
- e. ***Communication campaign with stakeholders initiated*** – completed, two stakeholder meetings held, as well as in communications with farmers, extension specialists, and County Governments.
- f. ***Study on impact of CBPP along cattle value chain ongoing*** – completed. A stochastic model was developed using @ Risk software and using data from Central Bureau of statistics of Kenya. Social Accounting Matrix (SAM) and computerized generalized

equilibrium (CGE) models were also applied. PERT analysis also carried out, 500 data iterations conducted.

- g. **Manuscript prepared on KAPP and WTP for the new vaccine** – not completed due to delayed formulation of the new vaccine. However similar studies of the current vaccine were used to facilitate the KAPP and WTP for the new product
- h. **Vaccine roll out strategy developed** – not completed because of delayed vaccine formulation. Estimated at 30%, needs manufacturer input and field trial data.
- i. **KAPP and WTP manuscript ready for the new vaccine** – not completed for the new vaccine because of delayed formulation and manufacturing
- j. **Impact on value chain study complete** – not completed because of delayed formulation and manufacturing
- k. **Manuscript prepared on socio-economic impact of CBPP and its control by vaccination – gendered analysis** – completed, manuscript ready for submission.
- l. **Communication campaign on the benefits of the new vaccine completed** – not completed. Communication will continue beyond project term because the vaccine needs support for product launch.

4. Synthesis of Research Activities and Results

Research activities are described below for each of the objectives as listed in the project proposal.

I. Define the vaccine formulation, including choice of adjuvant, route of delivery, dose and duration of immunity.

In Phase 1, we used reverse vaccinology to identify antigens and formulate a vaccine. This approach relies on genome analysis and on computational predictions of protein structure and immune reactions to reduce the inherent biases of traditional methods for antigen selection. In our case we identified three separate combinations of *Mycoplasma mycoides* antigens with protective potential which were selected for further examination.

During Phase 2 laboratory-produced vaccine prototypes were examined for their fitness for industrial production and sub-cloned in high-yield vectors, used to transform production strains.

Our attention focused on factors influencing the potential for success of the future vaccine

- potential for protection
- production cost of the antigens

- immunogenicity and potential for long-term protection.

Aiming to reduce the number of antigen components (and thus the cost per dose) in the new vaccine we constructed protein chimeras (fusion proteins). The proteins we used were YP_004400610.1, YP_004400581.1, YP_004400127.1, YP_00399790.1, and MSC_0160. They were expressed as fusion proteins because their size allowed the construction and expression of chimeric antigens. The other 2 groups which elicited protection in Phase 1 contained proteins which are larger in size and could be included as fusion proteins only after screening for immunogenic/protective epitopes.

The following fusion proteins were constructed (see Annex 2):

Leukotoxin-610-581

This chimera is composed of the *M. haemolytica* leukotoxin (LktA) fused in tandem to the *M. mycoides* subsp. *mycoides* YP_004400610.1 and YP_004400581.1 proteins.

The genes encoding the YP_004400610.1 and YP_4400581.1 proteins were synthesized by GenScript. The genes were synthesized so that the YP_004400610.1 and the YP_4400581.1 proteins were in tandem and separated by a six amino acid spacer (glycine). The protein was fused to a histidine tag for purification by affinity chromatography. The chimeric protein was expressed in *E. coli*.

The genes encoding the YP_004400610.1—YP_004400581.1 chimera were amplified by PCR without the histidine tag and cloned into a plasmid pAA352 encoding the *M. haemolytica* leukotoxin so that the chimeric protein was located in tandem and downstream of the leukotoxin protein to form the chimeric leukotoxin-610-581 protein.

Leukotoxin-127-790

This chimera is composed of the *M. haemolytica* leukotoxin fused in tandem to the *M. mycoides* subsp. *mycoides* YP_004400127.1 and YP_00399790.1 proteins.

Construction details were the same as for the Leukotoxin-610-581 fusion protein.

Leukotoxin-160

This chimera is composed of the *M. haemolytica* leukotoxin fused in tandem to the *M. mycoides* subsp. *mycoides* MSC_0160 protein.

Construction details were the same as for the Leukotoxin-610-581 fusion protein.

The team at VIDO-InterVac tested the immune responses to the chimeric proteins and compared them to the responses to individual proteins. Trial results showed very similar immune responses to a mixture of fused proteins and a mixture of individual proteins. The main difference was that LktA chimeras caused more balanced Th1/Th2 immune responses as judged by the IgG1:IgG2 ratio of antibody titres.

The vaccine efficacy trials carried out in Kenya involved experimental infection with *M. mycoides* and the results were evaluated using a scoring system which quantifies type and size of lesions and colonization of the host by the causative microorganism.

Since clinical signs of disease are often matter of subjective observation and are harder to quantify, the following procedure for objective pathology scoring was suggested by Hudson and Turner (3):

Lesion Scores:

- The presence of a resolving lesion with only fibrous tags or pleural fibrous adhesions only was rated 1;
- If other types of lesions were present, namely consolidation, acute, necrotic or sequestered tissue, these lesions were rated 2;
- In addition, if *MmmSC* was isolated a score of 2 was added.

Pathology Scores: The pathology score was calculated using a) the lesion score, and b) The total was multiplied by a factor determined by the average diameter of the lesion. i) 1 was used if the lesion size was under 5cm; ii) 2 was used if the lesion size was over 5 and under 20cm) and iii) 3 was used if the lesion size is 20cm and above.

From this, the maximum pathology score is $(2+2)3=12$ for each animal.

Protective efficacy was calculated as follows:

Vaccine efficacy = $1 - [\text{mean score of vaccinates}/\text{mean score of controls}] \times 100$

The **first** trial to define vaccine formulation and evaluate the protection efficacy induced by fusion proteins was conducted in Boran cattle. Groups of eight cattle each were injected with the fusion proteins and with a mixture of the proteins used for construction of fusions, but individually expressed in his-tagged vectors. Details and results of this trial are shown in Appendix 4. In summary, the fusion proteins failed to protect the treatment group. We believe that this unexpected finding could be a result of an underlying infection during or before the trial period, as we found abnormally high levels of some pro-inflammatory cytokines present in blood

samples. Other possible explanations include the effects of chimerization of the proteins used in this trial, and the selection of antigens from a group with lower score (group N instead of A or C).

A **second** trial to determine vaccine formulation and compare adjuvants was conducted in Boran cattle with a new set of vaccine antigens. The formulations tested in this trial included the LktA fusions of a combination of the antigens with strongest immune responses (3 from Phase1 group A and 1 from Phase1 group C) with 3 different adjuvants, plus two additional groups immunized with the formulations for groups A and C as used in Phase 1 (Annex 4).

The results showed that surprisingly very few animals developed clinical signs for short periods after inoculation with *M. mycoides*, and only one animal in the placebo group developed a lung lesion which was resolving at the time of necropsy. These mild symptoms did not allow us to derive any statistically significant difference in protective efficacy between the test groups.

The immunological analysis of this trial was conducted in KALRO laboratory in Muguga and in BecA at ILRI. Overall the immune responses of all vaccinated groups were similar, with Montanide ISA 61 VG showing best results. Considering the proven safety of the adjuvant, Montanide ISA 61 VG was selected as adjuvant for the final vaccine formulation. This adjuvant induces a balanced ratio of humoral and cell-mediated responses; has good value, availability and ease of use; and is accepted well by both manufacturers and registration authorities.

This trial also confirmed the immunogenicity and consequently the potential for good protective efficacy of the selected antigens (Phase 1 groups A and C), but the control group provided poor reproducibility of the infection. For this reason, and to obtain more complete data on the protective efficacy in the most common breeds of cattle in Kenya, the scientific management committee advised the team to conduct another trial in Zebu cattle. This suggestion was also supported by a small-scale test conducted in parallel with the second vaccine formulation trial. The test involved the main breeds – Boran and Zebu and 2 Afade isolates of *M. mycoides* (S from a lung lesion and P from pleural fluid). Isolate P showed levels of infection and clinical signs, which were similar to these in natural exposure infections. Zebu cattle in this trial showed susceptibility to the challenge with *M. mycoides* and was chosen for use in the third formulation trial.

The **third** formulation trial tested two recombinant formulations with Montanide ISA 61 VG as adjuvant. These formulations were compared to T₁/44 live attenuated vaccine, which necessitated the inclusion of 2 control groups – one with Montanide ISA 61 VG and no antigens and one with saline (since T₁/44 vaccine contains no adjuvant). One of the recombinant antigen groups consisted of his-tagged isolates of the four proteins (3 from Phase1 group A and 1 from Phase1 group C) as used in the second trial of this study, while the other antigen group was same as Phase 1 group A.

This trial resulted in reduced clinical symptoms and reduced pathology scores, observed in cattle inoculated with the combination of four proteins when compared to cattle in the groups inoculated with saline (negative control), and that inoculated with T₁/44 (the current vaccine group - positive control). The results showed that the new four-protein vaccine conferred 81% protection compared to the live attenuated vaccine that had a maximum protection of 22% in the same experiment (Appendix 6).

The IgG titers of the animals vaccinated with recombinant proteins were similar to those observed in trial two, and to the titers which the same proteins elicited in Phase 1 trials. These strong immune responses were not only result of vaccination but were also high in the sera of animals infected with CBPP, which indicates that in their native form these proteins are detectable by the immune system. The same proteins had some titre increase in the T₁/44 group, but the values were lower.

The results of our work were reported in 3 peer-reviewed publications and several posters and presentations at international scientific meetings.

Results were also presented and were well received at the Kenya Veterinary Association (KVA) Annual Scientific Conference (April 2018). KVA is the body whose members should administer the vaccine in Kenya. The presentation was in the context of expanding the use of the technology of reverse vaccinology to include other vaccines especially those caused by mycoplasmas, including CCPP.

II. Scale up of production of vaccine components from recombinant *E. coli*.

Activities on this objective aimed towards determining the conditions for expression and purification of the fusion proteins in laboratory setting, which is an important guideline for scaling up to pilot and industrial production. The experiments included optimization of growth media, induction conditions and plasmid stability.

We used one *E. coli* strain with proven efficiency in high-yield expression of recombinant proteins – BL21 STAR (DE3). This strain contains a genotype that promotes high mRNA stability and protein yield. Expression of proteins in this strain can be controlled by IPTG, which provides control of the strain growth and protein production. This strain also has a mutation in the RNaseE gene (*rne131*) that reduces levels of endogenous RNases and mRNA degradation, thereby increasing the stability of mRNA transcripts and increasing protein yield. Protein expression is further enhanced by the absence of the *lon* and outer membrane (*OmpT*) proteases, which reduces degradation of heterologous proteins.

Two plasmids were used in preparation for industrial production – pAA352 for the LktA fusion proteins and pGS21a for the his-tagged proteins. Of those pAA352 contains the *M. haemolytica* leukotoxin (LktA) which is adjacent to the cloning site, resulting in LktA fusions which induce increased immune responses. After optimization of expression conditions this plasmid produces 30-80 mg of recombinant proteins per litre of shaker-incubator culture. We achieved laboratory production at these levels for all our pAA352 constructs (data in Appendix 6).

Plasmid stability for the pAA352 constructs was also tested and showed high levels of retention of the constructs after 52-60 generations, especially in Phytone peptone broth.

The second plasmid we used – pGS21a is well characterized by itself and when expressing in BL21 STAR (DE3). For this reason, less optimisation and stability testing were required for the his-tagged constructs. The pGS21a vector is designed for cloning, high-level expression and convenient purification of proteins fused with both 6xHis and GST (glutathione S-transferase). The two 6xHis sequences in this vector allow for easy detection and purification of fused proteins. The first 6xHis is fused to GST and permits isolation by both Ni-infused beads and glutathione (Glu-Cys-Gly) beads. This fused tag 6xHis-GST can be cleaved using enterokinase. The second 6xHis can be used for detection and further purification after the cleavage of 6xHis-GST. This added flexibility of two independent isolation processes is an advantage to the manufacturer in pursuit of high-purity product.

The pGS21a in BL21 STAR (DE3) produces between 20 and 200 mg per litre of laboratory shaker-incubator culture. Because our yields were within these limits, and because fermenter cultures greatly improve the yield, we did not conduct extensive optimization of the his-tagged constructs.

Prototype vaccines and 35 recombinant *E. coli* strains were shipped to KALRO.

The laboratory stages in preparation for vaccine production were finalized and included quality control procedures – SDS-PAGE of the produced recombinant protein antigens, sterility testing of the vaccines and LAL assay for endotoxin.

Another significant advance towards production was the purchase, installation and commissioning of equipment in the new Product Development Centre in Muguga. A significant portion of this equipment was acquired using project funds and in the last years of the project was used for production of recombinant antigens, for production of analytical reagents, for assaying of clinical samples, and for quality analysis. This capacity was built in collaboration with VIDO-InterVac which provided consultations and training of laboratory personnel.

For the purpose of transition to manufacturing, an industrial consultation with a site visit was conducted in Nairobi in April 2016 by a team of experts from the vaccine manufacturer MCI Sante

Animale. The results of this audit were the basis for procurement of test-batch scale industrial production equipment (fermenter and filters). Further details of the transition to production involved collaboration between manufacturer (KEVEVAPI) and consultant (Merck), which resulted in developing the process for industrial production.

Another aspect of transition to production was achieving a consensus between the IP holders (VIDO-InterVac, KALRO and ILRI) and executing a license agreement with the manufacturer (KEVEVAPI). This process is currently in its second iteration. While preparing the first draft ILRI requested a non-exclusive license agreement, according to the mandate of CGIAR. After a consensus was reached between the IP owners, the draft was presented to KEVEVAPI for discussion. This draft suggested a non-exclusive license, royalty free for Kenya, no right to sub-license to third parties, and coverage by the manufacturer of patent protection costs. The manufacturer had reservations towards this version of the agreement and towards the possibility to gain the necessary approvals for signing.

Another round of consultations between KALRO, VIDO-InterVac and ILRI resulted in a consensus that a non-exclusive license could be avoided for a product that will require investments in both patent protection and manufacturing setup. The second consensus draft was a term- and territory-limited exclusive license with conditional rights to sub-license and sub-contract.

The license agreement was fully executed on February 26, 2018 by the three IP holder institutions (KALRO, ILRI and VIDO-InterVac) and the vaccine manufacturer KEVEVAPI. This agreement was based on the recommendation in the Memorandum of Grant Conditions for this project; C4.2 “Licenses to End Users in developing World”.

Extreme delays in KALRO procurement processes made impossible the completion of production scale up during the term of the project. Procurement office of a research organization is bound by the obligation to secure lowest cost and often lacks the experience to evaluate the long-term value projections of a manufacturing company.

III. Define the regulatory pathway for approval of the vaccine in Kenya and elsewhere in sub-Saharan Africa.

The regulatory pathway has been studied by project team members in parallel with the opportunities for product registration elsewhere in Sub-Saharan Africa. Towards this goal team members attended and presented the project at the 4-th Global Animal Health Conference - Regulatory Convergence (June 24-25, 2015 in Dar-es-Salaam) and at the FAO Technical Consultation on CBPP (October 14-16, 2015 in Rome).

We have obtained a list of documentation for registration of vaccines, which is harmonized with registration requirements in a number of countries in Sub-Saharan Africa. This list was further developed and formalized into fill-in templates by consultation with a professional trial monitor Dr. Collins Omugar. These templates were used to record all data from the trials conducted during the project term, which are now close to ready for submission format. As a result, all current and future documentation will comply with the registration requirements, including the recently developed requirements which are focused on regulatory convergence. These convergence requirements were developed based on recommendations by GALVmed, proceeding from UK product registration. Our team believes that the expertise of a monitor with years of practice in the UK was particularly valuable.

As part of this effort two stakeholder meetings were held on December 03, 2015 and on June 7, 2018 in Nairobi. Attendees included scientists from the project teams and representatives from a number of institutions responsible for vaccine registration: Kenya's Pharmacy and Poisons Board (PPB), the National Biosafety Authority (NBA) and Directorate of Veterinary Services (DVS). The objectives of the meetings were to create awareness among regulators on activities of the projects; to sensitise potential funding partners with interest in vaccine development; to discuss and agree on the product profile; and to address the requirements for product registration. During the first meeting it was agreed that a committee to oversee the animal trials for registration is to be established. The following composition of this committee was agreed upon: local Project PIs, with PIs abroad as co-opted; institutional animal welfare committee representative; Directorate of Veterinary Services representative; National Bio-safety Authority representative; Pharmacy and Poisons Board representative. However, it was later determined that what was required was a professional clinical research associate (CRA) also known as clinical monitor whom the project retained as a consultant. This committee may therefore be activated when the product is ready to be taken to the field.

One of the important results of the first stakeholder meeting and the following interactions with regulatory bodies was that the National Biosafety Board agreed that the source microorganism is a GMO, but the antigens derived from it do not require the scrutiny and permits associated with GMO for field testing. This agreement significantly simplifies the fulfillment of legal requirements for further vaccine testing and registration.

All other requirements for product registration were accounted for during preparation of the dossier documentation, which was developed according to the registration requirements in Kenya, and the convergence guidelines for simplified registration in other African countries.

The second stakeholder meeting elicited support from vaccine regulators. Over 40 people from regulatory and other interested organizations including AU-IBAR, the Kenya Veterinary Board,

National Biosafety Authority, National Council for Science and Technology, Directorate of Veterinary Medicines (DVM), FAO, GALVmed were in attendance. The regulators view the vaccine as key in the eradication of CBBP given the ability to distinguish between vaccinated and non-vaccinated animals with the new vaccine. Particularly useful was the interest of the new registration authority, the Veterinary Medicines Directorate, which are newly established and are willing to see through the registration of our vaccine according to the harmonized rules.

A business model for vaccine delivery includes one particular concept, the electronic voucher system (EVS). This concept aims at higher vaccine uptake, while enabling the sponsor(s) to track the use of money to the end users. The overall E-voucher conceptual framework and baseline study on viability was completed. It is based on use of SMS on mobile phones whose ownership and use was found to be 98% of the surveyed pastoralist population in the Maasai ecosystem. Random selection established 98% male headed households and 2% female headed households. This baseline data has resulted in preparation of a full proposal whose funding level is outside the value of the current project. ICT infrastructure to host the EVS was established at KALRO headquarters and key stakeholders on the EVS were identified. EVS payment module, communication component and data flow interactions were also established.

IV. Test the vaccine under field conditions.

Testing of the vaccine under field conditions was not done because it required a test-batch production, which would form the basis of industrial-scale production. Although many of the steps needed were taken in the activities described under the previous objectives, we could not conduct the field trial during the term of this project.

Particularly important for the progress of this activity were the vaccine formulation and delivery, which were developed based on the trials described in Objective 1. At the same time, the delays in these trials overwhelmed our plans to compensate and update the schedule. While we were eager to accommodate the field trial, we could not accept any shortcuts in vaccine formulation, because small oversights in formulation may have catastrophic consequences when taken to industrial scale. The socio-economic studies in particular the KAP and preferences studies helped us to define the product characteristics. Other activities towards field trial will continue after the end of the project. Scale-up to manufacturing, based on optimizing the growth conditions and yield of recombinant proteins, now continues through industrial consultation. The dialogue with the regulatory bodies and other stakeholders that showed interest in the product has stimulated the team to engage international, national and county authorities in search of additional funding.

V. Analyze the social and economic factors, including gender constraints that will influence the acceptability and adoption of the vaccine.

Analysis of social and economic factors has provided some interesting insights of the attitudes and perceptions concerning a new vaccine for CBPP. We were pleased to see that the vaccine attributes we are aiming to improve were ranked highly by livestock keepers in Ijara sub-County: pastoralists preferred a vaccine with a quality indicator, high efficacy and safety (both inherent to subunit vaccines compared to inactivated or attenuated viruses or bacteria), stable for more than 2 hours and administered by the government annually (one possible application of the proposed e-Voucher). Differences and similarities among male and female respondents with regard to preferences for vaccine and vaccination attributes were also investigated. Among the differences, females preferred a safer vaccine ($p < 0.05$), while males preferred a stable vaccine ($p < 0.05$). Further, whereas female respondents placed higher preference (Relative importance (RI) of 31.4%) on inclusion of a vaccine indicator for quality male respondents RI for both vaccine indicator and efficacy was 29.7% and 30.1% respectively. The RI for efficacy by female respondents was 23.1%. Similar preferences were observed on administration of the vaccine by government personnel; vaccinations carried out once annually and lower prices of the vaccine. The socio-economic determinants of preferences that were significant included marital status (monogamous, polygamous or widow), income, treatment of CBPP, perception on the effectiveness of CBPP treatment, education level of respondent, experience of vaccine adverse reactions and distance to animal health service providers. It appears that some of the significant factors are correlated to the gender of the respondent in this study: women pastoralists surveyed had lower education levels, less access to extension and lower income in comparison to their male counterparts. Incorporating the data on relative importance of preferred attributes into the new vaccine development, and further in vaccination programs, especially considering the socioeconomic factors influencing the vaccine and vaccination preferences and the gender differences can increase uptake of the new vaccine and enhance CBPP disease control.

Vaccination using the current vaccine and a second administration of questionnaires on perceptions and attitudes (KAPP) as well as preferences for delivery of CBPP vaccine took place in 2017. The study also aimed at testing the hypothesis that trypanosoma infections may limit control of CBPP by vaccination. Pre-vaccination sampling of 1017 animals in 2017 demonstrated that both CBPP and trypanosomoses were present in the study area at rates of 8.7% and 0.4% respectively. About 50,000 animals were vaccinated and ear-tagged for follow-up and 221 farmer questionnaires administered. Constraints in vaccine delivery that were identified included:

inadequate awareness on vaccination in spite of publicity conducted prior to commencement of the activity probably due to the vastness of the area; migration of animals due to severe drought, requiring teams to drive long distances to find the animals; resistance to vaccination by cattle owners for fear of adverse post-vaccination reactions by some and due to absence of Foot and Mouth Disease (FMD) vaccine for others. In subsequent vaccinations, constraints were minimized through improved publicity, vaccination of animals before migration and inclusion of the FMD vaccine in CBPP vaccinations. The vaccination follow-up in 2018 showed low sero-conversion (46%) of vaccinated animals and persistent trypanosomoses (0.9%). Data is under analysis on KAPP and to demonstrate the relationship between trypanosomoses and CBPP vaccination sero-conversion.

The adoption of the CBPP vaccine is deemed to be anchored on availability, accessibility, affordability and acceptability. To address these issues a policy study was carried out to map policies that are related to CBPP control and eradication which affect accessibility of the vaccine. Farmer responses regarding accessibility of the vaccine were also analysed. Like other livestock diseases, CBPP contributes to food insecurity, loss of wealth and income as well as increased household expenditure. Income and expenditure data were retrieved and analysed. Data on willingness to pay in view of household incomes was analysed to determine affordability of CBPP vaccination. Although about 70% of household incomes are from cattle related activities only 5% of expenditure is on CBPP and other livestock disease vaccinations. The most frequent reason for low vaccination was unavailability of the vaccine to the farmers and fear of post vaccination adverse reactions. Negative net incomes were recorded in 39.9% of households. Proportion of farmers willing to pay for a preferred vaccine at a benchmark price of KES. 34.6 (0.44CAD\$) was only 59%. Correlation between WTP and net incomes is low, meaning that ability of the farmers to pay for CBPP vaccination is low hence the need for intervention to ensure adequate vaccination coverage (above 80%).

Results of a recent national survey indicate that CBPP is still present in several counties at a prevalence of 14.8%. Desk top policy studies demonstrated persistence of CBPP to be due to KAP gaps and low vaccination coverage (20-60%) attributable to undesirable post-vaccination reactions, inadequate control resources, inadequate benefit-cost justification and inappropriate policies leading to inadequate vaccine delivery. Counties have strategic plans for vaccination against CBPP but have inadequate funding. The studies also showed that there are numerous stakeholders that can be involved in CBPP control and eradication at various levels albeit with various constraints. Two policy briefs were prepared on analyses of the problem that is being addressed by this project and who can be involved in CBPP control and eradication. Since 2010, there was devolution of disease control activities to subnational level (counties) in Kenya. The Counties are customizing national level policies for vaccination against CBPP for about 5-10 million cattle in the CBPP infected and protective zones. Although there is participation by the

national government in vaccinations in the counties, the linkages between national and county level policies are still informal yet there is need for coordination/regulation of County CBPP vaccinations. Vaccinations against CBPP in the counties remain free of charge. Some counties are not fully aware of CBPP control strategies and few are aware of the regional and international policies on CBPP control although there is synchronization of CBPP control with neighbouring countries. There are no organized livestock identification systems in the counties which makes documentation of evidence on vaccination a challenge. Control of CBPP remains a priority in infected counties in Kenya and adequate budgetary allocations are essential. There is strong need for awareness creation on national, regional and international CBPP control strategies. National government coordination and regulation in CBPP vaccination needs to be strengthened and collaboration with neighbouring countries maintained.

Towards evaluation of CBPP economic impact, existing literature and data for stochastic modeling of CBPP macro-economic impact was retrieved and modeling commenced. Data requirements for analysis of the impact of CBPP along the value chain and macroeconomic modeling were identified. Results indicated that the incidence of CBPP has been increasing and is predicted to reach 18% by 2030 (from the current 14%) at the current rate of control with the current vaccine. Similarly, mortality rates due to CBPP are on the increase. Regression models indicated that adoption of CBPP vaccination (current vaccine) was significantly conditioned by number of cattle owned, age of the livestock owner, household income and size as well as previous experience of CBPP in the herd.

A socio-economic team member participated in 2 webinars on gender integration in socio-economic studies; gender issues were incorporated in tools/studies in communication, E-Voucher, policy studies, economic impact and vaccine delivery.

5. Synthesis of results towards AFS themes

- **Increasing agricultural productivity (Availability).**

Studies on the impact of CBPP showed that the risk of CBPP and likely consequences were high in pastoral and mixed farming systems where most of the rural poor are located. Investigation of outbreaks of the disease (2014 and 2017) demonstrated that CBPP caused high production losses in one-county outbreak in terms of mortality, milk loss and abortions (up to CAD 73,000). Intervention costs added more expenses especially when several herds were involved (up to CAD 52,000). Therefore, the development of novel vaccine for CBPP - a disease with significant economic impact - will have direct positive effect on food productivity by reducing losses to cattle farming due to reduced performance and animal mortality. The risk of losses due to loss of

animals or loss of animal production quality and volume will be reduced by immunization, although the exact amount of reduction will not be known until field studies are carried out. This is due to the impact of vaccination on both, individual animal immunity, which we can predict, and also herd immunity for which no data is available yet. Gender inclusiveness in determining perceptions and attitudes towards the new vaccine is informing both product development and future vaccination campaigns. Environmental sustainability is also addressed by the approach we have chosen. Disease control programs by vaccination have the potential to significantly reduce antibiotic treatment, resulting in lowering the chance to select for drug-resistant infectious agents – a serious emerging problem for both animal and human health.

- **Improving access to resources, and/or markets and income (Accessibility).**

Given the economic loss caused by CBPP outbreak in one county, we believe that the developed product may have indirect impact on income improvement by reducing losses due to mortality, loss of quality, volume of production, and to international trade restrictions - all results of prevalence of animal infectious diseases.

- **Improving nutrition (Utilization).**

The project has a potential to improve nutrition indirectly by reduction of losses in animal production, thus increasing the availability of locally derived animal food products. In addition, a decrease in the use of antibiotics will ultimately lead to safer food due to less residue being present. Data in the project study area indicates that farmers often treated CBPP with 20ml of 10% oxytetracyclines and repeated treatment until the animal 'improves' which may take a long time. In addition, oxytetracyclines contribute a large proportion (26%) of the cost of treatment drugs used particularly in pastoralist areas of Kenya. Lack of response to treatment in CBPP affected animals is one contributor to development of resistance to antimicrobials. Often, this drug is used to treat 'respiratory syndromes' which would include CBPP.

- **Informing policy.**

Recombinant antigen vaccines have one of the highest impact potentials of all state-of-the-art disease control methods. In our dialogue with stakeholders, national regulatory bodies in Kenya (Pharmacy and Poisons Board, Department of Veterinary Services, National Biosafety Board, Directorate of Veterinary Medicines) have been engaged in discussions about the developed vaccine and have been informed about its features, safety, efficacy and potential impact during meeting held in December 2015 and June 2018 in Nairobi. Department of Veterinary Services and the national Biosafety Board of Kenya have shown interest and committed support for the project. The Pharmacy and Poisons Board and Department of Veterinary Services will be engaged in preparation for the field trial and ensuring that collected data can be used towards registration.

A committee to oversee the animal trials for registration includes local Project PIs, with PIs abroad as co-opted; institutional animal welfare committee representative; Directorate of Veterinary Services (DVS) representative; as well as Pharmacy and Poisons Board (PPB) representative.

6. Project Outputs

- A novel vaccine for CBPP with 81% efficacy against infection with CBPP. The vaccine was patented and licensed to a commercial vaccine manufacturer (KEVEVAPI).

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“Experimental evaluation of inactivated and live attenuated vaccines against *Mycoplasma mycoides* subsp. *mycoides*”, Martin Mwirigi, Isabel Nkando, Racheal Aye, Reuben Soi, Horace Ochanda, Emil Berberov, Andrew Potter, Volker Gerdts, Jose Perez-Casal, Jan Naessens, and Hezron Wesonga; [Veterinary Immunology and Immunopathology, Volume 169, January 2016, Pages 63-67](#)

“Capsular polysaccharide from *Mycoplasma mycoides* subsp. *mycoides* shows potential for protection against contagious bovine pleuropneumonia”, Martin Mwirigi, Isabel Nkando, Moses Olum, Samuel Attah-Poku, Horace Ochanda, Emil Berberov, Andrew Potter, Volker Gerdts, Jose Perez-Casal, Hezron Wesonga, Reuben Soi, and Jan Naessens; [Veterinary Immunology and Immunopathology, Volume 178, 1 October 2016, Pages 64-69](#)

“Development of a Subunit Vaccine for CBPP in Africa”, Jose Perez-Casal (presenter), Tracy Prysliak, Teresa Maina, Yeyun Wang, Hugh Townsend, Emil Berberov, Volker Gerdts, Andrew Potter, Isabel Nkando, Anne Liljander, Joerg Jores, Jan Naessens, Reuben Soi, Hezron Wesonga; Invited presentation at “Progress in Human and animal mycoplasmaology” meeting, 3-5th June 2015, Pendik, Istanbul.

“Evaluation of immunogenicity and protective potential of subunit Mmm proteins against contagious bovine pleuropneumonia (CBPP)” Isabel Nkando (presenter), Martin Mwirigi, Jose

Perez-Casal, Tracy Prysliak, Teresia Maina, Emil Berberov, Anne Liljander, Jorg Jores, Joseph Kuria, John Mugambi, Volker Gerds, Andrew Potter, Jan Naessens, Hezron Wesonga; presented at “Progress in Human and animal mycoplasmaology” meeting, 3-5th June 2015, Pendik, Istanbul.

“Developing of a subunit vaccine for CBPP in Africa”, Jose Perez-Casal (presenter), Tracy Prysliak, Teresa Maina, Yeyun Wang, Hugh Townsend, Emil Berberov, Volker Gerds, Isabel Nkando, Anne Liljander, Joerg Jores, JanNaessens, Reuben Soi, Hezron Wesonga, & Andrew Potter, **Invited presentation** at FAO-OIE-AU/IBAR-IAEA Consultative group on CBPP, Fifth meeting, Rome, 14-16 October 2015 Published in the proceedings: ISSN 1810-07321916126E.

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“Hope for improved livestock vaccines in Kenya using the technology of reverse vaccinology”, Wesonga H.O, Gicheru, N.N, Mungube E. O, Ndanyi, R. M, Kairu-Wanyoike, S and Maichomo, M.W. **Presentation initiated by the project** to the Kenya Veterinary Association 52nd Annual Scientific Conference, April 24-29, 2018, Green Hills Hotel, Nyeri, Kenya.

“Characterization of potential vaccine antigens from *Mycoplasma mycoides* subsp. *mycoides* based on homology with *Mycoplasma capricolum* subsp. *Capripneumoniae*”, Moses Omondi Orwe; Johnson Kinyua; Jane Ngaira; Martina Kyalo; Nimmo Gicheru; Harrison Lutta; Univer Chepleting; Maureen Luvanda; Hezron Wesonga; Roger Pelle; Jan Naessens. Manuscript submitted to BMC Veterinary Research BVET-D-18-00610, 2018.

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Reverse vaccinology approaches to disease in cattle, Potter, A. A. **Invited** presentation at the World Vaccine Congress, Washington, D.C., USA, March 29-31, 2016.

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7. Problems and challenges

There were several problems, which had negative impact on the project, mostly by affecting the timeline.

One problem area was the procurement process and requirements for time-consuming procedures to ensure lowest price. Such process is mandatory on many occasions and often cannot be expedited. Specifically affected were procurements by KALRO and KEVEVAPI, where the procedure for a bio-reactor (fermenter) was protracted and finally closed by procurement personnel without purchase. Similar delays were faced when procuring animals for the on-station trials, although the source of the problem there were not procurement procedures.

Another set of problems had its source in administrative procedures – both institutional and governmental. For example, one of the shipments of proteins from VIDO-InterVac to KALRO, under newly enacted regulations needed to be certified for product conformity by a Swiss company SGS in order to receive import permit for Kenya – a process which had much higher cost in time than in money. Similar obstacles occurred when reaching agreements between institutions - the vaccine manufacturing license agreement took almost two years to finalize.

We also faced the usual challenges inherent to working with living organisms – infected animals, unexpectedly low disease levels in control groups, and reactions to formulation changes. Those obstacles contributed to the delays in completing some milestones but would have had little effect if logistics of purchasing allowed us to apply faster our contingency plans. Fortunately, the team was experienced in dealing with such obstacles and we succeeded in developing the first recombinant vaccine for CBPP.

8. Overall assessment and recommendations

Overall the experience of working on this project was very positive. The relationship between research team and funding agency was excellent and the support for our R&D effort was enthusiastic. The project ideology was similar to the worlds most advanced international collaborations.

One area that deserves examination would be the improvement of purchasing. An approach like the one adopted by the “International Science and Technology Centre” (which provided instruments and reagents completely bypassing institutional procurement) may not be feasible, but discussions with institutional partners could reveal other opportunities to expedite procurement.

The project term is another area where extra flexibility could have strong positive impact. Longer no-cost extensions (of course agreed upon by research team and justified before the funding agency) may provide additional opportunity to optimize project efficiency and increase impact.

Finally, the highest impact of an innovative solution can not always be achieved in the initially planned timeframe. Even if a project has fulfilled all objectives in time, a follow-up involvement by IDRC could enhance the visibility of the Centre’s contribution to global change.

This field trial activity and others, including safety and shelf life studies are recommended for the future. These activities require a budget estimated at KSh 400 million. KALRO has indicated a willingness to source KSh200 million from a project already funded within KALRP but requiring competitive applications. It is proposed that the budget at KALRO is to be used for vaccine testing activities under field conditions because the budget is for activities in counties. Funds for laboratory and on station trials are therefore lacking. Laboratory activities include production of vaccine for more cattle as well as testing for efficacy of the vaccine under field conditions. The field testing includes testing of a business model using EVS. The source of funding for the business model and laboratory and on station activities is not yet established.

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1. Thomson, G.R., **Contagious bovine pleuropneumonia and poverty: a strategy for addressing the effects of the disease in sub-Saharan Africa**, DFID Animal Health Programme, 2004, Edinburgh: University of Edinburgh.
2. J.R. Hudson, A.W. Turner, **Contagious Bovine Pleuropneumonia: a comparison of the efficacy of two types of vaccine**, Aust. Vet. J., 39 (1963), pp. 373-385

9. Annexes

Annex 1 – AFS Themes

Increasing agricultural productivity (Availability)

- This project aims to improving productivity by preventing losses from contagious bovine pleuropneumonia. CBPP is the only bacterial infectious disease in the highest-priority group of OIE. With annual negative impact in excess of 44 million EUR, the effect of disease reduction on agricultural productivity and food security can be very significant.
- Reduced loss by the disease can contribute to stabilizing the market and reducing price volatility.
- Inherent feature of vaccination prophylaxis is the reduction of antibiotic use and antibiotic spread in the environment, which then reduces both the environmental contamination and the selection pressure towards new generations of drug-resistant microorganisms.

Improving access to resources, and/or markets and income (Accessibility)

- Impact can be indirect through reduction of losses

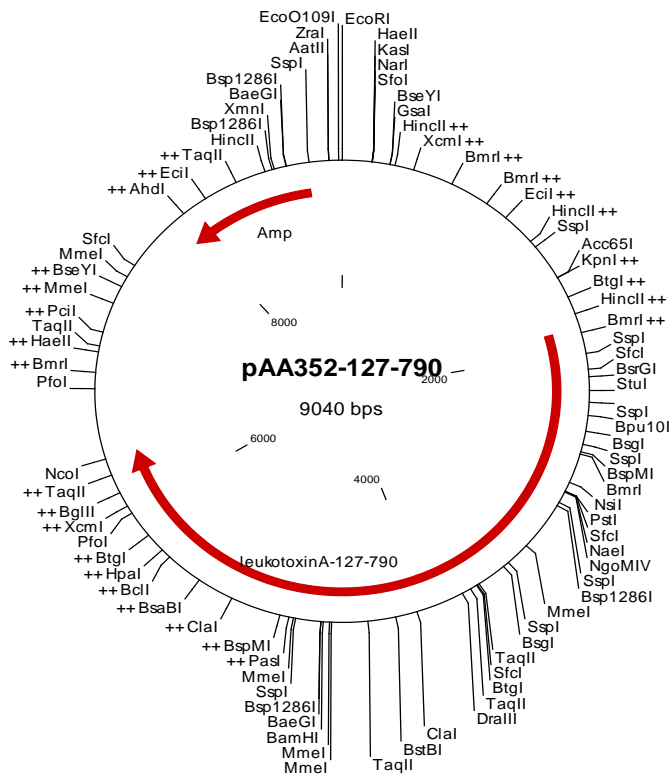
Informing policy

How is the project informing and/or influencing the development and implementation of food security policies? More specifically:

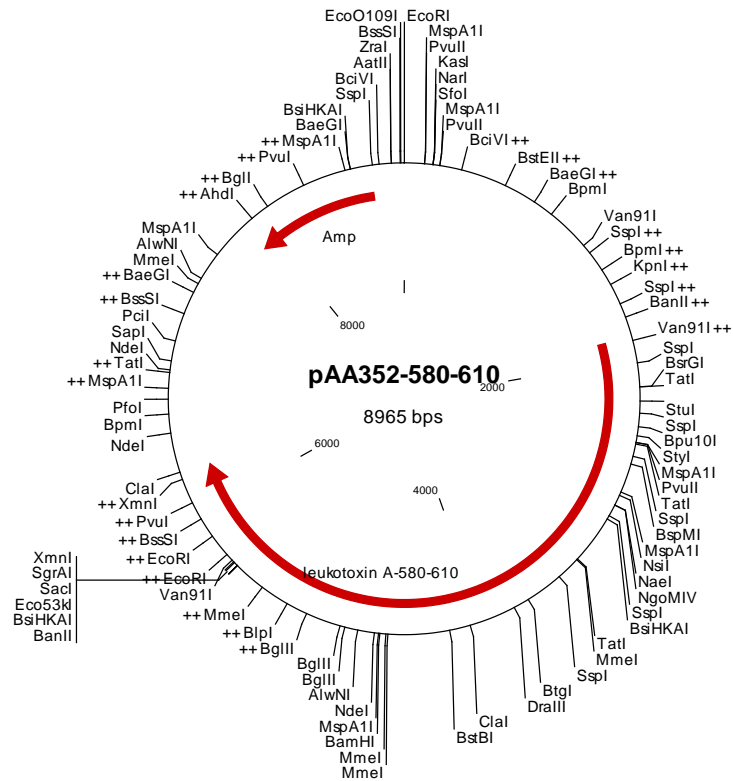
- Policy makers were engaged directly in stakeholder meeting in 2015 and 2018. Senior management of AU-IBAR, the Kenya Veterinary Board, National Biosafety Authority, National Council for Science and Technology, Directorate of Veterinary Medicines (DVM), FAO, and GALVmed were engaged in discussions and voiced support for the project.
- County government and County Veterinary officers were routinely engaged during stakeholder meetings as well as during project field activities.

Annex 2 – Antigens for the first vaccine formulation trial

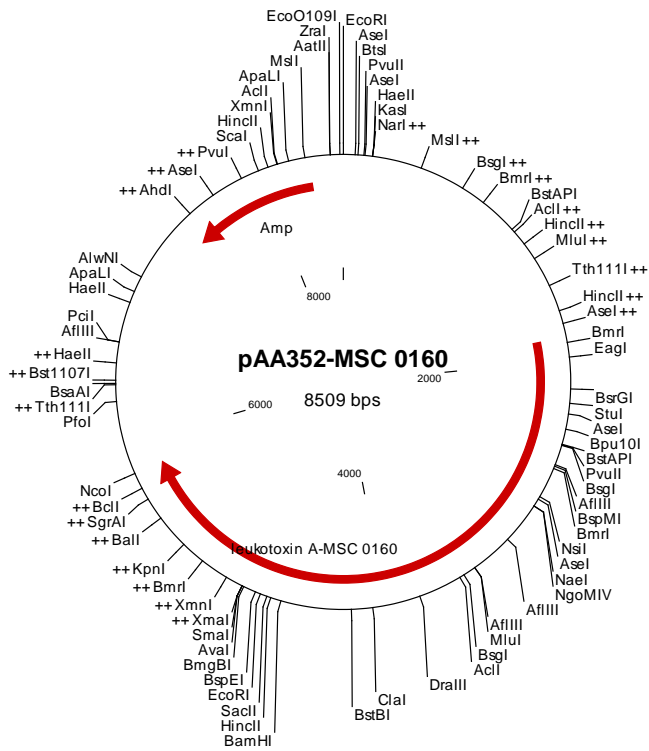
We constructed fusions of proteins YP_004400127.1 and YP_00399790.1 cloned in frame with leukotoxin A protein of *M. haemolytica* by insertion in *BamHI*-digested plasmid pAA352. The latter contains the *lktA* gene and has been used commercially in Canada as part of a shipping fever vaccine.



We constructed fusions of proteins YP_004400610.1—YP_004400581.1 cloned in frame with leukotoxin A protein of *M. haemolytica* by insertion in *BamH1*-digested plasmid pAA352. The latter contains the *lktA* gene and has been used commercially in Canada as part of a shipping fever vaccine.



We constructed fusions of protein MSC 0160 cloned in frame with leukotoxin A protein of *M. haemolytica* by insertion in *BamHI*-digested plasmid pAA352. The latter contains the *lktA* gene and has been used commercially in Canada as part of a shipping fever vaccine.



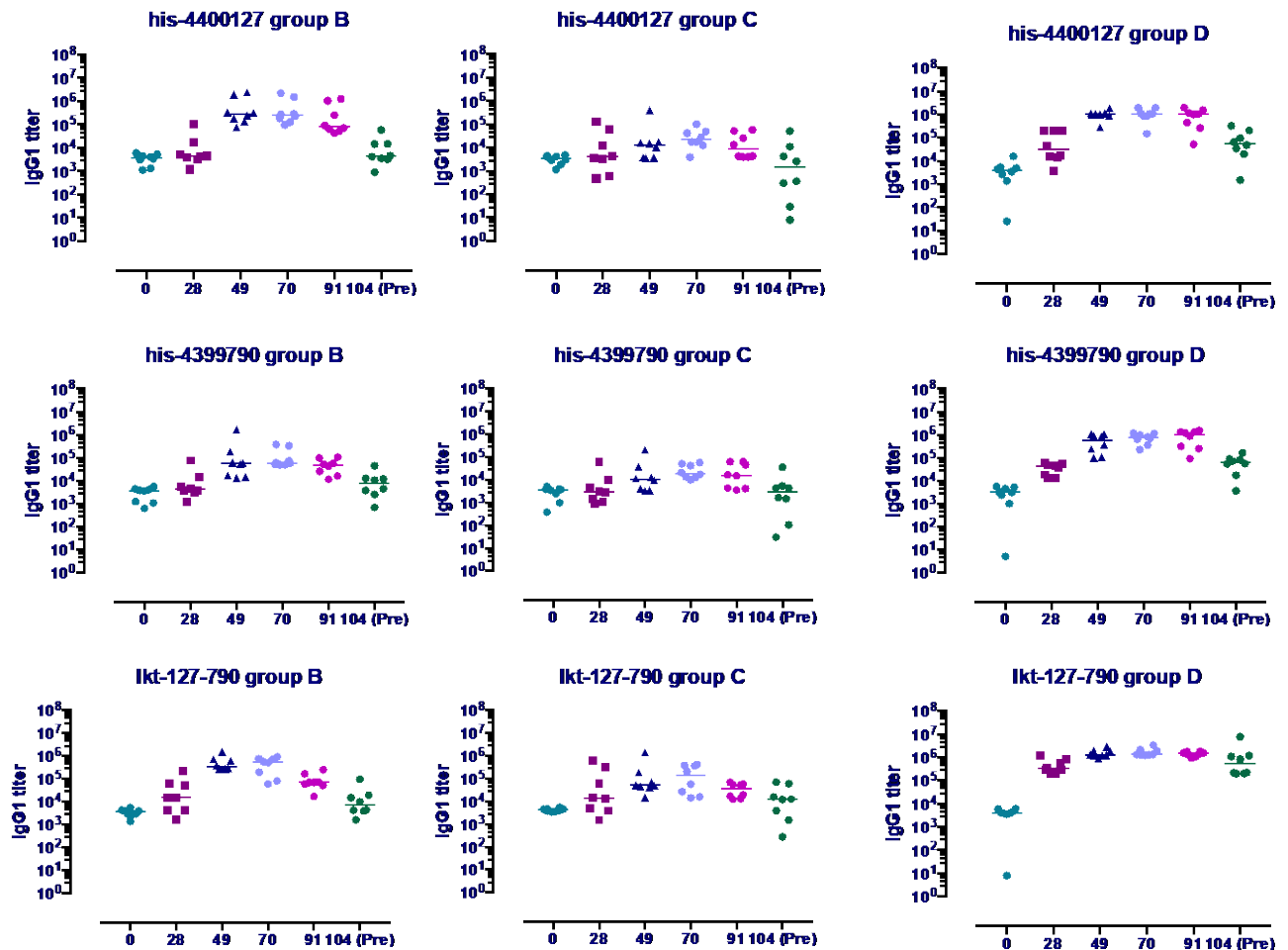
Annex 3 – First vaccine formulation trial

32 Boran cattle, 8 animals per group

Group	Antigens (50 µg/dose)	Adjuvants
A	NONE	30% Emulsigen CpG2007 (250 µg)
B	LtxA-YP_004400127.1-YP_004399790.1 LtxA-YP_004400580.1-YP_004400610.1 LtxA-MSC_0160	30% Emulsigen CpG2007 (250 µg)
C	LtxA-YP_004400127.1-YP_004399790.1 LtxA-YP_004400580.1-YP_004400610.1 LtxA-MSC_0160	VIDO Adjuvant (EP3 + CpG2007 + IDR1002)
D	LtxA-YP_004400127.1-YP_004399790.1 LtxA-YP_004400580.1-YP_004400610.1 LtxA-MSC_0160	Montanide ISA61VG

Two vaccinations, 28 days apart. 2 ml by subcutaneous route.
Challenge 104 days after initial vaccination

Comparison of IgG1 titers in the different treatment groups, showing stronger immune responses to leucotoxin (LktA) fusion proteins. In the **first row** titers to protein YP 4400127.1 and are shown for different adjuvants: left column (Group B):30% Emulsigen with CpG 2007; middle column (Group C):EP3 with CpG 2007 and IDR 1002; right column (Group D):Montanide ISA61VG. **Second row** shows titers to the protein YP 4399790.1, with the same adjuvants in the respective columns. **Third row** shows titers to Leucotoxin fusion proteins containing both YP 4400127.1 and YP 4399790.1, with the same adjuvants in the respective columns.



Annex 4 – Second vaccine formulation trial

48 Boran cattle, 8 animals per group

Group	Vaccine antigens 50µg each/dose	Adjuvant
Group A	placebo (no antigens)	CpG ODN 2007 and emulsigen
Group B	LKT-MSC 0136, LKT-MSC 0431, LKT-MSC 0499, LKT-MSC 0775	Emulsigen, and CpG
Group C	LKT-MSC 0136, LKT-MSC 0431, LKT-MSC 0499, LKT-MSC 0775	CpG, host defence peptide, and EP3
Group D	LKT-MSC 0136, LKT-MSC 0431, LKT-MSC 0499, LKT-MSC 0775	Montanide ISA61VG
Group E	his-MSC 0136, his-MSC 0431, his-MSC 0499, his-MSC 0776, MSC 0957	Emulsigen and CpG
Group F	his-4399807, his-4400559, his-MSC 0775, his-MSC 0816, his-MSC 0160	Emulsigen and CpG

Two vaccinations, 28 days apart. 2 ml by subcutaneous route.

Group A was control. Groups B, C, D were formulated to test the adjuvants and contained the antigens with strongest immune responses in previous trials. Groups E and F were controls to compare to the Phase 1 trial where they were protective. At the end of the trial the animals were challenged with *M. mycoides* to assess possible differences in protective efficacy.

Annex 5 – Third vaccine formulation trial

40 Zebu cattle, 8 animals per group

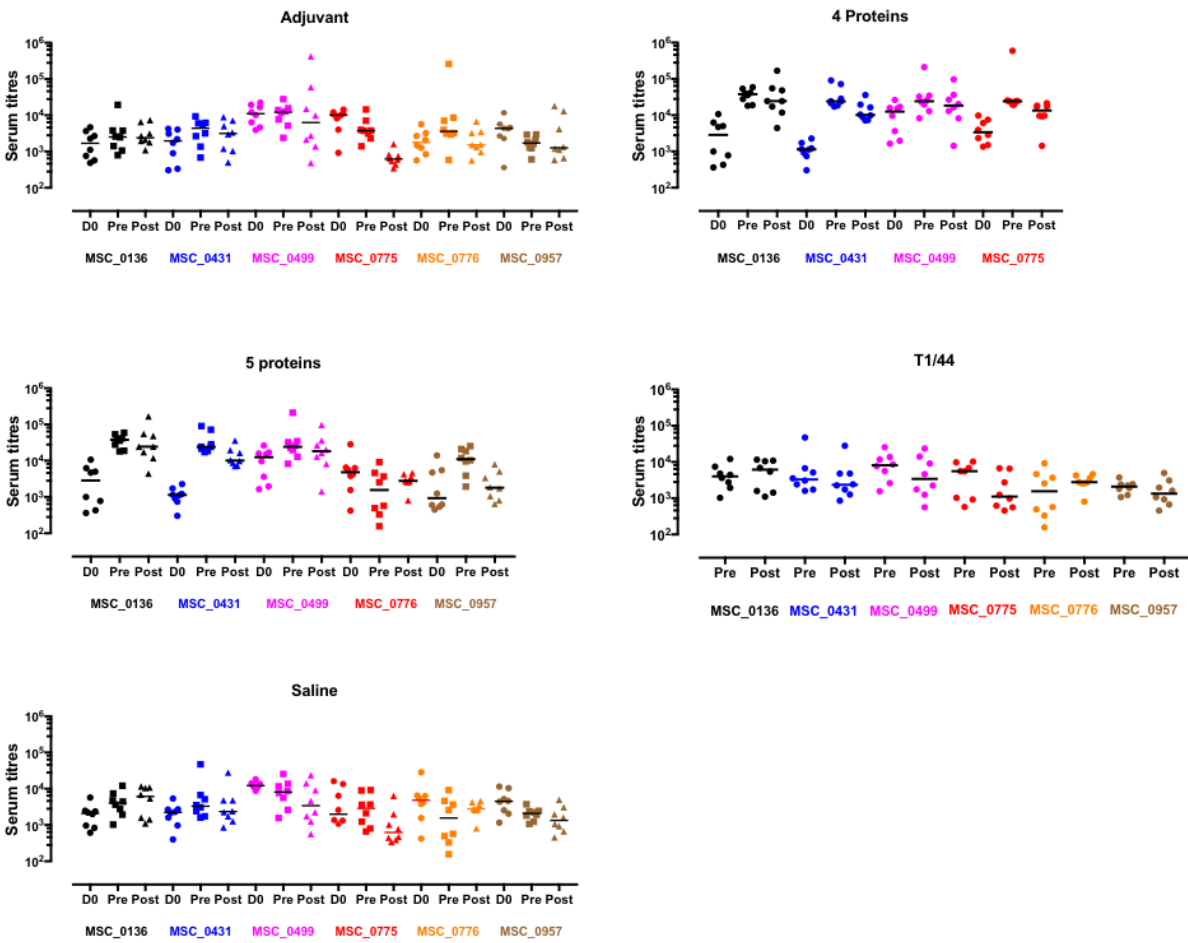
Group	Vaccine antigens 50µg each/dose	Adjuvant
Group A	placebo (no antigens)	Montanide ISA61VG
Group B	his-MS C 0136, his-MS C 0431, his-MS C 0499, his-MS C 0775	Montanide ISA61VG
Group C	his-MS C 0136, his-MS C 0431, his-MS C 0499, his-MS C 0776, his-MS C 0957	Montanide ISA61VG
Group D	T 1/44	Montanide ISA61VG
Group E	Placebo (no antigens, no adjuvant)	None

Two vaccinations, 28 days apart (one vaccination for the T1/44). 2 ml by subcutaneous route.

Lesion and Pathology scores for the treatment groups

Group	Antigen	Lesions	Mean lesion score	Mean pathology score	% Protection
A (Adjuvant)	None	1 / 8	1.5	18	33
B (Proteins)	4 proteins	1 / 8	0.75	5	81
C (Proteins)	5 proteins	3 / 8	2.25	30	-11
D (T1/44)	Attenuated	5 / 8	1.375	21	22
E (Saline)	None	4 / 8	2.125	27	*

IgG titers of the treatment groups



Annex 6 - Expression conditions and plasmid stability

As part of the process of optimizing gene expression in recombinant E. coli strains, we examined several parameters including the effect of different growth media, optimal cell density at which to induce cultures, length of induction, concentration of IPTG, and growth temperature. The best conditions, based on purity of protein and total yield, are listed below.

Growth in LB broth (animal components present)

BL21 DE3 (pAA352-127-790)

- induce at A600 of 0.5 (mid log)
- induce with 0.5mM IPTG
- induce for 2 hours
- grow at 37C
- total yield is approximately 60mg/L

BL21 DE3 (pAA352-580-610)

- induce at A600 of 0.8 (late log)
- induce with 0.5mM IPTG
- induce for 2 hours
- grow at 37C
- total yield is approximately 80mg/L

BL21 DE3 (pAA352-MSC 0160)

- induce at A600 of 0.2 (early log)
- induce with 1.0mM IPTG
- induce for 3 hours
- grow at 37C
- total yield is approximately 30mg/L

Growth in phytone peptone broth (no animal components present)

BL21 DE3 (pAA352-127-790)

- induce at A600 of 0.1 (early log)
- induce for 3 hours
- total yield is approximately 80mg/L

BL21 DE3 (pAA352-580-610)

- induce at A600 of 0.1 (early log)
- induce for 3 hours
- total yield is approximately 50mg/L

BL21 DE3 (pAA352-MSC 0160)

- induce at A600 of 0.35 (mid log)
- induce for 3 hours
- total yield is approximately 50mg/L

Plasmid stability of the fusion proteins

Cultures of strains, containing the plasmids pAA352-127-790, pAA352-580-610 and pAA352- MSC 0160 were grown in non-selective media: LB broth, and phytone peptone. Dilutions of the cultures were plated on non-selective media and numbers were compared with the same dilutions plated on selective media. The ratio of colonies growing on selective media versus the colonies growing on non-selective media (expressed in percentage) is shown as plasmid stability for the given number of generations (left columns of each table).

grown in LB broth

pAA352-127-790	
# of generations	% stability
2	99.7
3.5	100
5	99.7
16	96
36	99
56	83.3

pAA352-580-610	
# of generations	% stability
2	99.3
3.5	99.1
5	99.3
16	69.3
36	55.6
56	37.3

pAA352-MSC 0160	
# of generations	% stability
2	99.9
3.5	100
5	99.3
16	100
36	100
56	100

grown in phytone peptone broth

pAA352-127-790	
# of generations	% stability
1.5	100
4.5	99.2
7.5	99.8
16	100
32	100
52	100

pAA352-580-610	
# of generations	% stability
1	97.4
2.5	93
4.5	97.5
19	97
38	99
60	99

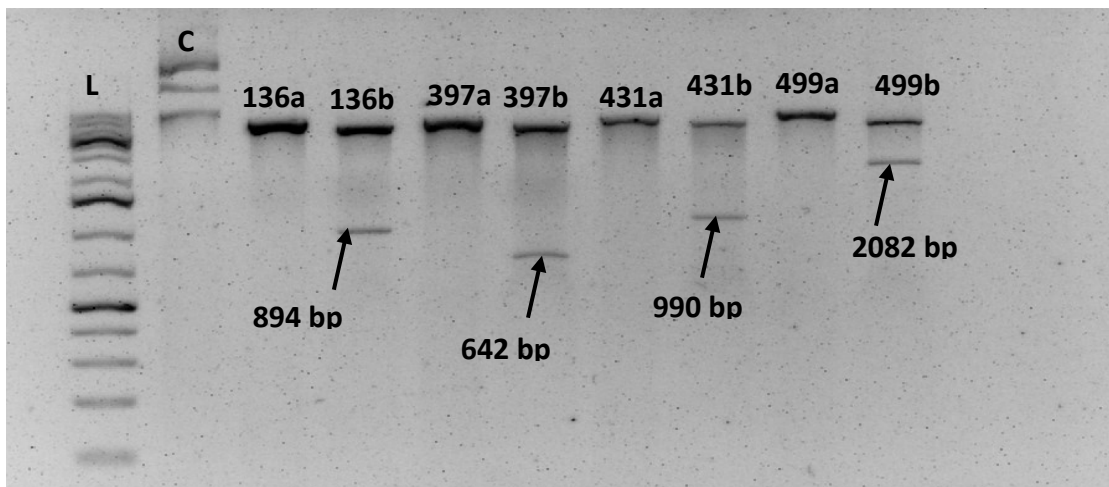
pAA352-MSC 0160	
# of generations	% stability
4	100
7	100
10	100
17	99
35	98
57	98

Annex 7 – Summary of his-tagged strains for antigen production.

The genes encoding the antigens listed in the second column were individually cloned in plasmid pGS21a and transformed in BL21 STAR (DE3) strain. Plasmid pGS21a contains histidine residues, which are fused with the expressed proteins and used for protein purification in both laboratory and industrial conditions.

Description	Name	Insert	Origin Insert	Protein MW	Strain #	Plasmid
lipoprotein	MSC_0136	894 bp	Mmm-Shawawa/PG1	34kDa	EC2935	pMSC 0136
Elongation factor Tu	MSC_0160	1185 bp	Mmm-Gladysdale/Shawawa/PG1	43kDa	EC2937	pMSC 0160
lipoprotein	MSC_0431	990 bp	Mmm-Shawawa	38kDa	EC2945	pMSC 0431
lipoprotein	MSC_0499	2082 bp	Mmm-Gladysdale/Shawawa/PG1	79kDa	EC2948	pMSC 0499
lipoprotein	MSC_0775	2106 bp	Mmm-Shawawa	81kDa	EC2956	pMSC 0775
lipoprotein	MSC_0776	2265 bp	Mmm-Shawawa	88kDa	EC2957	pMSC 0776
lipoprotein	MSC_0816	1146 bp	Mmm-Gladysdale/Shawawa	44kDa	EC2963	pMSC 0816
lipoprotein	MSC_0957	1230 bp	Mmm-Gladysdale/Shawawa/PG1	47kDa	EC2965	pMSC 0957

Example of gel electrophoresis showing the size of inserts in several pMSC plasmids. Numbers above each lane denote the respective MSC number, plasmids in lanes marked with “a” were digested with one (*BamHI*) restriction enzyme, and in lanes marked with “b” were digested with two restriction enzymes (*BamHI* and *HindIII*), which released the cloned insert.



Annex 8 - Strains shipped from VIDO-InterVac to KALRO

Strains expressing his-tagged antigens

EC2935 BL21 STAR DE3 (pMSC 0136)	EC2974 BL21 STAR DE3 (pET15b-4399807)
EC2937 BL21 STAR DE3 (pMSC 0160)	EC2991 BL21 STAR DE3 (pET15b-4400559)
EC2945 BL21 STAR DE3 (pMSC 0431)	EC3008 BL21 STAR DE3 (pET15b-127-790)
EC2948 BL21 STAR DE3 (pMSC 0499)	EC3010 BL21 STAR DE3 (pET15b-580-610)
EC2956 BL21 STAR DE3 (pMSC 0775)	EC2994 BL21 STAR DE3 (pET15b-4400610)
EC2957 BL21 STAR DE3 (pMSC 0776)	EC2992 BL21 STAR DE3 (pET15b-4400580)
EC2963 BL21 STAR DE3 (pMSC 0816)	EC2979 BL21 STAR DE3 (pET15b-4400127)
EC2965 BL21 STAR DE3 (pMSC 0957)	EC2971 BL21 STAR DE3 (pET15b-4399790)
	EC2952 BL21 STAR DE3 (pMSC 0610)
	EC2933 BL21 STAR DE3 (pMSC 0052)

Strains expressing leukotoxin fusions

EC3017 BL21 DE3 (pAA352-127-790)	EC3050 W1485 (pAA352-MSC 0776)
EC3018 BL21 DE3 (pAA352-580-610)	EC3051 W1485 (pAA352-MSC 0499)
EC3019 BL21 DE3 (pAA352-MSC 0160)	EC3054 W1485 (pAA352-MSC 0160)
EC3032 BL21 DE3 (pAA352-4400559)	EC3055 W1485 (pAA352-MSC 0816)
EC3034 BL21 DE3 (pAA352-MSC 0136)	EC3056 W1485 (pAA352-MSC 0431)
EC3036 BL21 DE3 (pAA352-MSC 0499)	EC3059 W1485 (pAA352-4399807)
EC3038 BL21 DE3 (pAA352-MSC 0431)	EC3060 BL21 DE3 (pAA352-MSC 0957)
EC3040 BL21 DE3 (pAA352-MSC 0816)	EC3062 W1485 (pAA352-MSC 0957)
EC3043 BL21 DE3 (pAA352-MSC 0776)	EC3064 W1485 (pAA352-580-610)
EC3045 BL21 DE3 (pAA352-4399807)	EC3066 BL21 DE3 (pAA352-MSC 0775)
EC3048 W1485 (pAA352-127-790)	EC3067 W1485 (pAA352-MSC 0775)
EC3049 W1485 (pAA352-4400559)	EC3068 W1485 (pAA352-MSC 0136)