Vaccine 42 (2024) 1868–1872

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Short communication

Deep sequencing and variant frequency analysis for the quality control of a live bacterial vaccine against contagious bovine pleuropneumonia, strain T1

François Thiaucourt [a,b], Antoni Exbrayat [a,b], Etienne Loire [a,b], Anne Boissière [a,b], Nick Nwankpa [c,1], Lucía Manso-Silván [a,b,*]

[a] CIRAD, UMR ASTRE, F-34398 Montpellier, France
[b] ASTRE, Univ Montpellier, CIRAD, INRAE, F-34398 Montpellier, France
[c] Pan-African Veterinary Vaccine Centre of the African Union (AU -PANVAC), PO Box 1746, Bishoftu, Ethiopia

ARTICLE INFO

Keywords:
Live bacterial vaccine
Quality control
Variant frequency analysis
Mycoplasma mycoides subsp. *mycoides*
Contagious bovine pleuropneumonia

ABSTRACT

Vaccination is the most cost-effective tool to control contagious bovine pleuropneumonia. The vaccines currently used in Africa are derived from a live strain called T1, which was attenuated by passage in embryonated eggs and broth culture. The number of passages is directly correlated to the degree of attenuation of the vaccinal strains and inversely correlated to their immunogenicity in cattle. Current quality control protocols applied to vaccine batches allow the assessment of identity, purity, and titers, but cannot assess the level of genetic drift form the parental vaccine strains. Deep sequencing was used to assess the genetic drift generated over controlled *in vitro* passages of the parental strain, as well as on commercial vaccine batches. Signatures of cloning procedures were detected in some batches, which imply a deviation from the standard production protocol. Deep sequencing is proposed as a new tool for the identity and stability control of T1 vaccines.

1. Introduction

Contagious bovine pleuropneumonia (CBPP), caused by the bacterium *Mycoplasma mycoides* subsp. *mycoides* (Mmm), is a severe infectious disease affecting *Bovidae* [1]. CBPP is a disease notifiable to the world organization for animal health (WOAH, founded as OIE). It is widely distributed in Africa, South of the Sahara, down to the north of the SADC (Southern African Development Cooperation) [2]. Its control is mainly based on vaccination, since antibiotic treatments are officially discouraged due to the risk of antimicrobial resistance. The efficacy of current control strategies relies on two conditions: (i) the vaccines are of good quality, and (ii) mass vaccination campaigns are correctly implemented, so that a significant proportion of the cattle population is able to develop the expected protective immune response. The most widely used vaccine strain, T1/44, recommended by the WOAH [1], was attenuated by 44 serial passages in embryonated eggs [3]. T1/44 induces a protection lasting for approximately one year but, due to its residual virulence, it occasionally induces adverse post-vaccinal reactions at the injection site [4]. This local inflammation is known as the "Willems reaction" in memory of Dr Willems, who described this phenomenon resulting from "inoculation" trials with virulent strains [5]. A streptomycin-resistant derivative of T1/44, called T1sr, was developed by adding a few serial *in vitro* passages, and this strain was used when combined campaigns against rinderpest and CBPP were implemented in the 70ies [6]. The T1sr strain induces shorter protection, estimated at around six months but, conversely, it does not induce the Willems' reaction at the injection site [7]. This illustrates the fact that the empirical development of an effective live vaccine strain is dependent on a delicate balance between achieving sufficient attenuation and preserving enough virulence to stimulate the immune system and induce protection [8]. This critical

Abbreviations: CBPP, Contagious bovine pleuropneumonia; Mmm, *Mycoplasma mycoides* subsp. *mycoides*; WOAH, world organization for animal health; SNP, single nucleotide polymorphism.

* Corresponding author at: UMR CIRAD-INRAE ASTRE, CIRAD, Département Bios; TA A-117/E Campus International de Baillarguet, 34398 Montpellier Cedex 5, France.

E-mail addresses: antoni.exbrayat@cirad.fr (A. Exbrayat), etienne.loire@cirad.fr (E. Loire), anne.boissiere@cirad.fr (A. Boissière), lucia.manso-silvan@cirad.fr (L. Manso-Silván).

[1] Present address: National Veterinary Research Institute (NVRI), Vom, Nigeria.

https://doi.org/10.1016/j.vaccine.2024.02.031
Received 8 December 2023; Received in revised form 2 February 2024; Accepted 8 February 2024
Available online 15 February 2024

point may be achieved after a variable number of passages, as illustrated by historical vaccine strains such as KH3J, obtained following 89 passages, and T2, after 32 passages. Interestingly, this delicate balance is still critical today for the successful development of genetically engineered vaccines [9].

The current quality control of CBPP vaccines is mainly based on the verification of the identity, purity and titer of the final product [1]. The identity can be determined using a conventional PCR assay [10], the purity by culture in appropriate media, and the titer by mycoplasma titration procedures. In the case of CBPP vaccines, the potency of each batch is not verified, since there is no laboratory animal model allowing a cost-effective assay. The potencies of the T1/44 and T1sr grand-parental strains were re-evaluated in cattle some years back [7,11]. It is considered that the final vaccine batches should have a potency similar to the grand-parental strains, as long as producers strictly adhere to WOAH production guidelines [1]. These guidelines state clearly that (i) there must be a limited number of passages between the parental strain stock and the final vaccine batch and (ii) cloning procedures must be avoided, as the selection of variants may lead to products of unknown quality. Unfortunately, there is no quality control procedure available at present to verify that these guidelines are strictly respected and to reveal an eventual genetic drift of the vaccine strain.

The objective of this study was to develop a quality control tool to evaluate the genetic drift that may occur when passaging T1/44 *in vitro* and to analyze a representative number of commercial CBPP vaccine batches using this tool. For this purpose, we adopted a deep sequencing approach for variant frequency analysis along the T1 strain genomes.

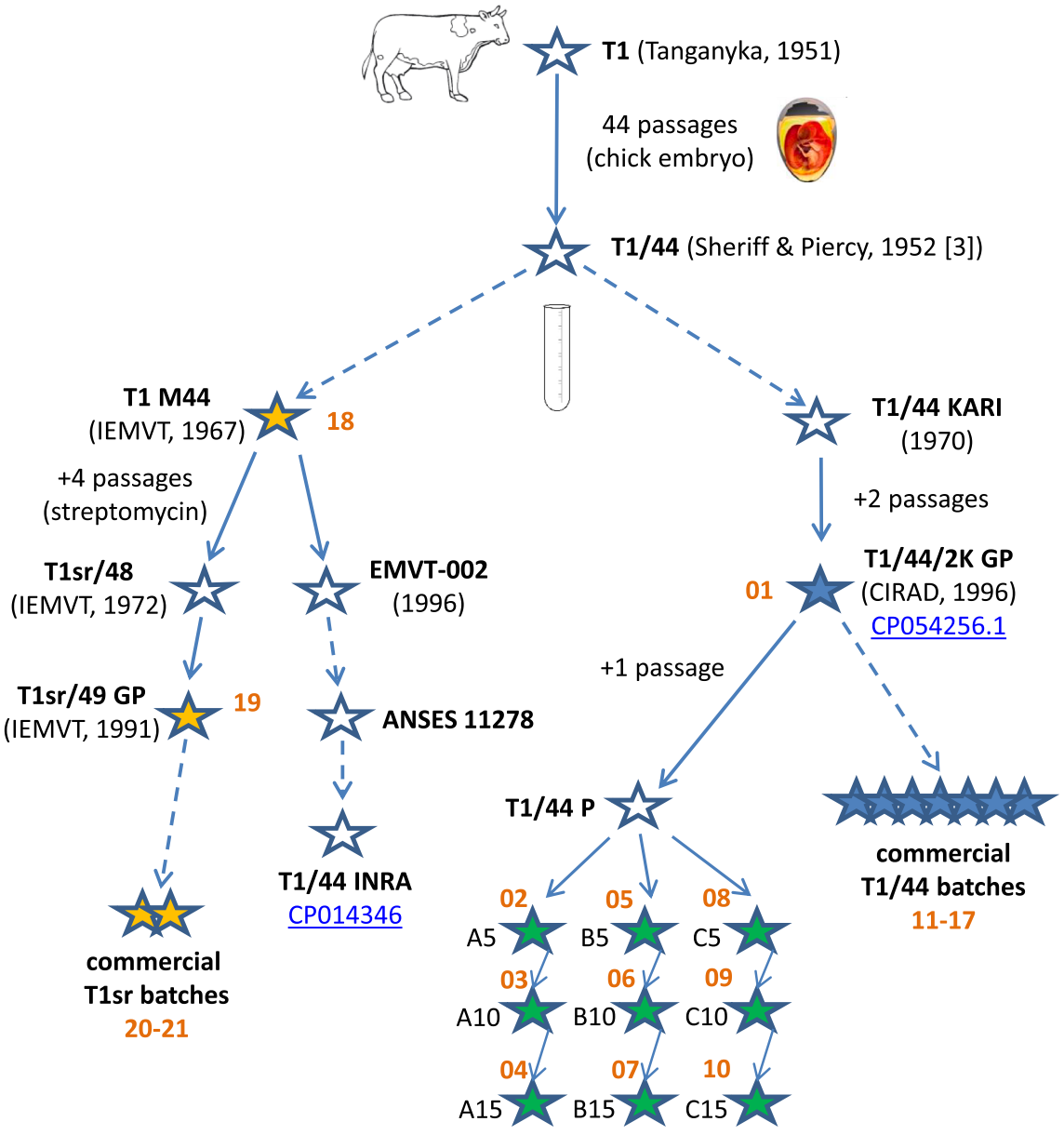

Fig. 1. T1 strain history The origin and passage history of T1 strain stocks, passages and commercial batches analyzed in this study is shown. T1 strains are represented by star symbols, and those from which the whole genome sequence was obtained in this study are colored: in blue, T1/44/2K grand parental stock and commercial vaccine batches; in green, three independent cultures (named A, B and C) derived from the T1/44/2K grand-parental stock and their respective passages 5, 10 and 15; and in yellow, T1sr parental strains and commercial vaccine batches. Dashed arrow lines indicate unknown passage history. Sequenced samples are numbered in orange. GenBank accession numbers of available whole genome sequences are provided. Abbreviations: ANSES: Agence Nationale de Sécurité Sanitaire de l'Alimentation de l'Environnement et du Travail; EMVT: Département "Elevage et Médecine vétérinaire du CIRAD"; IEMVT: "Institut d'élevage et de médecine vétérinaire des pays tropicaux"; INRA: "Institut National de Recherche Agronomique"; KARI: Kenya Agricultural Research Institute.

2. Materials and methods

2.1. Strains and culture conditions

The history of the T1 strains analyzed in this study is summarized in Fig. 1. T1, isolated in "Tanganyika", Tanzania, was described by Sheriff and Piercy as an Mmm strain of mild virulence; it was passaged 44 times in embryonated eggs until it was sufficiently attenuated to be considered a safe attenuated vaccine, which was then named T1/44 [3]. In 1996, CIRAD was asked by the FAO to produce a grand-parental stock for potency re-confirmation and distribution to vaccine producing laboratories. For that purpose, a stock of T1/44, freeze-dried in 1970, was obtained from KARI Muguga, Kenya. It was passaged twice and freeze-dried before being submitted to AU-PANVAC for quality control. This stock, named T1/44/2K, is now considered the grand-parental stock.

T1sr was developed at the "Institut d'élevage et de médecine vétérinaire des pays tropicaux (IEMVT), Maisons-Alfort, France" in 1972 by passaging a stock of T1/44 (T1 M44 obtained from Pr. Lindley in 1967) in a medium containing streptomycin. After 4 passages, the resulting streptomycin-resistant strain was called T1sr/48. In 1991 a stock of grand-parental strain was produced with an additional passage, T1sr/49 [6].

To study the *in vitro* evolution of T1/44 under vaccine production conditions [1], three independent cultures (named A, B and C) of a "parental stock (T1/44P)" produced by a single passage from the T1/44/2K GP stock (Fig. 1) were performed and passaged fifteen times. Each passage consisted in applying a 1/100 dilution (50 µl in 5 ml) in modified Hayflick's medium [12] and incubating it for about 24 h until turbidity was observed. Aliquots of passages 5, 10 and 15 were collected and stored at −80 °C for whole genome sequence analysis.

To study the level of genetic drift in CBPP vaccines presently used in Africa, the AU-PANVAC selected 7 batches of T1/44 and 2 batches of T1sr vaccines from different manufacturers that had been sent for quality control. One vial of each batch was sent to CIRAD, after being coded to respect confidentiality.

2.2. Whole genome sequencing

Five milliliters of end of exponential phase cultures in modified Hayflick's medium were pelleted and washed in PBS, with centrifugation for 10 min at 12,000 *g*, and DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). DNA samples were sent to Macrogen (Seoul, Republic of Korea) for analysis. Illumina TruSec DNA PCR-free libraries were constructed before analysis through an Illumina 2500 platform (2X250bp).

2.3. Reference T1 genomes

The T1/44 genome sequence available at NCBI prior to this study (CP014346) [13] was obtained from a T1/44 stock at INRAE, derived from the "T1 M44" stock held at CIRAD (Fig. 1). This stock was transferred via a third laboratory to INRAE-Bordeaux, where its chromosome was sequenced. Thus, the culture history of the sequenced stock differs from that of the grand-parental stock held at CIRAD. The genome sequence of the grand-parental strain (T1/44/2K GP) was obtained in this study, as described above, and reads were mapped on the T1/44 INRAE genome sequence available at NCBI (CP014346). The T1/44/2K GP genome was annotated using Prokka [14], deposited in GenBank (CP054256.1) and used as reference for subsequent diversity analyses. The genome of T1 M44 (Fig. 1), also obtained in this study, was used as reference for diversity analysis concerning T1sr/49 grand-parental and T1sr commercial strains.

The raw genomic sequences analyzed in this study were deposited in the NCBI Sequence Read Archive PRJNA1050060.

2.4. Determination of allelic frequency variations among T1 stocks

The pipeline grenepipe [15] was used to analyze raw Illumina sequencing data. Briefly, adapter and low-quality sequences were first removed with TRIMMOMATIC, reads were mapped on the genome sequence of the grand-parental strain T1/44/2K GP (CP054256.1) with BWA-MEM, duplicates were filtered with PICARD and variants were called using GATK HAPLOTYPECALLER, filtering variants with less than 500X coverage and a calling quality of 100. Additional filtering steps were undertaken to remove variants with low frequency (5 % and below). The remaining SNPs were evaluated to remove those located on repetitive sequences. This was done by copying the 30 bases upstream the SNP and searching for duplicates in the original reference sequence. SNPs resulting from misalignments near tandem repeats or homopolymers were also removed. The reads from T1sr/49 and commercial T1sr strains were mapped on the genome of T1 M44 (Fig. 1), also obtained in this study.

3. Results and discussion

The genome sequences of subsequent *in vitro* passages (5, 10 and 15) from three independent cultures of a T1/44 parental stock performed at CIRAD, as well as seven commercial batches of T1/44, were analyzed. SNP calling was performed using as reference the genome of the T1/44/2K grand-parental strain (Fig. 2).

The three independent control cultures of T1/44/ (A, B and C) underwent a progressive genetic drift that was characterized by the accumulation of a limited number of mutations. Such evolution is compatible with Mmm's mutation rate, which was estimated around 10^{-8}/base/duplication [16].[11] Three identical polymorphic positions were detected in all passages of the three independent cultures (Fig. S1). Their frequency increased from around 30 % (after 5 *in vitro* passages) to 82–89 % (after 10 and 15 passages). A few additional polymorphic positions were observed. However, these were all at very low frequencies. These results illustrate the stability of cultures when passages are performed according to good manufacturing practices (*i.e.*, reduced number of passages and no cloning procedures).

Contrasted results were obtained on the commercial T1/44 vaccine batches (Fig. 2, Fig. S1). Two batches (11 and 15) retained high similarity to the grand-parental stock. They only presented low numbers of low frequency SNPs (well below 50 %), similar to the results obtained in the 5th passage of cultures A, B and C, indicating that these vaccines were performed according to good manufacturing practices. The other five batches differed significantly from the original stock. Batch 17 was the least divergent, though the number and frequency of mutations in this batch were higher than in the 10th passage of cultures A, B and C, which may indicate the implementation of additional subcultures from the T1/44 parental stock before batch production. Batches 13 and 16, on the other hand, presented several SNPs at very high frequency (over 90 %), which may either reflect the use of a different grand-parental stock or the rapid expansion of a variant within the culture. The other two (batches 12 and 14) presented several fixed SNPs (found at a frequency of 100 %). Batch 12 had the highest number of totally fixed mutations (N = 5). Mutations fixed in the population may indicate the selection of variants, which occur during population bottlenecks. This may happen when seeding a parental stock in primary dilution and selecting the last tube showing turbidity to seed the production medium. In such situations, the seed culture is likely to derive from a few mycoplasmas in which mutations may have been fixed. Again, the fixed mutations may also indicate the use of a different parental stock.

Although T1sr is rarely used, commercial T1sr vaccine batches are still sent to PANVAC for quality control. Two batches were thus included in this analysis. However, since T1sr vaccines have a different origin, SNP calling analyses were performed using as reference the genome of T1 M44 (Fig. 1). The comparison of T1sr sequences (Fig. S2) revealed the presence, in the T1sr/49 grand-parental stock, of a fixed, non-

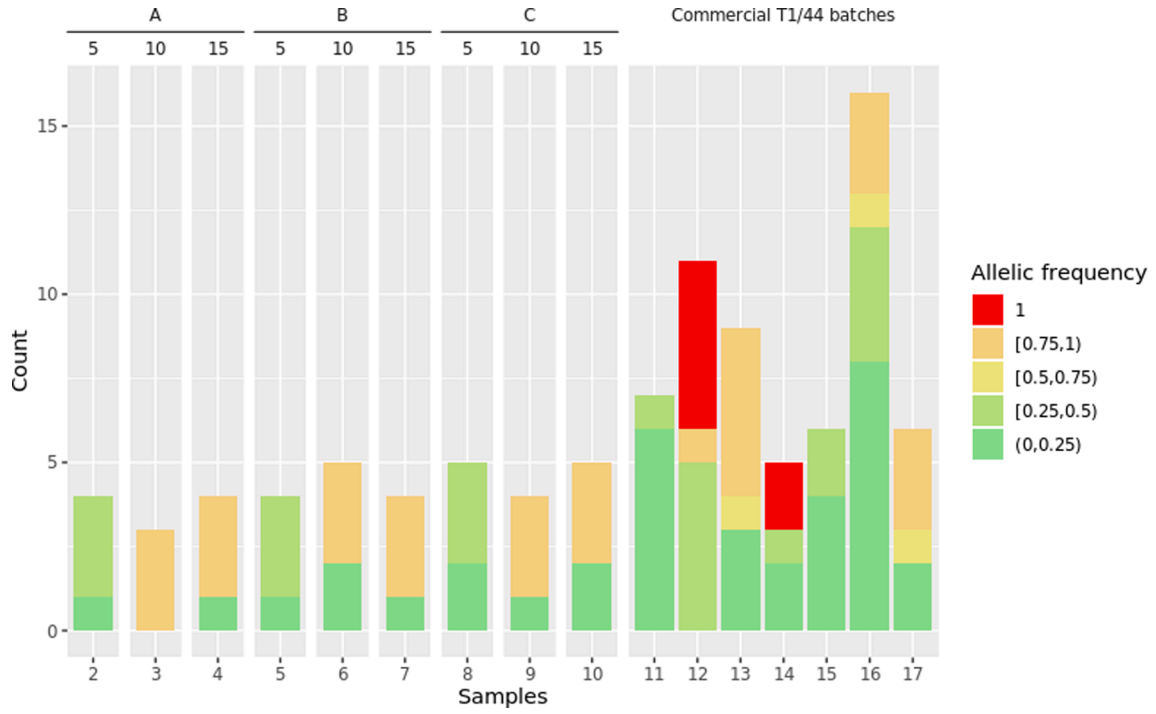

Fig. 2. Variant analysis among T1/44 independent culture passages and commercial vaccine batches The genome sequences of passages 5, 10 and 15 from three independent cultures (named A, B and C) of T1/44, as well as seven commercial T1/44 vaccine batches, were subjected to diversity analysis using as reference the T1/44/2K GP genome (CP054256.1). The number of polymorphisms identified in each sample is shown, with their respective allelic frequency range indicated according to the color code provided in the figure legend. Sample numbers as indicated in Fig. 1.

synonymous mutation affecting the *rplS* gene (HR079_04940; Tab. S1). In bacteria, mutations in this gene, encoding 30S ribosomal protein S12, are often correlated with streptomycin resistance, but also reduced growth rate and virulence [17]. Six additional mutations at frequencies ranging from 79 to 82 % were found in the T1sr/49 grand-parental stock in comparison with the T1 M44 reference genome. For what concerns the two T1sr commercial vaccine batches, they differed notably from the grand-parental stock by loss of the seven characteristic T1sr positions mentioned above, including the *rplS* mutation. This may be an indication that vaccine manufacturers are not adding streptomycin to the culture medium, presumably to improve the growth in culture. Again, the genetic drift observed in these vaccines is not compatible with good manufacturing practices and indicates either the use of alternative parental stocks or excessive passage and cloning during vaccine production.

4. Conclusion

A live vaccine is not a genetically uniform entity but consists of a more or less heterogeneous population determined by the characteristics of the grand-parental vaccine stock, the production history of the batches, and the evolutionary mechanisms at stake for the organism. Analysis of deep sequencing data can elucidate strain diversity at very high resolution and enable the comparison of live vaccine stocks that may have evolved differently [18,19]. This is particularly important for attenuated vaccine strains, such as T1, since divergent populations may have different immunizing properties. In this work, we show that the genetic drift occurring in live CBPP vaccine batches can be easily evaluated by the proposed genome variant analysis. This is essential for vaccines that cannot be routinely checked for potency and for which quality control relies mainly in the evaluation of viable titers. Such genome variant analysis may lead to standardized procedures and be incorporated into regulatory frameworks for vaccine batch quality compliance. The cost of such analysis is certainly minimal compared to the cost of using vaccines with reduced potency.

Authorship Statement

All authors attest they meet the ICMJE criteria for authorship.

Authors Contributions

FT, LM-S & NN conceived and designed the study and acquired the data; FT, AE, EL, AB & LM-S analyzed and interpreted the data; FT, AE & LM-S drafted the article; all the authors revised it critically and approved the final version to be submitted.

Conflict of interest

The authors declare that they have no conflicts of interest.

https://declarations.elsevier.com/?journalAcronym=JVAC (each author to follow).

CRediT authorship contribution statement

François Thiaucourt: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Writing – original draft. **Antoni Exbrayat:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Etienne Loire:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Anne Boissière:** Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nick Nwankpa:** Conceptualization, Resources, Validation, Writing – original draft. **Lucía Manso-Silván:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw genomic sequences analyzed in this study were deposited in the NCBI Sequence Read Archive PRJNA1050060.

Acknowledgements

The work described here was financially supported by the Bill & Melinda Gates Foundation (United States) and the Foreign, Commonwealth and Development Office of the Government of the United Kingdom, through the Global Alliance for Livestock Veterinary Medicines (GALVmed) and the research project Ben-1: "Contagious Bovine Pleuropneumonia BEN-1 Vaccine Evaluation for Africa" (December 2013-October 2017). More specifically, this research was comprised in the work-package 2, including "In vitro evolution of T1 vaccine strain genome", coordinated by the French Agricultural Research Centre for International Development (CIRAD) in France, in collaboration with the Pan-African Veterinary Vaccine Centre of the African Union (AU-PANVAC). The work of FT, AE, EL, AB and LM-S was financially supported by CIRAD in France. The donors did neither have a role in the study design, data collection, analysis and interpretation, nor in the writing of the report and decision to submit the article for publication.

Submission declaration and verification

The work described here has not been published previously and is not under consideration for publication elsewhere. Its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically, without the written consent of the copyright holder.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.02.031.

References

[1] WOAH. Contagious bovine pleuropneumonia (Infection with *Mycoplasma mycoides subsp. mycoides)* In: World Organization for Animal Health, editor. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2021. Chapter3.4.8.
[2] Thiaucourt F. Contagious bovine pleuropneumonia, update. In: Coetzer JAW, Thomson GR, MacLachlan J, Michel A, Botha C, editors. Infectious Diseases of Livestock 2018.
[3] Sheriff D, Piercy SE. Experiments with an avianised strain of the organism of contagious bovine pleuropneumonia. Vet Rec 1952;64:615–21.
[4] Revell SG. Local reactions following C.B.P.P. vaccination in Zambia. Trop Anim Health Prod 1973;5:246–52.
[5] Willems L. Mémoires sur la pleuropneumonie épizootique du gros bétail. Recl Méd Vét Pratique (Maisons Alfort) 1852;3:401–34.
[6] Provost A. Principes de production d'un vaccin mixte associé antibovipestique-antipéripneumonique inoculé en un seul temps. Bull epizoot Dis Afr 1969;17:7–10.
[7] Wesonga H, Thiaucourt F. Experimental studies on the efficacy of T1sr and T1/44 vaccine strains of *Mycoplasma mycoides subsp. mycoides* SC against a field isolate causing contagious bovine pleuropneumonia in Kenya- Effect of a revaccination. Rev Elev Med Vet Pays Trop 2000;53:313–8.
[8] Provost A. Prophylaxie et vaccination dans la péripneumonie bovine. Evolution des techniques et applications pratiques actuelles. Rev Elev Med Vet Pays Trop 1974; 27:145–61.
[9] Galen JE, Curtiss 3rd R. The delicate balance in genetically engineering live vaccines. Vaccine 2014 Jul 31;32(35):4376–85. https://doi.org/10.1016/j.vaccine.2013.12.026. 1.
[10] Lorenzon S, David A, Nadew M, Wesonga H, Thiaucourt F. Specific PCR identification of the T1 vaccine strains for contagious bovine pleuropneumonia. Mol Cell Probes 2000 Aug;14(4):205–10. https://doi.org/10.1006/mcpr.2000.0305.
[11] Yaya A, Golsia R, Hamadou B, Amaro A, Thiaucourt F. Essai comparatif d'efficacité de deux souches vaccinales T1/44 et T1sr contre la péripneumonie contagieuse bovine. Rev Elev Med Vet Pays Trop 1999;52:171–9.
[12] Bonnefois T, Vernerey MS, Rodrigues V, Totté P, Puech C, Ripoll C, et al. Development of fluorescence expression tools to study host-mycoplasma interactions and validation in two distant mycoplasma clades. J Biotechnol 2016 Oct;20(236):35–44. https://doi.org/10.1016/j.jbiotec.2016.08.006.
[13] Gourgues G, Barré A, Beaudoing E, Weber J, Magdelenat G, Barbe V, et al. Complete Genome Sequence of *Mycoplasma mycoides subsp. mycoides* T1/44, a Vaccine Strain against Contagious Bovine Pleuropneumonia. Genome Announc 2016;4:e00263–316. https://doi.org/10.1128/genomeA.00263-16.
[14] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014; 30:2068–9. https://doi.org/10.1093/bioinformatics/btu153.
[15] Czech L, Exposito-Alonso M. grenepipe: a flexible, scalable and reproducible pipeline to automate variant calling from sequence reads. Bioinformatics 2022 Oct; 38(20):4809–11. https://doi.org/10.1093/bioinformatics/btac600.
[16] Lee DH, Miles RJ, Inal JR. Antibiotic sensitivity and mutation rates to antibiotic resistance in *Mycoplasma mycoides ssp. mycoides*. Epidemiol Infect 1987;98:361–8. https://doi.org/10.1017/s0950268800062129.
[17] Maisnier-Patin S, Berg OG, Liljas L, Andersson DI. Compensatory adaptation to the deleterious effect of antibiotic resistance in *Salmonella typhimurium*. Mol Microbiol 2002;46:355–66. https://doi.org/10.1046/j.1365-2958.2002.03173.x.
[18] Deng Y, Cai W, Li J, Li Y, Yang X, Ma Y, et al. Evaluation of the genetic stability of Sabin strains and the consistency of inactivated poliomyelitis vaccine made from Sabin strains using direct deep-sequencing. Vaccine 2019;37:130–6. https://doi.org/10.1016/j.vaccine.2018.11.020.
[19] High-definition viral vaccine strain identity and stability testing using full-genome population data–The next generation of vaccine quality. Vaccine. 2015 Oct 26;33 (43):5829-5837. doi: 10.1016/j.vaccine.2015.08.091.