

A vaccine based on recombinant modified Vaccinia Ankara containing the nucleoprotein from Lassa virus protects against disease progression in a guinea pig model

E.M. Kennedy^{*}, S.D. Dowall, F.J. Salguero, P. Yeates, M. Aram, R. Hewson

Public Health England, Porton Down, Salisbury, Wiltshire SP4 0JG, England



ARTICLE INFO

Article history:

Received 15 February 2019

Received in revised form 2 July 2019

Accepted 6 July 2019

Available online 19 July 2019

Keywords:

Lassa

Modified Vaccinia Ankara (MVA)

Vaccine

Nucleoprotein

Immunogenicity

Efficacy

Recombinant vaccine

ABSTRACT

Lassa fever remains the most imported viral haemorrhagic fever in Europe and is responsible for 5000 deaths per year throughout Western Africa. There is no vaccine and treatment is often ineffective. We have developed a vaccine based on modified Vaccinia Ankara expressing the nucleoprotein from Lassa virus (MVALassaNP). This study investigated the immunogenicity (in mice) and efficacy (in guinea pigs) of the MVALassaNP vaccine as a prime/boost or single vaccination regime. ELISA and ELISpot assays confirmed humoral and T-cell immunity following both a prime and prime/boost vaccination, with the prime/boost regime producing a statistically increased response compared to a prime only vaccine ($P < 0.0001$). The vaccine offered protection in guinea pigs against disease manifestations after challenge with virulent Lassa virus. Clinical signs, weight loss and temperature increases were observed in all animals receiving a control MVA vaccine, after challenge with Lassa virus. In contrast, no clinical signs, fever or weight loss were observed in any of the MVALassaNP vaccinated animals demonstrating that both a single immunisation, and prime/boost regime confer protection against disease progression.

In conclusion, the MVALassaNP vaccine candidate elicits an immune response, demonstrates efficacy against Lassa virus disease and is suitable for further preclinical and clinical development.

Crown Copyright © 2019 Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

1. Background

Lassa virus (LASV), the causative agent of Lassa fever is endemic in Western Africa, particularly in Nigeria, Sierra Leone, Guinea and Liberia [1]. Sporadic and often large outbreaks are common with evidence of outbreaks in non-endemic neighbouring countries including; Benin, Togo, Ghana, Ivory Coast, Burkina Faso and Mali. During early 2018, Nigeria observed its largest outbreak to date with approximately 420 confirmed cases resulting in 106 deaths [2]. There are estimated to be up to 500,000 cases of Lassa fever per year with 5000 deaths attributed to the virus. The overall mortality rate is relatively low as many infections are mild or even asymptomatic, but during outbreaks the mortality rate can increase to 65% [3]. The disease is especially severe in late pregnancy with maternal death and or foetal loss in up to 90% of cases in the third trimester [4]. The majority of mild cases recover fully; however, deafness occurs in approximately 25% of positive cases with only half of these cases regaining hearing [2]. There is currently no vaccine for Lassa fever – the antiviral Ribavirin is the only treatment

for LASV infection, but it is only effective when administered in early infection. Diagnosis is often slow and as symptoms are similar to other diseases misdiagnosis is high. Disease prevention is based on controlling and limiting exposure to the host reservoir – the multimammate rat – *mastomys natalensis* [5]. Transmission of infection is predominantly through contact with the urine/faeces of the host rodent, although consumption of rodent meat is commonplace in many regions increasing the risk of infection [6]. Human-to-human transmission is observed through contact with infected bodily fluids [3].

LASV belongs to the Old World (OW) group (former lymphocytic choriomeningitis virus, LCMV-LASV sero-complex), genus *Mammarenavirus* family *Arenaviridae* [7]. Its genome consists of two single stranded segments of RNA termed L and an S, each encoding two open reading frames in ambisense polarity separated by a highly structured intergenic region. The L segment encodes the viral polymerase and zinc binding protein (Z) whilst the S segment encodes for the structural proteins – the nucleoprotein (NP) and the glycoprotein (GP) precursor. The glycoprotein is cleaved post translation to glycoproteins GP1 and GP2, and SSP (stable signal protein) [8]. The NP and GP are, historically and currently, proteins of significant interest in the development of a suitable vaccine candidate for LASV.

^{*} Corresponding author.

E-mail address: emma.kennedy@phe.gov.uk (E.M. Kennedy).

There have been many attempts to develop a suitable vaccine for Lassa. According to the WHO there are over 14 Lassa vaccine candidates in the pipeline, but only one has reached clinical trials [9]. The different vaccine approaches include: virus-like particles [10]; DNA vaccines [11]; live-attenuated vaccines [12] and vectored vaccines [13,14]. For the assessment of vaccine efficacy, guinea pig and non-human primate models are the most common animals models used for LASV studies [15]. There are often cost and ethical implications when choosing a suitable animal model which can limit the data availability and it is still yet to be determined whether the efficacy of animal models will truly reflect a human vaccine [16]. The correlate of protection is still poorly understood for Lassa fever. Although antibodies specific to LASV are usually produced in immunisation studies, in human disease they are more often identified at the convalescent stage of infection and not during the acute phase [15,17,18]. T-cell responses are believed to be the main correlate of protection as they are detected early in infection and memory T-cells that recognise NP and GPC epitopes can be maintained for years after infection [19].

Previous attempts to use Vaccinia virus (VV) as a viral vaccine vector for LASV proteins have described success [14,20,21]. Full protection against a lethal challenge model (using LASV strain GA391) was observed in guinea pigs immunised with LASV nucleoprotein derived from GA391 inserted into the VV Lister strain [14]. LASV NP and GP derived from the Josiah strain of LASV inserted into VV (New York Board of Health strain) have demonstrated similar observations [22]. Interestingly, parallel studies in non-human primates (NHPs) have shown that NP offers less protection [18] than observed in a guinea pig model. However, immunisation using GP (specifically GP1 and GP2 combined) or a GP/NP combination seem to offer the highest level of protection [18]. Despite promising data, there has been limited progression into pre-clinical trials and there is still no vaccine available.

The use of Vaccinia as a vector is not novel, it was successfully used in the eradication campaign of smallpox virus, but safety complications arising particularly in immunocompromised individuals halted its use as a suitable vaccine vector candidate. As Lassa fever is endemic in an area of the world where the numbers of immunocompromised people are high, other strains of the Vaccinia virus and modified versions have been investigated to overcome the incompatibility of the vector for high risk groups [23]. One method was serial passage of the Vaccinia Ankara strain in Chicken Embryo Fibroblasts (CEFs) and continual passages (over 500 times) have led to the production of the highly attenuated Modified Vaccinia Ankara (MVA) virus strain [23,24]. MVA does not fully replicate in mammalian cells and is avirulent in animal models. However, it results in high levels of expression of recombinant proteins and induces both a humoral and cell-mediated immune response. These characteristics make it a useful vaccine vector platform [23,25].

Recombinant MVA (rMVA) has been shown to be efficacious with antigens other than LASV. It was used as a successful smallpox vaccine [26] and is in different stages of development and clinical trials for viruses including influenza [27], HIV [28] and CCHF [29].

This study initiated the pre-clinical development of a Lassa fever vaccine candidate using rMVA as a vector and assessed the immunogenicity and the efficacy of the vaccine candidate in a rodent model.

2. Materials and methods

2.1. Cells

Baby Hamster Kidney-21 (BHK-21) cells (European Collection of Cell Cultures, UK) cultured in modified essential eagle medium (MEM) (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS), 2 mM L-Glutamine, 100U penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich).

Chicken Embryo Fibroblast (CEF) cells were received from the Institute of Animal Health (Pirbright, UK) and cultured in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, 100U penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich).

VeroE6 cells (European Collection of Cell Cultures, UK) were cultured in Leibovitz's L-15 medium containing glutamax (Life Technologies, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich).

2.2. Viruses

MVA strain 1974 was kindly donated by Prof B Moss (NIH, USA). The virus titre was determined by plaque assay in CEF cells.

LASV (strain GA391) was isolated from a case in Northern Nigeria [30]. Virus was grown and titered by TCID₅₀ assay on VeroE6 cells (European Collection of Cell Cultures, UK).

LASV for the guinea pig challenge studies was passaged twice to ensure the virus was capable of causing disease and to increase disease severity, with virus from spleen homogenates being used for passaging the virus into new animals. A dose of 10³ TCID₅₀ was used for efficacy testing since that demonstrated uniform disease progression post-challenge.

2.3. Animals

For immunogenicity studies, 24 adult female BALB/c aged 5–8 weeks were supplied from Charles River. For efficacy studies, adult female guinea pigs with weights of 300–350 g were supplied from Marshall BioResources. These studies were approved by the ethical review process of PHE, Porton Down, UK and the Home Office, UK via project licence number 30/2993. Work was performed in accordance with the Animals (Scientific Procedures) Act 1986 and the Home Office (UK) Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989).

Animals infected with LASV were housed within flexible film isolators under climate-controlled conditions in an animal containment level 4 (CL4) room.

2.4. Plasmid construction

A DNA cassette for MVALassaNP was generated by GeneArt (Thermo Fisher) to contain an orthopoxvirus P11 promotor, Green Fluorescence Protein (GFP) and mH5 promotor followed by an appropriate Kozak sequence upstream of the NP ORF. A 24-residue linker sequence followed by a V5 epitope and stop codon was engineered downstream of NP. The cassette was inserted into an SfiI/SfiI cloning site of plasmid pMK-RQ to produce plasmid 16ADHWFP_2037865QAD_MVALassaNP (pMVALassaNP). The plasmid DNA was transformed and amplified in bacteria (*E. coli* K12 DH10B™ T1R) and subsequently purified. Its concentration was determined by GeneArt (Thermo Fisher) using UV spectroscopy.

2.5. Vaccine candidate production

BHK-21 cells were infected with MVA 1974 at a multiplicity of infection of 0.05. Infected cells were transfected with pMVALassaNP using lipofectamine (Life Technologies) as directed by the manufacturer. The resulting recombinant MVALassaNP was serially plaque-purified 4 times in CEF cells, based on GFP expression. MVALassaNP was amplified in CEF cells, purified by sucrose cushion centrifugation and titrated by plaque assay using CEF cells prior to *in vivo* use. Plaques were visualised using GFP fluorescence and by immunostaining with rabbit anti-vaccinia antibody (AbD Serotec, UK) and Vectastain Universal ABC-AP kit (Vector laboratories, USA). Genomic DNA from infected cells was extracted using the

Wizard SV genomic DNA purification system (Promega, USA) and used as a template in PCR with KAPA2G Fast HotStart PCR Kit (KAPA Biosystems, USA) for genotype analysis.

2.6. Detection of protein expression

CEF cells were infected with MVALassaNP at a multiplicity of infection of 0.05 and incubated at 37 °C in MEM supplemented with 2% FBS (Sigma-Aldrich, UK). The medium was removed after 48 h once GFP fluorescence and CPE was observed microscopically. Cells were lysed with 1x LDS Nupage® reducing sample buffer (Nupage® LDS sample buffer containing 1x Nupage® sample reducing buffer) (Thermo Fisher, UK), transferred to Eppendorf tubes and heated at 70 °C for 10 min. Uninfected cells were treated in the same manner as a negative control. MVALassaNP lysates were subjected to SDS-PAGE on a 4–12% Bis-Tris gel (Life Technologies) and proteins were transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked using 5% milk powder (Merck Millipore), then incubated in the presence of a primary antibody (rabbit anti-V5 polyclonal (Invitrogen) at 1/1000 in phosphate buffered saline (PBS) containing 0.05% Tween (PBS-0.05% Tween) for 1–2 h with rocking, before being washed 3 times in PBS-0.05% Tween (Sigma-Aldrich). Membranes were incubated in the presence of a HRP-conjugated secondary antibody (anti-rabbit IgG peroxidase (Sigma-Aldrich) at 1/5000 in PBS-0.05% Tween) for 1 h with rocking and washed as before. Protein expression was determined by detection of bound antibody using a Pierce ECL WB substrate kit (Thermo Fisher) according to the manufacturer's instructions and visualised in a Chemi-Luminescent Imager (Syngene). Molecular weights were determined using the molecular ladder MagicMark XP Western Protein Standard (Invitrogen) as a reference.

2.7. Immunogenicity

Vaccination studies and scheduling for prime and boost regimes were based on standard MVA protocols and previous MVA studies [29,31], 24 female 5–8 week old BALB/c mice were randomly divided into 4 groups. Group 1 received a single vaccine shot of MVALassaNP in endotoxin-free PBS at 1×10^7 plaque forming units (pfu) per animal on day 14. Group 2 received a two dose vaccination of MVALassaNP in endotoxin free PBS at 1×10^7 pfu per animal on days 0 and 14. Group 3 received a two dose vaccination of MVA 1974 (wild-type) in endotoxin-free PBS at 1×10^7 pfu per animal on days 0 and 14. Group 4 received a two dose vaccination of endotoxin-free PBS as a negative control on days 0 and 14. All mice were injected intramuscularly into the caudal thigh. 100 µl was administered at each vaccination (50 µl into each thigh). Animal weights were recorded daily throughout the study. Animals were euthanised and spleen tissue and blood were collected on day 28 after the primary vaccination. All efforts were made to minimise animal suffering.

2.8. ELISpot

Spleens from test animals were collected aseptically and homogenised, and the red blood cells were lysed. Splenocytes were resuspended in RPMI medium (Sigma-Aldrich) supplemented with 5% FBS, 2 mM L-Glutamine, 100U penicillin & 0.1 mg/ml streptomycin, 50 mM 2-mercaptoethanol and 25 mM HEPES solution (Sigma-Aldrich). Splenocytes were assessed for their antigen recall response via IFN-γ ELISpot (Mabtech, Sweden), performed as per the manufacturer's instructions. Cells were seeded in PVDF microtiter plates at 2×10^5 per well and re-stimulated with peptide pools (JPT, Berlin). Peptides spanning the Lassa NP sequence were 15 residues long, with an overlap of 10 residues between peptides. They were applied to cells at a final concentration of 25 µg/ml per

peptide, with 20 peptides per pool. Plates were developed after 18 h at 37 °C, 5% CO₂ in a humidified incubator. Spots were counted visually on an automated ELISpot reader (Cellular Technologies Limited, USA). Background values from wells containing cells and medium but no peptides were subtracted, and data are presented as the response to individual pools or summed across the target protein. Results are expressed as spot forming units (SFU) per 10^6 cells.

2.9. ELISA

Recombinant Lassa NP as a crude lysate (Native Antigen Company, UK) was diluted in 0.2 M carbonate-bicarbonate buffer pH 9.4 (Thermo Scientific, UK) and used to coat Maxisorp 96-well plates (Nunc, Denmark) at 10 µg/ml in 100 µl. Plates were incubated at 4 °C overnight, then washed with PBS + 0.01% Tween-20 (Sigma-Aldrich, UK) and blocked with 100 µl of 5% milk powder (Merck, Millipore, UK) in PBS + 0.01% Tween-20 at 37 °C for 1 h, before re-washing in PBS + 0.01% Tween-20. Samples were diluted 1:50 in 5% milk powder in PBS + 0.01% Tween-20 buffer, added to the plates in triplicate (100 µl per well) and incubated at 37 °C for 1 h. A polyclonal anti-Lassa virus hyper immune mouse ascitic fluid sample (supplied through an agreement with BEI resources, USA) was used as a positive control sample. Plates were washed with PBS + 0.01% Tween-20 and 100 µl of a polyclonal IgG anti-mouse HRP conjugate (Sigma-Aldrich, UK) at a 1: 20,000 dilution in PBS + 0.01% Tween-20 containing 5% milk powder was added to each well. Following a further 1-hour incubation at 37 °C, plates were washed with PBS + 0.01% Tween-20 and 100 µl of TMB substrate (Surmodics, USA) was added to each well and then incubated at 20 °C for 1 h. The reaction was stopped by the addition of 100 µl of stop solution (Surmodics, USA) prepared according to the manufacturer's instructions. Plates were read at 450 nm using an absorbance reader and analysed with Softmax Pro version 5.2 software (Molecular Devices, USA). Background absorbance values were subtracted from the sample values and results are reported as Absorbance (450 nm) at a 1:50 dilution. Data was illustrated and analysed using Graph Pad Prism 7.

2.10. Efficacy

40 female Dunkin-Hartley guinea pigs were randomly divided into 4 groups. Group 1 received a single vaccination with MVALassaNP in endotoxin-free PBS at 2×10^7 pfu per animal on day 14. Group 2 received a two dose vaccination of MVALassaNP in endotoxin-free PBS at 2×10^7 pfu per animal on days 0 and 14. Group 3 received a two dose vaccination of MVA 1974 (wild-type) in endotoxin-free PBS at 2×10^7 pfu per animal on days 0 and 14. Group 4 received a two dose vaccination of endotoxin-free PBS as a negative control on days 0 and 14. All animals were injected intramuscularly into the caudal thigh – 200 µl was administered at each vaccination (100 µl into each thigh). All animals were anaesthetised and challenged with 200 µl of LASV at 1×10^3 TCID₅₀ delivered subcutaneously 24 days after the last vaccination. All efforts were made to minimise suffering and any animal that reached a humane endpoint (body weight < 20% or < 10% with moderate clinical signs) was removed from the study early. Clinical observations other than weight and temperature were assigned a numerical value depending on their severity to allow the data to be graphically illustrated [29]; normal/no observations = 0; ruffled fur = 2; lethargy = 3; hunched = 3; laboured breathing = 5; culled as met humane endpoint = 5.

All remaining animals were euthanised and tissues were collected on day 21 post challenge. Liver and spleen were removed for histology and viral load analysis.

2.11. Pathological studies

Liver and spleen samples were collected and fixed by immersion in 10% neutral buffered formalin (NBF) for at least 21 days before being routinely processed into paraffin wax. Sections of 4 μ m were cut, stained with haematoxylin and eosin (H&E) and examined by light microscopy. The severity of LASV-associated lesions in the sections of spleen and liver from each animal was assessed and scored subjectively. 'RNAscope' (an in-situ hybridisation method used on formalin fixed paraffin embedded tissues [32]) was used to identify LASV nucleic acid in the liver and spleen. Tissues were pre-treated with hydrogen peroxide for 10 mins (room temperature), with target retrieval buffer for 30 mins (98–101 °C) and with protease plus for 30 mins (40 °C) (all Advanced Cell Diagnostics, USA). A V-LASV-GPC probe (Advanced Cell Diagnostics, USA) was incubated with the tissues for 2 h at 40 °C. Amplification of the signal was carried out following the RNAscope protocol (RNAscope 2.5 HD Detection Reagent – Red) using the RNAscope 2.5 HD Red kit (Advanced Cell Diagnostics, USA). The presence of cells showing LASV RNA was assessed subjectively by a board-certified veterinary pathologist. Slides with an occasional single positive cell scored 1, slides with scattered positive cells scored 2, slides with frequent positive cells scored 3, slides with marked patchy to diffuse presence of positive cells scored 4.

2.12. Illustrations and statistics

All data illustrations and statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad, USA). A two-way ANOVA (Tukey comparison) was used to compare data from different test groups in both the immunogenicity and efficacy studies.

3. Results

3.1. Vaccine constructions and production

A quality-controlled plasmid suitable for progressing into vaccine production was developed (Fig. 1). Infection/transfection assays produced GFP fluorescing plaques that were easy to identify and purify. PCR confirmed the presence of rMVALassaNP with no evidence of wild-type MVA after purification (Fig. 2a). Next Generation Sequencing (NGS) using an Illumina HiSeq method showed passaged picks to be congruent with the initial reference sequence. Pick P4(5.5.3.2) was chosen to take forward as determined by sequencing data. Western blot analysis confirmed the expression of protein with the V5 tag (Fig. 2b) and, following amplification and purification, the vaccine was titrated. A concentration of 1×10^8 pfu/ml was stored at -80 °C as a stock vaccine.

3.2. Immunogenicity – ELISpot

The vaccine candidate was observed to be well tolerated during the immunogenicity study. All mice gained weight and no clinical signs were observed (Supplementary data Fig. 1). The ELISpot method was performed to measure IFN- γ secreting cells in response to a Lassa nucleoprotein stimulus and to determine if the response is enhanced in vaccinated animals compared to control animals. Stimulation of IFN- γ production in splenocytes by Lassa NP peptide pools was significantly increased in cells from mice vaccinated with a prime/boost regimen ($P < 0.0001$). However, even the single vaccine regime induced a significantly increased response compared to unvaccinated mice ($P < 0.0001$). Splenocytes from the MVA-WT and PBS control mice were negative for IFN- γ production when stimulated with the Lassa NP peptides.

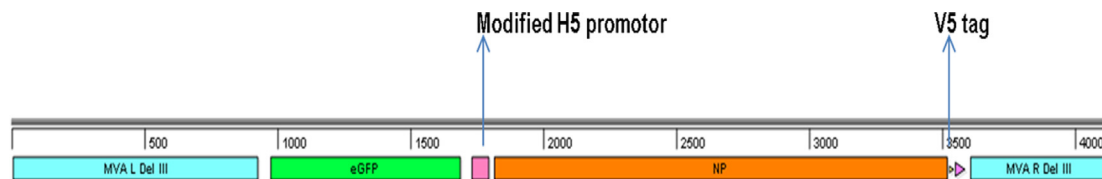


Fig. 1. A cassette for MVALassaNP was generated by GeneArt (Thermo Fisher) to contain a P11 promotor, Green Fluorescence Protein (GFP) and an MH5 promotor followed by a Kozak sequence upstream of the nucleoprotein sequence. A 24-residue linker sequence followed by a V5 epitope and stop codon was engineered downstream of NP.

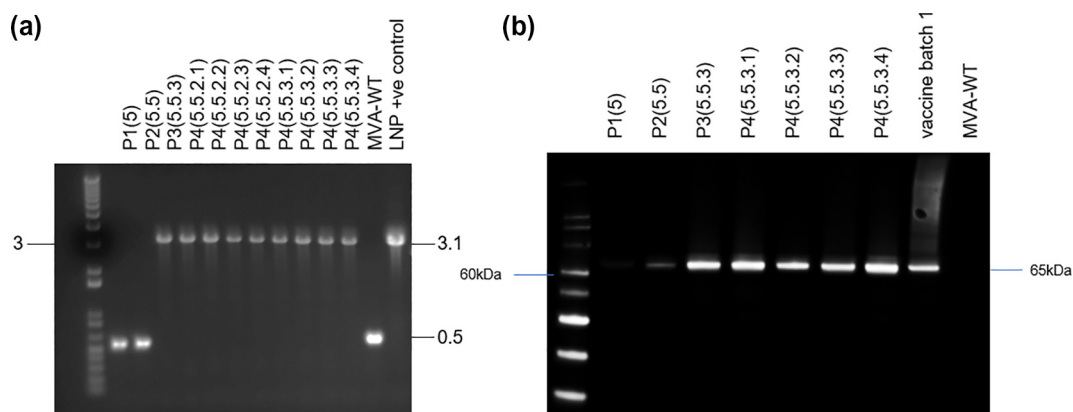


Fig. 2. PCR confirmation of the rMVALassaNP by passage 3 (a). Passage 1 and 2 picks show the presence of MVA-WT (band at 0.5 kb as expected for MVA-WT and congruent to that of the MVA-WT control); all other passages show bands at the expected size of 3.1 kb. Western blot analysis of vaccine preparations (b). Pre-purified samples from the first pick through to passage 4 picks and the purified vaccine batch indicate the expression of the V5 tag upstream of the LASV sequence. The expected size of 65 kDa was observed for all samples.

Splenocytes from vaccinated groups specifically displayed IFN- γ responses to two distinct regions of the nucleoprotein as shown in Fig. 3. A two-way ANOVA revealed significant differences between the prime and prime/boost vaccinated groups of mice in pool 2 ($P < 0.0001$) and 4 ($P < 0.0001$) suggesting that a prime/boost vaccination regime elicits a stronger immune response compared to a prime vaccination only. However, even a single dose of MVALassaNP induced a significantly increased response ($P < 0.0001$) compared to the control groups of wild type-MVA and PBS. There were small (non-significant) responses detected to pools 1, 3, 5, 6 and 7 (particularly groups 1, 6 and 7) for the prime/boost and prime groups compared to unvaccinated groups.

3.3. Immunogenicity – ELISA

Serum collected from all mice (vaccinated and un-vaccinated) was analysed for the presence of antibodies specifically to LASV nucleoprotein. IgG response was measured as set out in the methods section. The mean optical density (OD) obtained with the PBS mice serum plus 3 standard deviations ($OD = 0.09$) was used to determine a positive/negative cut off for the study. Sera from all mice in the MVA-WT group gave ODs below the 0.09 cut off and the responses of all mice in the prime and the prime/boost vaccinated groups were greater than 0.09 (Fig. 4). Sera from the single dose mice recorded an average OD of ~ 0.75 and the prime/boost an average OD of ~ 1.2 . The difference between the response in the prime/boost group and that in the single dose group was significant (< 0.0001 using a one-way ANOVA with multiple comparisons); however, the single dose also gave a significantly increased response compared to both control groups ($P < 0.0001$).

3.4. Efficacy

Guinea pigs were vaccinated with a single dose of MVALassaNP or two doses in a prime-boost regime, spaced two weeks apart. Negative control groups consisted of animals injected with wild-type MVA or PBS buffer. At a time-point 24 days after the last vaccination, all animals were challenged with LASV. Guinea pigs

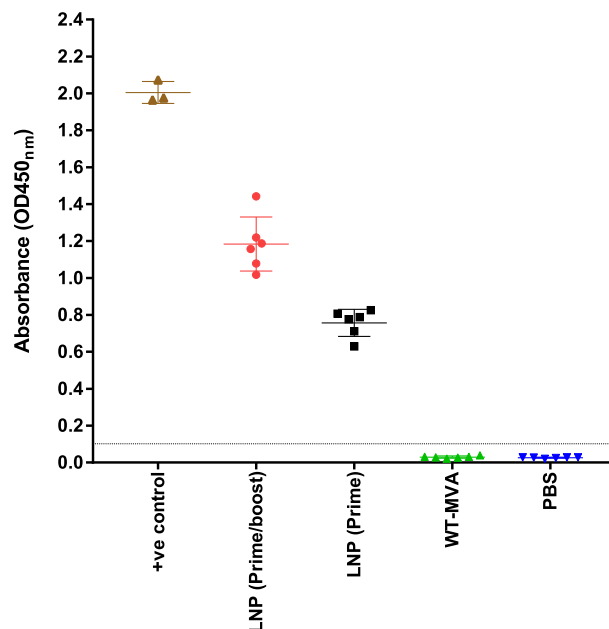


Fig. 4. Humoral (IgG) response to vaccination with MVALassaNP in BALB/c mice. Sera from animals vaccinated with MVALassaNP single dose (black), MVALassaNP prime/boost dose (red), WT-MVA (green) or PBS (blue) were analysed in an indirect ELISA to determine antibody responses. Error bars represent mean $\pm$ SD. An enhanced IgG response was identified in both vaccinated groups with the unvaccinated groups negative to LASV NP.

were weighed and had their temperatures recorded daily; assessments of clinical symptoms and scoring were performed by experienced veterinary technicians. Results demonstrated that all animals given MVALassaNP were protected against disease progression (Figs. 5 and 6). All MVALassaNP vaccinated animals were scored as healthy throughout the course of the experiment. In contrast, animals given wild-type MVA or PBS buffer all exhibited weight loss, temperature rises and showed clear clinical signs of disease including laboured breathing, hunched prostration and

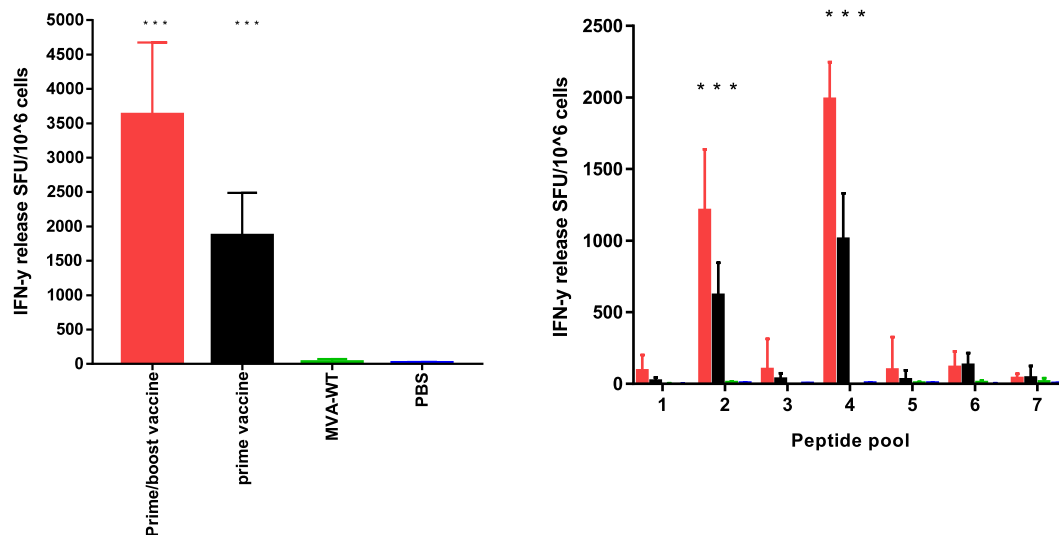


Fig. 3. IFN- γ ELISpot responses from vaccinated BALB/c mice, 14 days after booster vaccination. Splenocytes from animals vaccinated with MVALassaNP single dose (black), MVALassaNP prime/boost dose (red), WT-MVA (green) or saline (blue) were restimulated with peptides derived from the LASV NP. Data is illustrated as the summed antigen response from all peptide pools or individual peptide responses. Error bars indicate mean and standard deviation, stars highlight results significantly different to the control groups ($P < 0.001$). Error bars indicate mean with standard deviation. T-cell responses were identified in both vaccinated groups, with some peptide regions more immunogenic than others (specifically pools 2 and 4).

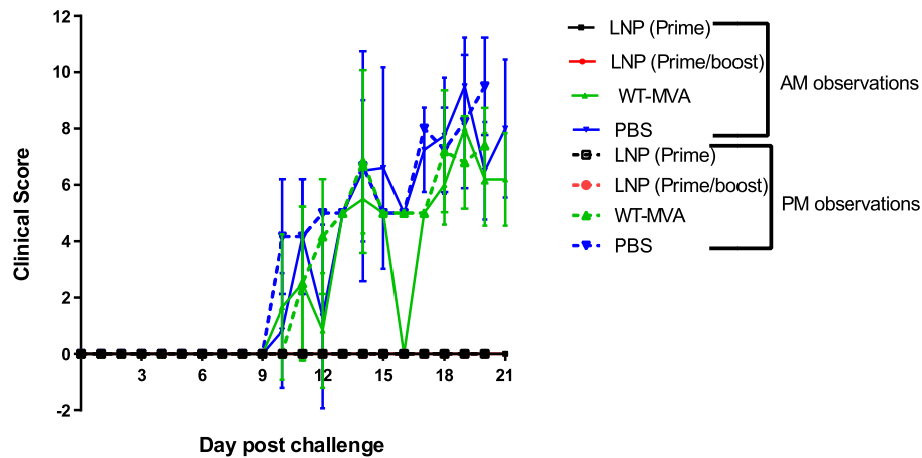


Fig. 5. Clinical scores based on severity (e.g. laboured breathing: 5, ruffled fur: 2). Guinea pigs were assessed twice a day (solid line: AM observations; dotted line: PM observations). Group 1: single vaccine (black), Group 2: prime/boost vaccine (red), Group 3: MVA-wild type (green) and Group 4: negative control (blue). Error bars represent mean $\pm$ SD.

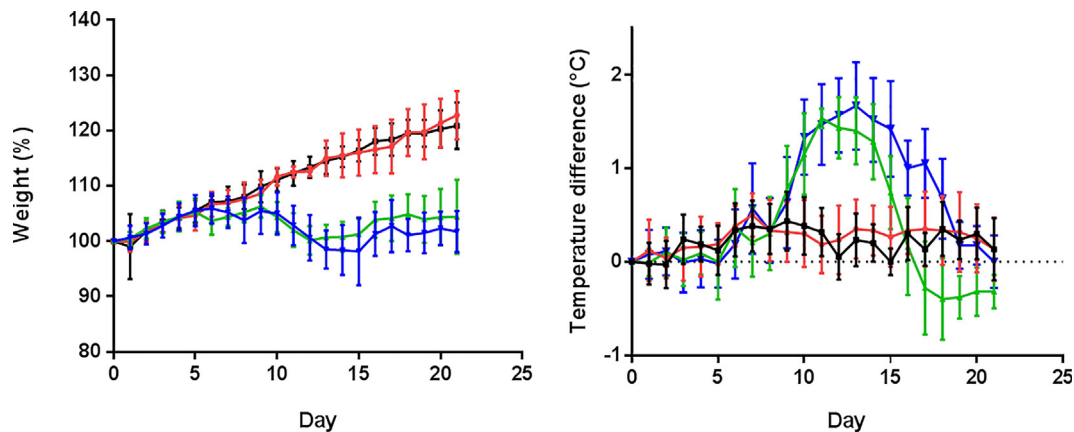


Fig. 6. Daily weight gain (day 0 = 100%) and daily temperature monitoring of animal's post challenge with LASV. Group 1: single vaccine (black), Group 2: prime/boost vaccine (red), Group 3: MVA-wild type (green) and Group 4: negative control (blue). Error bars represent mean $\pm$ SD.

general lethargy after challenge (Fig. 5). In addition, one and two animals in the wild-type MVA and PBS groups, respectively, met humane clinical endpoints and were culled during the study.

3.5. Histopathology

Splenic lesions attributed to LASV infection comprised: (a) heterophilic splenitis (an increased number of heterophils in the splenic red pulp); (b) depletion of lymphocytes from the splenic follicles (white pulp); (c) *peri*-follicular monocytopia and a diffuse increase in the pale-staining monocytes in the red pulp; and (d) diffuse monocytopia of the red pulp. Liver lesions attributed to Lassa virus comprised: (a) individual hepatocyte cell death, frequently accompanied by small numbers of mononuclear (lymphocytes, macrophages and plasma cells) inflammatory cells; (b) foci of mononuclear inflammatory cells within hepatic sinusoids (sinusoidal inflammation); (c) infiltration of portal triads by mononuclear inflammatory cells; and (d) the presence of crisp edged vacuoles in hepatocytes. Bell et al. have previously described vacuolation resulting from intracellular lipid accumulation [33]. All the lesions were scored as 0 = none; 1 = minimal; 2 = mild; 3 = moderate and 4 = marked. Lesion scores were more pronounced in splenic tissue compared to liver tissue. Although all animals were scored for lesions to some degree, the scores were

higher in the tissues of non-vaccinated groups compared to the vaccinated animals (Fig. 7). Tables 1 and 2 document all lesions and severity for all animals. RNAscope analysis demonstrated that

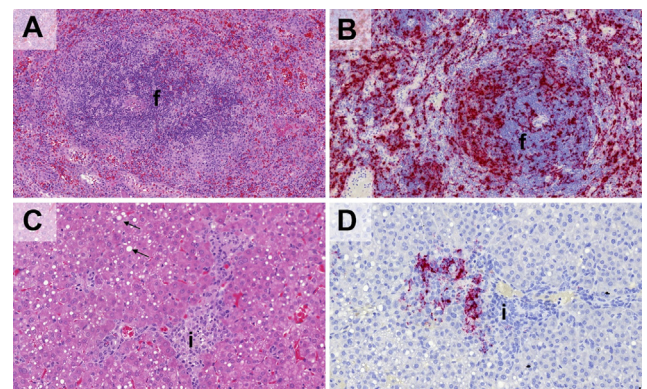


Fig. 7. Histopathological changes in the spleen (A and B) and liver (C and D) in control animals. A. H&E. Lymphoid depletion in spleen observed mainly within the follicles (f). B. RNAscope ISH LASV. Marked diffuse positive staining within the follicles (f) and the red pulp. C. H&E. Foci of mononuclear cell infiltration (i) and presence of crisp-edge vacuoles (arrows) within hepatocytes. D. RNAscope ISH LASV. Positive staining within periportal inflammatory cell infiltrate (i).

Table 1

Severity scores for spleen histopathological lesions and Lassa virus ISH in guinea pigs killed 7 and 32 days post challenge (dpc). Severity scores: - None observed, + Minimal, ++ Mild, +++ Moderate, ++++ Marked. Guinea pigs were euthanised at 32 dpc unless otherwise stated; * euthanised 7 dpc, **reached humane endpoint and removed prior to scheduled study end date.

Group	Animal	Heterophilic splenitis	Lymphocyte depletion in white pulp	Perifollicular monocyctosis	Diffuse monocyctosis of red pulp	Lassa ISH score
1 Prime vaccine	11816*	+	–	–	++	0
	54845*	+	+	–	–	0
	12545*	++	–	–	++	0
	11918*	–	–	–	+++	1
	54832	–	–	–	+++	0
	11716	–	++	+++	+++	0
	11923	–	–	–	–	0
	54846	–	+	+	+	0
	54833	–	–	–	–	0
	12707	–	++	++	+++	0
2 Prime/boost vaccine	12365*	++	–	+++	+++	0
	12362*	–	–	–	–	0
	54831*	++	+	–	++	2
	54844*	–	++	+++	+++	0
	54830	++	++	–	–	0
	54843	–	–	–	+++	0
	12022	–	–	–	–	0
	11768	++	+	+	+++	0
	12442	++	+++	+++	++	0
	12857	+++	++	+++	+++	0
3 MVA-WT Control	12319*	+++	++	++	–	3
	12109*	++	+	++	–	4
	12672*	–	–	–	–	4
	11808*	+	–	–	++	4
	12394**	+	++++	++++	++++	4
	12840	++	++	+++	+++	3
	12097	++	+++	+++	+++	1
	12561	+	+++	+++	+++	1
	12686	++	++	+++	+++	2
	12309	++	++	++	++	2
4 PBS Control	11954*	+++	–	–	–	3
	30529*	+++	+++	++	+++	4
	12366*	+++	–	–	–	3
	54835*	–	–	–	–	4
	26298**	+++	++++	++++	++++	4
	24767**	+++	++++	++++	+++	4
	24822	++	++++	++	++	3
	29412	++	++++	++++	++++	1
	26033	+++	++++	+++	+++	3
	25034	+++	++	++	++	3

the vaccinated groups had less LASV RNA present in both the liver and spleen, with a number of animals displaying no presence of LASV RNA. In comparison, LASV RNA was detected in all non-vaccinated animals.

4. Discussion

This study has demonstrated that MVA is a suitable viral vector for Lassa NP encoded from Nigerian strain GA391. The Lassa NP gene was inserted into the MVA with ease and sequencing data suggested stability after multiple passages. PCR confirmation of the MVALassaNP showed bands of the expected size of 3.1 kb compared to the expected size of MVA–WT (0.5 kb). Next Generation Sequencing (NGS) using an Illumina HiSeq also provided evidence that no wild type MVA was present in the final vaccine batch and there were no Single Nucleotide Polymorphisms (SNPs) or other mutations suggesting that a pure recombinant vaccine is easy to produce and maintain. Western blot analysis provided evidence that the nucleoprotein was expressed from an early stage of production (as early as passage 1, although the faint observed band would suggest a low concentration of MVALassaNP in the sample and this would be expected) and its expression continued throughout all subsequent passages. The purified vaccine batch observed good expression of the nucleoprotein with no breakdown. The

Western blot used antibody detection of the V5 tag added to the construct. In clinical studies the vaccine will undergo alterations; GFP and V5 markers will be removed prior to human studies to ensure only essential proteins are included; therefore, it would be preferable in subsequent assays to detect the LASV NP itself and this should be explored in future studies.

Previous use of MVA has proved its safety and suitability as a vaccine candidate [29] and the MVALassaNP vaccine in this report was well-tolerated in rodents (see [supplementary data](#)).

The MVALassaNP vaccine has provided useful immunogenicity data suggesting that humoral and cell-mediated immune responses were triggered. Elevated IgG responses were demonstrated in all vaccinated mice and the response was further elevated in mice that received a two-dose immunisation regime. The role of the humoral arm of the immune system is debatable; whilst some have shown that antibodies have little effect on reducing viral load [18,22], others suggest an important role for neutralising antibodies in protection [15] or that non-neutralising antibodies (specific to glycoprotein in this case) correlate with protection [34]. Nevertheless, we have demonstrated that MVALassaNP is able to induce an IgG response which may be linked to protection – however further data is required to determine the significance of the antibody response and to determine whether protection is conferred from the humoral arm of the immune

Table 2

Severity scores for hepatic histopathological lesions and Lassa ISH in guinea pigs killed 7 and 32 dpc. Severity scores: - None observed, + Minimal, ++ Mild, +++ Moderate, ++++ Marked. Guinea pigs euthanised at 32 dpc unless otherwise stated; *euthanised 7 dpc, **reached humane endpoint and removed prior to scheduled study end date.

Group	Animal	Scattered single cell death	Portal inflammation. Mononuclear cells +/- heterophils	Sinusoidal inflammation (mononuclear cells)	Crisp-edge vacuolation	Lassa ISH score
1 Prime vaccine	11816*	-	++	++	-	0
	54845*	-	++	++	+	0
	12545*	-	-	-	-	0
	11918*	-	++	-	-	0
	54832	-	+	-	-	0
	11716	-	-	-	-	0
	11923	-	++	++	-	0
	54846	+	+++	-	++	0
	54833	-	-	-	-	0
	12707	-	++	+	-	0
2 Prime/boost vaccine	12365*	-	+	+	-	0
	12362*	-	-	-	-	0
	54831*	++	+++	++	-	0
	54844*	-	++	+	-	0
	54830	-	++	++	-	0
	54843	-	-	-	-	0
	12022	-	++	++	-	0
	11768	-	+	+	-	0
	12442	-	-	-	-	0
	12857	-	-	-	-	0
3 MVA-WT Control	12319*	-	++	++	-	1
	12109*	+++	+++	++	++	1
	12672*	-	-	-	-	1
	11808*	-	+	-	-	0
	12394**	++++	++++	++++	++++	2
	12840	+	+++	+++	+	0
	12097	-	+	+	-	0
	12561	-	++	++	-	0
	12686	+++	+++	+++	-	0
	12309	-	++	+	-	0
4 PBS Control	11954*	-	++	+	-	0
	30529*	-	++	-	++	1
	12366*	-	+	-	-	0
	54835*	-	++	++	++	1
	26298**	++	++	++	+++	0
	24767**	+	+++	++	++++	3
	24822	+	++++	+++	+	0
	29412	-	+++	++	-	0
	26033	-	++	-	+++	1
	25034	-	++	+	-	1

system. Due to the NP antigen being an internal viral antigen the mechanism of the antibody may be through antibody dependant cellular cytotoxicity (ADCC).

Induction of a cell-mediated immune response is likely to provide protective immunity to LASV [14,20,22] and this study demonstrated a significantly heightened IFN- γ stimulation in all MVALassaNP vaccinated animals compared to non-vaccinated animals ($P < 0.001$). A two-dose vaccine regime offered a more enhanced T-cell response; however, encouragingly even a single vaccine was able to stimulate a significantly increased T-cell response compared to the non-vaccinated groups ($P < 0.001$). This suggests that the LASV NP alone can trigger the necessary immune responses to LASV infection and could act as a standalone vaccine without the need for the LASV glycoprotein, as others have previously suggested [18]. To our knowledge there is not a predicted immunodominant peptide region in the NP pools that gave the strongest response, but our data may lead to identification of immunodominant peptides in the future. The results provided from our ELISpot assays suggest 2 regions of interest to research further. One common sequela in human's post LASV infection is deafness. The association of deafness and LASV infection was first determined in 1990 [35] and since then a number of case studies have reported hearing loss in patients following LASV infection [36–38]. The cause of hearing loss is still not fully determined; most studies suggest it to be either attributed to the individual's immune response [35], although viral damage [39], or a combina-

tion of the two are also reported. If a T-cell mediated response is the cause of deafness in infected patients, clinical studies in humans will require close observations to ensure the vaccine (which enhances the T-cell response) does not also cause sensorineural hearing loss. Vaccination studies using a murine model developed by Yun et al. [40] could provide vaccine safety data with regards to hearing loss.

The MVALassaNP vaccine has demonstrated protection against disease progression in guinea pigs when challenged with a homologous LASV strain in a non-uniformly lethal challenge model. All animals in the control groups showed clinical signs with two from the PBS group and one from the MVA-WT group removed from the study early because they reached humane endpoints. The MVALassaNP vaccine demonstrated similar protection to that described for a similar vaccine developed at Porton Down in 1987 using Vaccinia virus as the antigen delivery vehicle [14]. Our data is the first to demonstrate that an MVA vaccine containing Lassa NP is sufficient to protect at least against progression of disease in a challenge model in guinea pigs. It would be beneficial to follow up these studies to show that this MVALassaNP vaccine can protect against a fully lethal Lassa challenge in guinea pigs prior to human clinical studies. It would also be beneficial to determine immunogenicity and efficacy of this vaccine in an NHP model as it has been previously shown that protection and cell-mediated responses differ between guinea pigs and NHP's [14,18]. The NHP model may align better with human infection and as such provide more telling

results prior to vaccination in humans. This study used a non-lethal LASV challenge and clinical parameters (weight, temperature and clinical scores) were the readout of protection. Use of a lethal dose of challenge virus would allow protection to be ascertained; however, as the mortality rate for Lassa fever does not reach 100%, the model system we used here is representative of natural disease severity. One further limitation of the animal model is the route of challenge material. Due to safety implications of handling a high consequence pathogen, working with animal models in a high containment laboratory, and ensuring conformity of challenge in all animals, LASV was administered subcutaneously, whilst this route does not mimic most natural human infections, it is representative of animal bite and potential exposure through hospital/medical incidents.

Histology results provided further analysis that the vaccine protected against LASV challenge. Although lesions were observed in splenic tissue of the vaccinated guinea pigs, they were markedly lower in numbers and less severe than those of the unvaccinated animals. The observations in guinea pig livers were similar to those described previously [33,41] with lesions that were less severe although still present in vaccinated animals. In previous studies, virus concentration ($\log_{10}$ PFU/g) was greater in the spleen compared to the liver and RNA presence in this study corroborate these findings [42]. The guinea pig model is a valuable *in vivo* model for assessment of interventions prior to advancement to NHP and human trials [33].

In this study the prime and boost vaccinations were homologous to the challenge strain (GA391). Future work is planned with a heterologous LASV challenge. LASV strains are separated into distinct lineages: GA391 is from lineage III (distributed in Nigeria), lineages I and II are also distributed throughout Nigeria, and lineage IV strains are closely related and found among the other Lassa endemic countries of Western Africa [43]. The majority of LASV studies concentrate on the Josiah strain (from lineage IV – Sierra Leone [15]); however, this strain and the GA391 strain used in our studies are not currently circulating strains, so it may be beneficial to use circulating strains in future studies to ensure a more likely successful vaccine. LASV NP has been shown to have some variability (12% at the amino acid level) across the different lineages [8]. As variability is relatively low, LASV NP is a useful antigen candidate for vaccine production that could be efficacious across all endemic regions.

MVA is a useful vaccine vector for use with LASV antigens as it demonstrates many favourable qualities for use in Lassa endemic regions. Its limited replication in mammalian cells and high capacity to accommodate foreign genes make it a good vaccine candidate [23,44]. It does not require a cold chain so can be used in low income countries, and it is capable of large scale GMP manufacture [44,45].

This research has demonstrated exciting potential for a vaccine against Lassa fever using an MVA vaccine candidate. Our vaccine has demonstrated strong immunogenicity and protection against disease progression in a small animal model, thus suggesting that it has the potential for advancement to pre-clinical and clinical trials.

Declaration of Competing Interest

Author declares that there is no conflicts of interest.

Acknowledgments

The authors would like to thank the Biological Investigations Group and the Histology department (particularly Kirsty Emery for her involvement in the histology laboratory) at PHE Porton for their work during the project.

This report is work commissioned by Innovate UK and the Department of Health and Social Care (Project title “SRBI New Vaccines for Global Epidemics: LassaVacc; File Ref. 972223) and is funded through Official Development Assistance (ODA). The views expressed in this publication are those of the author(s) and not necessarily those of employing institutions or funding bodies.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.07.023>.

References

- [1] Yun NE, Walker DH. Pathogenesis of Lassa fever. *Viruses* 2012;4:2031–48. <https://doi.org/10.3390/v4102031>.
- [2] WHO. Lassa fever outbreak contained but continual vigilance needed. 2018.
- [3] Fisher-Hoch SP, Tomori O, Nasidi A, Perez-Oronoz GI, Fakile Y, Hutwagner L, et al. Review of cases of nosocomial Lassa fever in Nigeria: the high price of poor medical practice. *BMJ. Br Med J* 1995;311:857–9.
- [4] Price ME, Fisher-Hoch SP, Craven RB, McCormick JB. A prospective study of maternal and fetal outcome in acute Lassa fever infection during pregnancy. *BMJ. Br Med J* 1988;297(6648):584–7.
- [5] Salazar-Bravo J, Uedas LA, Yates TL. Mammalian reservoirs of arenaviruses. *Curr Top Microbiol Immunol* 2002;262:25–63.
- [6] ter Meulen J, Lukashevich I, Sidibe K, Inapogui A, Marx M, Dorlemann A, et al. Hunting of peridomestic rodents and consumption of their meat as possible risk factors for rodent-to-human transmission of Lassa virus in the Republic of Guinea. *Am J Trop Med Hyg* 1996;55(6):661–6.
- [7] Radoshitzky SR, Bao Y, Buchmeier MJ, Charrel RN, Clawson AN, Clegg CS, et al. Past, present and future of arenavirus taxonomy. *Arch Virol* 2015;160(7):1851–74. <https://doi.org/10.1007/s00705-015-2418-y>.
- [8] Bowen MD, Rollin PE, Ksiazek TG, Hustad HL, Bausch DG, Demby AH, et al. Genetic diversity among Lassa virus strains. *J Virol* 2000;74(15):6992–7004.
- [9] Inovio First to Advance Lassa Fever Candidate Vaccine Into a Clinical Trial [Internet]. 201 Available from: <https://www.prnewswire.com/news-releases/inovio-first-to-advance-lassa-fever-candidate-vaccine-into-a-clinical-trial-300853937.html>.
- [10] Branco LM, Grove JN, Geske FJ, Boisen ML, Muncy IJ, Magliato SA, et al. Lassa virus-like particles displaying all major immunological determinants as a vaccine candidate for Lassa hemorrhagic fever. *Virol J* 2010;7:279. <https://doi.org/10.1186/1743-422X-7-279>.
- [11] Rodriguez-Carreno MP, Nelson MS, Botten J, Smith-Nixon K, Buchmeier MJ, Whitton JL. Evaluating the immunogenicity and protective efficacy of a DNA vaccine encoding Lassa virus nucleoprotein. *Virology* 2005;335:87–98. <https://doi.org/10.1016/j.virol.2005.01.019>.
- [12] Lukashevich IS, Carrion Jr R, Salvato MS, Mansfield K, Brasky K, Zapata J, et al. Safety, immunogenicity, and efficacy of the ML29 reassortant vaccine for Lassa fever in small non-human primates. *Vaccine* 2008;26(41):5246–54.
- [13] Jiang X, Dalebout T, Bredendiek P, Carrion Jr R, Brasky K, Patterson J, et al. Yellow fever 17D-vectored vaccines expressing Lassa virus GP1 and GP2 glycoproteins provide protection against fatal disease in guinea pigs. *Vaccine* 2011;29(6):1248–57. <https://doi.org/10.1016/j.vaccine.2010.11.079>.
- [14] Clegg JCS, Lloyd G. Vaccinia recombinant expressing Lassa-virus internal nucleocapsid protein protects guinea pigs against Lassa fever. *Lancet* 1987;2(2):186–7.
- [15] Hallam HJ, Hallam S, Rodriguez SE, Barrett ADT, Beasley DWC, Chua A, et al. Baseline mapping of Lassa fever virology, epidemiology and vaccine research and development. *npj. Vaccines* 2018;3(11):1–12. <https://doi.org/10.1038/s41541-018-0049-5>.
- [16] WHO. Efficacy trials of Lassa Vaccines: endpoints, trial design, site selection” WHO Workshop Final Report 2018. Available from: https://www.who.int/blueprint/what/norms-standards/LassaVxeval_Final_MeetingReport.pdf?ua=1.
- [17] Lloyd G, Barber G, Clegg J, Kelly P. Identification of Lassa fever virus infection with recombinant nucleocapsid protein antigen. *Lancet* 1989;334:1222.
- [18] Fisher-Hoch SP, Hutwagner L, Brown B, McCormick JB. Effective vaccine for Lassa fever. *J Virol* 2000;74(15):6777–83.
- [19] ter Meulen J, Badusche M, Satoguina J, Strecker T, Lenz O, Loeliger C, et al. Old and new world arenaviruses share a highly conserved epitope in the fusion domain of the glycoprotein 2, which is recognized by lassa-specific human CD4+ T-cell clones. *Virology* 2004;321:134–43.
- [20] Fisher-Hoch SP, McCormick JB, Auperin DD, Brown BG, Castor M, Perez G, et al. Protection of rhesus monkeys from fatal Lassa fever by vaccination with a recombinant vaccinia virus containing the lassa virus glycoprotein gene. *PNAS* 1989;86:317–21.
- [21] Geisbert TW, Jones S, Fritz EA, Shurtleff AC, Geisbert JB, Liebscher R, Grolla A, Ströher U, Fernando L, Daddario KM, Guttieri MC. Development of a new vaccine for the prevention of Lassa fever. *PLoS Med* 2005;2(6):e183. Epub Jun 28.
- [22] Morrison HG, Bauer SP, Lange JV, Esposito JJ, McCormick JB, Auperin DD. Protection of guinea pigs from Lassa fever by Vaccinia virus recombinants

- expressing the nucleoprotein or the envelope glycoproteins of Lassa virus. *Virology* 1989;171:179–88.
- [23] Blanchard TJ, Alcami A, Andrea P, Smith GL. Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. *J General Virol* 1998;79:1159–67. PubMed PMID: AF016273.
- [24] Meyer H, Sutter G, Mayr A. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J Gen Virol* 1991;72:1031–8.
- [25] Stittelaar KJ, Kuiken T, de Swart RL, van Amerongen G, Vos HW, Niesters HGM, et al. Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. *Vaccine* 2001;19:3700–9.
- [26] von Krempelhuber A, Vollmar J, Pokorny R, Rapp P, Wulff N, Petzold B, et al. A randomized, double-blind, dose-finding Phase II study to evaluate immunogenicity and safety of the third generation smallpox vaccine candidate IMVAMUNE. *Vaccine* 2010;28(5):1209–16. <https://doi.org/10.1016/j.vaccine.2009.11.030>. PubMed PMID: 19944151.
- [27] Kreijtz JHCM, Goeijenbier M, Moesker FM, van den Dries L, Goeijenbier S, De Gruyter HLM, et al. Safety and immunogenicity of a modified-vaccinia-virus-Ankara-based influenza A H5N1 vaccine: a randomised, double-blind phase 1/2a clinical trial. *Lancet Infect Dis* 2014;14(12):1196–207. [https://doi.org/10.1016/S1473-3099\(14\)70963-6](https://doi.org/10.1016/S1473-3099(14)70963-6).
- [28] Guimarães-Walker A, Mackie N, McCormack S, Hanke T, Schmidt C, Gilmour J, et al. Lessons from IAVI-006, a Phase I clinical trial to evaluate the safety and immunogenicity of the pTHr.HIVA DNA and MVA.HIVA vaccines in a prime-boost strategy to induce HIV-1 specific T-cell responses in healthy volunteers. *Vaccine* 2008;26(51):6671–7. <https://doi.org/10.1016/j.vaccine.2008.09.016>.
- [29] Buttigieg KR, Dowall SD, Findlay-Wilson S, Miloszevska A, Rayner E, Hewson R, et al. A novel vaccine against Crimean-Congo haemorrhagic Fever protects 100% of animals against lethal challenge in a mouse model. *PLoS ONE* 2014;9(3):e91516. doi:10.1371.
- [30] Grundy DJ, Bowen ETW, Lloyd G. Isolated case of Lassa fever in Zaria, Northern Nigeria. *Lancet* 1980;316(8195):649–50. [https://doi.org/10.1016/S0140-6736\(80\)90322-0](https://doi.org/10.1016/S0140-6736(80)90322-0).
- [31] McCurdy L, Rutigliano J, Johnson T, Chen M, Graham B. Modified vaccinia virus Ankara immunization protects against lethal challenge with recombinant vaccinia virus expressing murine interleukin-4. *J Virol* 2004;78(22):12471–9. <https://doi.org/10.1128/JVI.78.22.12471-12479.2004>.
- [32] Wang F, Flanagan J, Wang LC, Bui S, Nielson A, Wu X, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mole Diagn* 2012;14(1):22–9. <https://doi.org/10.1016/j.jmoldx.2011.08.002>.
- [33] Bell TM, Shaia CL, Bearss JJ, Mattix ME, Koistinen KA, Honnold SP, et al. Temporal progression of lesions in guinea pigs infected with Lassa virus. *Vet Pathol* 2017;54(3):549–62.
- [34] Abreu-Mota T, Hagen K, Cooper K, Jahrling P, Tan G, Wirblich C, et al. Non-neutralizing antibodies elicited by recombinant Lassa-Rabies vaccine are critical for protection against Lassa fever. *Nat Commun* 2018. <https://doi.org/10.1038/s41467-018-06741-w>.
- [35] Cummins D, McCormick JB, Bennett D, Samba J, Farrar B, Machin S, et al. Acute sensorineural deafness in Lassa fever. *J Am Med Assoc* 1990;264:2093–6.
- [36] Okokhere P, Ibekwe T, GO A. Sensorineural hearing loss in Lassa fever: two case reports. *J Med Case Rep* 2009;3:36. <https://doi.org/10.1186/1752-1947-3-36>.
- [37] Grahm A, Bråve A, Lagging M, Dotevall L, Ekqvist D, Hammarström H, et al. Imported case of Lassa fever in Sweden with encephalopathy and sensorineural hearing deficit. *Open Forum Infect Dis* 2016;3(4). <https://doi.org/10.1093/ofid/ofw198>.
- [38] Ibekwe T, PO O, Asogun D, Blackie F, Nwegbu M, Wahab K, et al. Early-onset sensorineural hearing loss in Lassa fever. *Eur Arch Otorhinolaryngol* 2011;268(2):197–201.
- [39] Liao B, Byl F, Adour K. Audiometric comparison of Lassa fever hearing loss and idiopathic sudden hearing loss: evidence for viral cause. *Otolaryngol Head Neck Surg* 1992;106(3):226–9. <https://doi.org/10.1177/019459989210600303>.
- [40] Yun N, Ronca S, Tamura A, Koma T, Seregin A, Dineley K, et al. Animal model of sensorineural hearing loss associated with Lassa virus infection. *J Virol* 2016;90:2920–7. <https://doi.org/10.1128/JVI.02948-15>.
- [41] Walker DH, Wulff H, Lange JV, MF A. Comparative pathology of Lassa virus infection in monkeys, guinea-pigs and *Mastomys natalensis*. *Bull World Health Organ* 1975;52:523–34.
- [42] Jahrling PB, Smith S, Hesse RA, B RJ. Pathogenesis of Lassa virus infection in guinea pigs. *Infection and Immunity*. 1982; 37(2): pp. 771–778.
- [43] Leski TA, Stockelman MG, Moses LM, Park M, Stenger DA, Ansumana R, et al. Sequence variability and geographic distribution of Lassa virus, Sierra Leone. *Emerg. Infect. Dis.* 2015;21(4):609–18. <https://doi.org/10.3201/eid2104.141469>. PubMed PMID: 25811712.
- [44] Cottingham MG, Carroll MW. Recombinant MVA vaccines: dispelling the myths. *Vaccine* 2013;31:4247–51.
- [45] Amato RJ, Hawkins RE, Kaufman HL, Thompson JA, Tomczak P, Szylyk C, et al. Vaccination of metastatic renal cancer patients with MVA-5T4: A randomised, double-blind, placebo-controlled phase, III study. *J Clin Cancer Res* 2010;16(22):5539–47. <https://doi.org/10.1158/1078-0432.CCR-10-2082>.