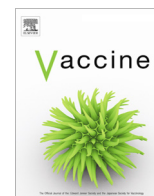


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## Dual route vaccination for plague with emergency use applications <sup>☆</sup>

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### ABSTRACT

Here, we report a dual-route vaccination approach for plague, able to induce a rapid response involving systemic and mucosal immunity, whilst also providing ease of use in those resource-poor settings most vulnerable to disease outbreaks. This novel vaccine (VypVaxDuo) comprises the recombinant F1 and V proteins in free association. VypVaxDuo has been designed for administration via a sub-cutaneous priming dose followed by a single oral booster dose and has been demonstrated to induce early onset immunity 14 days after the primary immunisation; full protective efficacy against live organism challenge was achieved in Balb/c mice exposed to  $2 \times 10^4$  median lethal doses of *Yersinia pestis* Co92, by the sub-cutaneous route at 25 days after the oral booster immunisation. This dual-route vaccination effectively induced serum IgG and serum and faecal IgA, specific for F1 and V, which constitute two key virulence factors in *Y. pestis*, and is therefore suitable for further development to prevent bubonic plague and for evaluation in models of pneumonic plague. This is an essential requirement for control of disease outbreaks in areas of the world endemic for plague and is supported further by the observed exceptional stability of the primary vaccine formulation in vialled form under thermostressed conditions (40 °C for 29 weeks, and 40 °C with 75% relative humidity for 6 weeks), meaning no cold chain for storage or distribution is needed.

In clinical use, the injected priming dose would be administered on simple rehydration of the dry powder by means of a dual barrel syringe, with the subsequent single booster dose being provided in an enteric-coated capsule suitable for oral self-administration.

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### 1. Introduction

Plague is an ancient disease which is endemic today in many global regions [1], with demonstrated potential for epidemic spread. Indeed, one of the worst outbreaks, featuring a high rate (70%) of confirmed and suspected pneumonic plague, occurred from August–November 2017 in Madagascar [2], with >2500 cases and a case fatality rate of 8.6% [2]. Whilst antibiotic therapy is effective, this needs to be initiated early and is not ideal in a plague-endemic area, because of the potential emergence of

anti-microbial resistance, as observed previously in Madagascar and Peru [3,4].

Historically, research has shown that plague is preventable by vaccination with live attenuated strains (EV76) or killed whole cell vaccines (KWCV) [5]. Although EV76 vaccines are highly immunogenic, they also carry some risk [6]. The KWCV are hazardous to produce and whilst thought effective against bubonic plague [7], they are deficient against pneumonic plague [8]. Currently there is no readily available licensed vaccine, although a variety of plague vaccine candidates have been proposed, reviewed in [9,10]. The F1 and V proteins have emerged as the predominant protective antigens [11,12], whilst other proteins e.g. YscF [13], YadC and OppA [14,15] have demonstrated partial efficacy. Previously, using liquid formulations, we demonstrated that the recombinant F1 and V proteins were safe and immunogenic in >160 volunteers [16] and protective in a range of animal models of the disease [17,18]. The induction of functional antibody [19] and cell-mediated immunity [20] are both important for protection against plague.

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We have designed novel formulations of the F1 and V proteins, intended for emergency use authorisation (EUA) [21], inducing rapid immunity and with simplified delivery involving an injected prime and oral boost to induce systemic and mucosal immunity to counter bubonic and pneumonic plague [22,23]. This vaccine (VypVaxDuo) comprises two formulations: (i) F1 and V proteins embedded in calcium phosphate coated onto amino-acid microcrystals [24] for primary immunisation; and (ii) the same antigens dispersed in an oil vehicle [25] for oral boosting. Our aim is to provide an efficacious vaccine, easily administered in resource-poor, low- to- middle income countries (LMIC), without requirement for cold chain distribution or storage.

Here, each arm of the vaccination regimen has been optimised for eventual clinical use, entailing the selection of excipients, particularly for the oral formulation, to overcome the tolerance which protects humans from exposure to dietary antigens [26]. Enteric-coating will ensure gastric transit; subsequent uptake by Peyer's Patches (PP) in the small intestine [27] will be promoted by incorporated excipients [28] such as vitamin A.D. and E derivatives, which enhance dendritic cell (DC) activation, which in turn induces effector T-cells [29,30]. Vitamin A or retinoic acid (RA) stimulates gut-resident DC to induce T-cell expression of CCR9 and  $\alpha 4\beta 7$  [31] and promote gut-homing of T-cells through binding to MAdCAM-1 and CCL25, selectively expressed on intestinal cells [32]. Gut-resident DC also actively secrete RA, whilst skin-resident DC produce the active form of vitamin D, 1,25(OH)2D3; the latter imprints CCR10 expression on T-cells and homing to skin inflammation sites through binding to CCL37, selectively expressed on keratinocytes [33]. Alpha tocopheryl acetate (ATA), a non-toxic derivative of vitamin E, also activates DC and has adjuvant properties [34].

We have also incorporated dimethyldioctadecylammonium bromide (DDAB), cholera toxin B sub-unit (CTB) and trehalose dibehenate (TDB) in the oral formulation. DDAB is a cationic surfactant with similar action to chitosan [35–37] promoting electrostatic contact with PP cells and vaccine uptake. TDB, a synthetic analog of the mycobacterial trehalose dimycolate, is a potent macrophage activator [38], whilst CTB is anti-inflammatory and immunomodulatory [39].

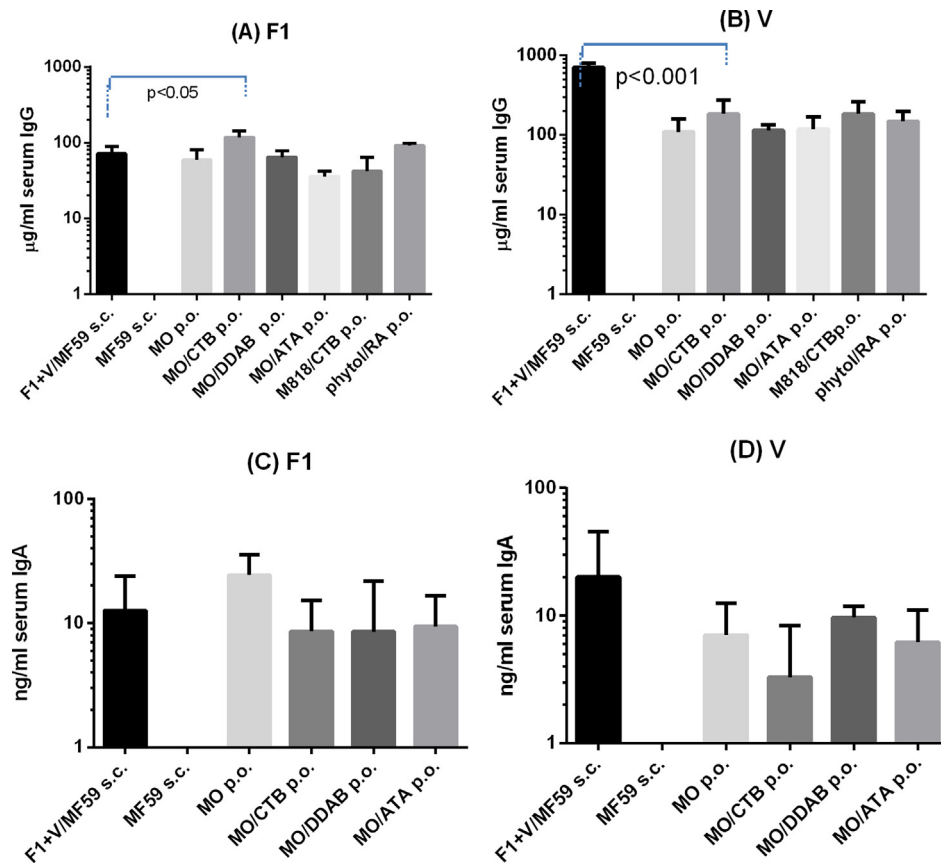
## 2. Materials and methods

### 2.1. Protein antigens, vaccine formulation and stability

Recombinant F1 and V antigens were expressed from *E. coli*, purified as previously described [39] and supplied in PBS or ammonium acetate buffer for formulation.

For initial sub-cutaneous (s.c.) testing, F1 and V were admixed in PBS and MF59 (Novartis) was added 1:1 (v/v). For subsequent studies, the injected formulations of F1 and V were prepared as calcium phosphate microcrystals, using published methodology [24]. Briefly, aqueous mixtures of F1 and V with sodium orthophosphate and glutamine (Gln) or histidine (His) were precipitated as calcium phosphate protein-coated microcrystals (CaP-PCMC), by addition into a 19- fold excess of isopropanol containing dissolved calcium chloride. The resultant suspension contained self-assembled microcrystals comprising an amino-acid core with the protein embedded in a thin surface layer of CaP. The CaP-PCMC were isolated by vacuum filtration and dried to a powder. Protein content and integrity was determined by ELISA and SDS-Page.

The preparation of oral formulations of F1 and V was adapted from published methodology [25]. In brief, F1 and V, dissolved in



**Fig. 1.** Immunogenicity of F1 + V in 3 dose (days 0, 10, 31) dual route schedule. Mice were administered 10 µg F1 + 10 µg V formulated in MF59 (s.c.) and boosted with either the same formulation (s.c.) or with 25 µg F1 + 25 µg V in oil per oral (p.o.), with excipients as shown. Negative control mice received MF59 (s.c.) on days 0, 10 and 31. Mean serum IgG and IgA titres per group to F1 (A) and (C) and to V (B) and (D) were determined at day 67.

0.1 M ammonium acetate solution, were added to an amphiphile solution in cyclohexane, comprising a combination of soya phosphatidyl choline, Brij 52 and sodium docusate. This mixture was vortexed to give a water-in-oil emulsion, cooled to  $-30^{\circ}\text{C}$  and lyophilised overnight at  $4^{\circ}\text{C}$ . Next day, a clinical grade oil containing the remaining excipients, was added to the dry residue. Iterations of the oral formulation comprising either mineral oil (MO), M818 (Huls AG, Germany) or phytol and containing RA, ATA, vitamin D, TDB (Invivogen, UK), CTB, and DDAB were prepared. All reagents were purchased from Sigma-Aldrich UK, unless stated otherwise. The optimum combination for efficacy testing comprised F1 and V in MO with all the excipients above (termed Formulation B), which was compared with Formulation A (identical, except for the omission of CTB).

The formulations were subjected to a temperature range of ( $4^{\circ}\text{C}$ – $40^{\circ}\text{C}$ ) for defined time periods and relative humidity (RH). A relative humidity (RH) of 75% was maintained by using saturated sodium chloride in a closed container housing samples in screw-topped vials. Changes to F1 and V integrity were probed by SDS-PAGE, size-exclusion chromatography–HPLC (SEC-HPLC) and ELISA. SDS-Page gels were run under reducing conditions with dithiothreitol (Sigma). Bolt 4–12% Bis-Tris Plus gels were used with Bolt reagents and were silver-stained (Silver Xpress silver staining kit). All electrophoresis reagents were obtained from ThermoFisher Scientific.

## 2.2. Animals, immunisation, immunogenicity and efficacy

SPF female Balb/c mice (>6 weeks) were supplied by a commercial breeder. Mice were randomised for allocation to treatment group with food, water and environmental enrichment *ad libitum* and acclimatised to the facility prior to any procedures, which were performed in accordance with the UK Animal (Scientific Procedures) Act 1986. Mice were immunised in groups of 5 with either (1): a s.c. priming dose followed either by two s.c. or two per oral (p.o.) booster doses, given at 10 and 31 d. after the prime; or (2) a s.c. priming dose followed by a single p.o. or s.c. booster dose 21 d. after the prime. For s.c. immunisation, mice received  $10\ \mu\text{g}$  F1 +  $10\ \mu\text{g}$  V in 0.1 ml, whereas for p.o. dosing, mice received  $25\ \mu\text{g}$  F1 +  $25\ \mu\text{g}$  V in 0.1 ml, by gavage. For assay of antibody titre, serial blood samples were collected from the tail vein, whilst final samples were collected by cardiac puncture under terminal anaesthesia. Faecal pellets were collected from each treatment group and rapidly frozen ( $-80^{\circ}\text{C}$ ).

F1- and V-specific serum IgG, IgA, IgG1 and IgG2a titres were determined for individual mice by ELISA [40]. Faecal pellets (FP), amalgamated by treatment group, were extracted for assay of IgA. In brief, to 10 ml of cold PBS, 1 tablet of complete mini protease inhibitor cocktail (Sigma) and  $5\ \mu\text{l}$  Tween 20 were added. One ml of supplemented PBS was added per 0.1 g FP, incubated (RT, 5 min), vortexed, incubated on ice (20 min) and centrifuged (15,000g, 5 min). Supernatants were retained and stored at  $-80^{\circ}\text{C}$  pending assay. Antibody concentrations were determined from the standard curves included on each ELISA plate, using Ascent software with four parameter logistic curve-fitting and were reported in ng/ml or  $\mu\text{g}/\text{ml}$  of sample.

At 20 d. after the booster immunisation, mice were transferred to ACDP3 biocontainment for live organism challenge and acclimatised in their treatment groups. *Y. pestis* Co92, a clinical isolate [41], was cultured in blood agar broth and then diluted in PBS to estimated challenge doses of  $2 \times 10^7/\text{ml}$  or  $2 \times 10^5/\text{ml}$ . The challenge dose was quantified retrospectively by 48 h culture of the inoculum on blood agar. Groups of mice were challenged with the appropriate inoculum (0.1 ml/mouse s.c.) and then closely monitored for 14d post-infection (p.i.), with those showing clinical signs representing the humane endpoint, being culled by cervical

dislocation. At 14d p.i., surviving mice were terminally anaesthetised, blood was collected by cardiac puncture and mice were culled, with splenectomy.

Splenocyte suspensions or sera were prepared in sterile PBS and serially diluted; 0.1 ml of each suspension was cultured in duplicate on blood agar ( $37^{\circ}\text{C}$ , 48 h) before colony forming units (CFU) were enumerated.

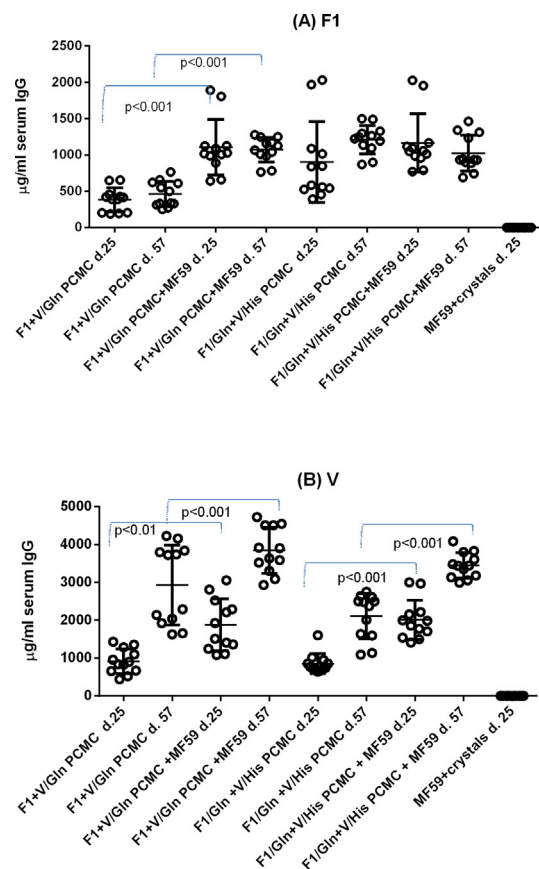
## 2.3. Statistical analysis

All data were analysed using Graph Pad Prism software v.6 and expressed as mean  $\pm$  s.e.m. Statistical comparisons were made

**Table 1**

Immunogenicity of F1 + V in a 3 dose (days 0, 10, 31) dual route schedule. All priming doses with F1 + V were administered s.c. in MF59; the formulations for oral booster doses were varied in terms of different amphiphiles, oils, and immunostimulants. IgA was measured on faecal pellets, collected at day 42 and amalgamated by treatment group prior to extraction.

Treatment groups	IgA measured in extracts of faecal pellets (0.1 g), amalgamated by treatment group	
	F1	V
F1 + V/MF59 s.c./s.c	0	0
F1 + V/MF59 s.c./MO p.o.	6.3	3.02
F1 + V/MF59 s.c./MO + CTB p.o.	0	0
F1 + V/MF59 s.c./MO + DDAB p.o.	5.98	6.25
F1 + V/MF59 s.c./MO + ATAp.o.	8.29	2.18



**Fig. 2A.** Immunogenicity of F1 + V formulated on the same PCMC (F1 + V/Gln PCMC) or on PCMC of different composition (F1/GlnPCMC + V/His PCMC), with and without MF59, following immunisations in a 3 - dose s.c. only schedule (days 0, 10, 31) with  $10\ \mu\text{g}$  F1 +  $10\ \mu\text{g}$  V. Mean serum IgG titres to (A) F1 and (B) V were determined at days 25 and 37.

using one-way ANOVA or unpaired *t*-test. The survival data were expressed as Kaplan-Meier survival curves and statistical significance was determined by Log-rank test.  $P < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Vaccine formulation for dual route dosing

Initially, the formulation for the s.c. priming dose of F1 and V was a simple mixture with MF59 adjuvant, whilst the formulation for the oral booster doses (d.10 and 31) was varied in terms of excipients and oil. The effect of varying the oral formulation on serum IgG titres to F1 and V at the end of the immunisation schedule (day 67) can be seen in Fig. 1 (Panels A-B). Oral boosting with F1 + V formulated in MO with added CTB, significantly enhanced IgG responses to F1 but not to V, compared with s.c. boosting with F1 + V in MF59. Since MO was better tolerated by the mice than either M818 or phytol, and since there was little advantage of these alternative oil vehicles on IgG titres, MO was down selected for subsequent studies. RA was also included, since similar IgG responses were seen when presented in phytol, in the absence of CTB.

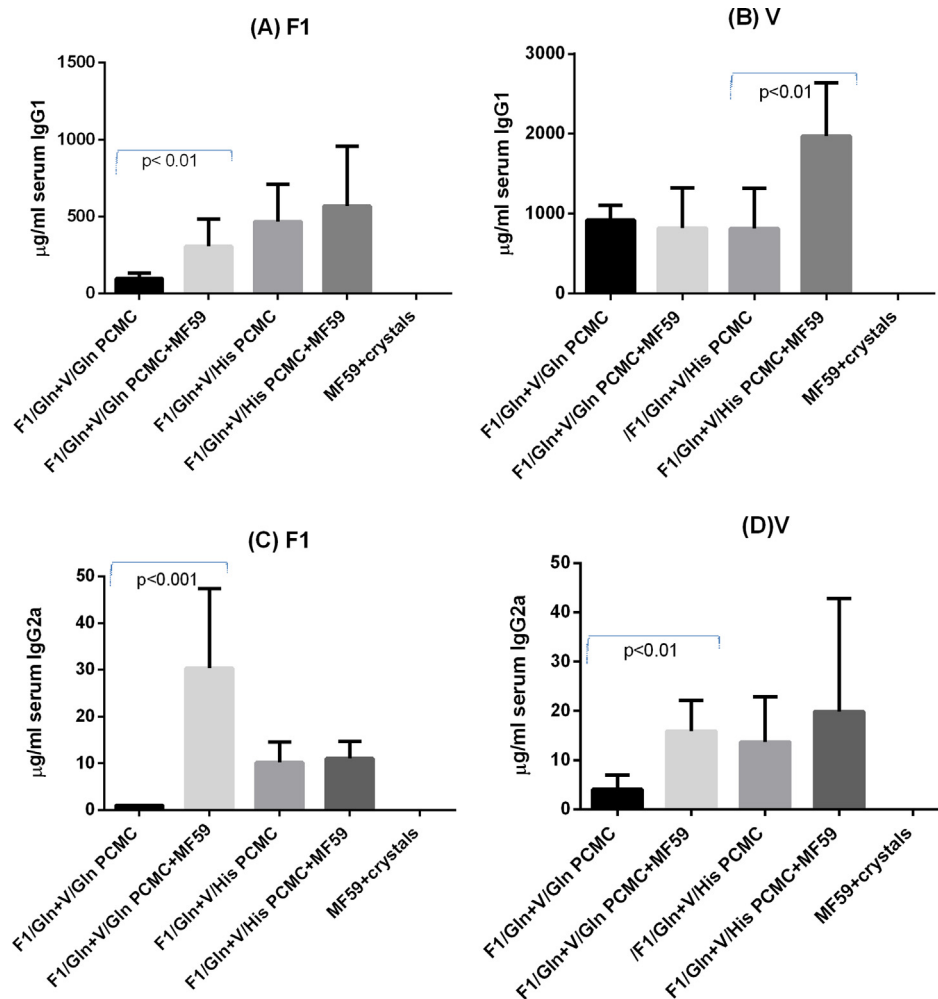
Analysis of IgA in faecal extracts at d. 42 (11 d. after the oral dose) indicated a benefit of including DDAB and ATA, but not CTB, in the MO vehicle (Table 1). However, day 67 serum IgA titres

for each group primed with F1 + V/MF59 and boosted orally with MO containing the individual immunostimulants (CTB/DDAB/ATA) did not differ significantly from those achieved by s.c. boosting (Fig. 1C and D). Intra-group, titres of serum IgA were more variable than IgG. Since the excipients CTB, DDAB and ATA each contribute differently to enhance immunity, and were compatible in MO, they were combined into the oral formulation for subsequent studies.

#### 3.2. Optimisation of the sub-cutaneous dosing formulation

For the s.c. arm of the dual route vaccine, permutations of F1 and V, either co-formulated on the same CaP-PCMC particle or immobilised on separate particles, with or without MF59, were trialled, initially in the 3-dose regimen (d.0,10,31) used above. The development of specific IgG titres was determined at d. 25 and 57 (Fig. 2A). The absolute titre induced to the V antigen was greater than that induced to the F1 antigen. However the specific titre to either F1 or V, when both were formulated together on glutamine PCMC (F1 + V/Gln), was significantly enhanced at d. 25 and 57 by the addition of MF59. However, when different PCMC were used F1 on Glutamine (F1/Gln) with V on Histidine (V/His) (F1/Gln + V/His), only the response to V was significantly enhanced by the inclusion of MF59.

At d. 57 of this s.c. dosing regimen, the predominant isotype to either F1 or V, by 3 to 6-fold, was IgG1 (Fig. 2B). However, the addi-



**Fig. 2B.** Determination of IgG isotypes induced to F1 and V at day 57, by PCMC formulations with and without MF59 in a 3 dose s.c. cutaneous only schedule (days 0, 10, 31) are predominantly IgG1-biased (A and B) with some IgG2a (C and D) in the presence of MF59.

tion of MF59 to the PCMC significantly enhanced IgG2a titres, particularly where F1 and V were presented together on the same glutamine PCMC.

3.3. Optimisation of the dual route dosing regimen

Optimised formulations were combined in a dual route dosing regimen. For the s.c. priming dose, F1/Gln + V/His PCMC ± MF59 were used. For the oral booster dose at day 21, F1 and V were combined in formulation B. The serum IgG response was determined after each dose at d.14 and 35 (Fig. 3A and B). In this dual route, 2-dose regimen, whilst the effect of adding MF59 to the s.c. priming dose was to reduce variability in the IgG response, it also limited the anti- F1 and V titres achieved at d.35, compared with groups primed with the PCMC formulations without MF59 and

boosted with oral formulation B. However when MF59 was included in the priming formulation, it increased serum IgG2a relative to IgG1, so that the IgG1: IgG2a ratios were 28 and 20 for F1 and V respectively, whereas in the absence of MF59, serum IgG1 predominated, with IgG1: IgG2a ratios of 102 and 170 for F1 and V (data not shown).

3.4. Characterisation of the effect of MF59 on the immune response to F1 and V

The effect of MF59 in the priming formulation was investigated in a subsequent study, in which mice were primed with PCMC ± MF59 and either no booster dose, or one of two different oral formulations (A or B) were administered. Here we again observed that inclusion of MF59 in the priming formulation suppressed the anti-

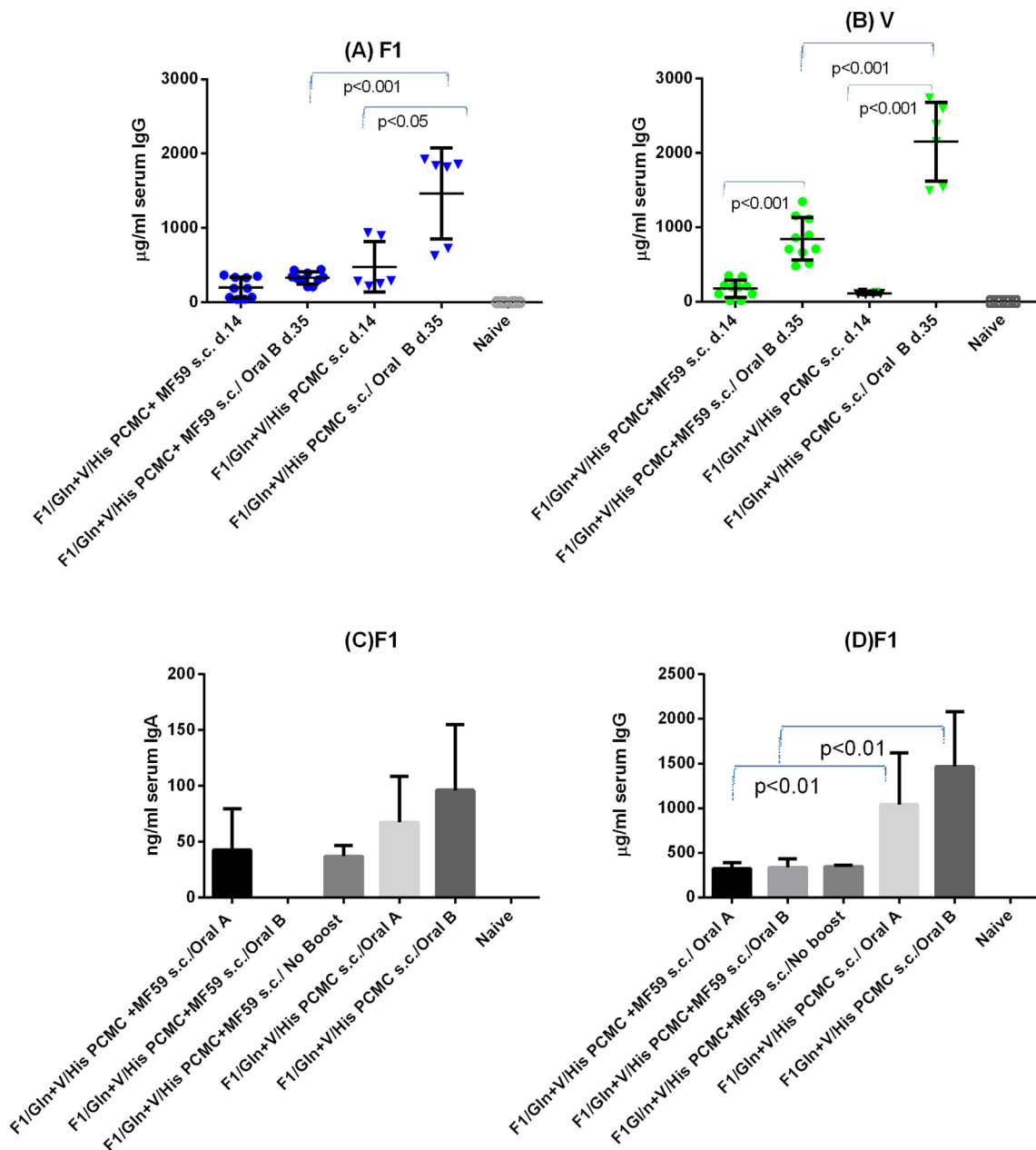


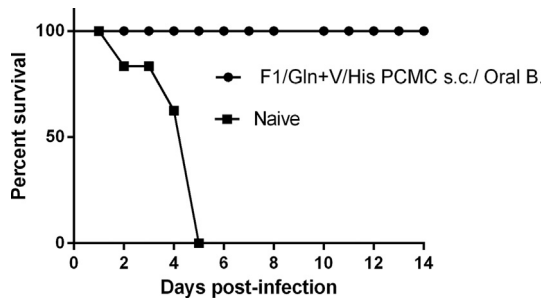
Fig. 3. Immunogenicity of F1 + V in dual route, 2-dose schedule (s.c. prime on day 0, with oral booster on day 21). Mean serum IgG titres are shown for F1 (A) and V (B) at day 14 (14 days after the primary immunisation) and day 35 (14 days after the oral booster). In a subsequent study, mean serum IgA (C) and IgG (D) titres to F1 are shown at day 35 after boosting with either of 2 different oral formulations (oral A or B) or not boosting.

F1 serum IgG or IgA titres achieved after oral boosting (Fig. 3C and D); suppression of titre was independent of the exact oral formulation used and was observed for the V antigen also (data not shown).

From these studies, we selected candidate formulations for an efficacy study: F1/Gln + V/His PCMC as the priming formulation; formulation B for the oral booster.

### 3.5. Protective efficacy against *Y. pestis* challenge

Mice immunised in the dual route dosing regimen on d. 0, 21 with F1/Gln + V/His PCMC s.c. and boosted orally with formulation B, were challenged with *Y. pestis* Co92 by the s.c. route on d. 46 with  $2.2 \times 10^4$  MLD ( $2.2 \times 10^4$  cfu) (Fig. 4). By d. 5p.i., all naïve mice had died, whereas all immunised mice survived to d.14p.i. In order to determine the limits of protective efficacy, 2 further groups of mice were challenged on d.46 with  $2.2 \times 10^6$  MLD *Y. pestis*, and 90% survival was observed at 14 days p.i., with no survivors in the naïve group. The blood and spleens of all mice surviving at d.14p.i. were culture-negative for *Y. pestis*.



**Fig. 4.** Protective efficacy of 2-dose dual route immunisation against live organism challenge. Mice were immunised s.c. with the PCMC formulation on day 0 and boosted with the oral formulation on day 21; they were challenged s.c. on day 46 with  $2.2 \times 10^4$  cfu ( $2.2 \times 10^4$  MLD) *Y. pestis* Co92. The plot shows survival at day 14p.i.

### 3.6. Stability assessment

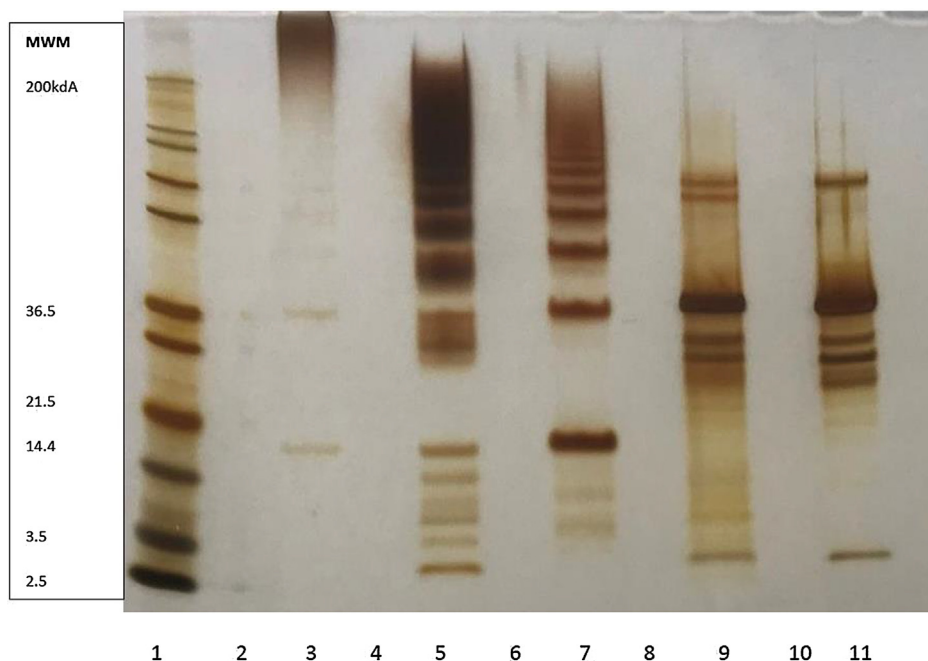
CaP-PCMC formulations held at 40 °C for 29 weeks, were examined by SDS-Page (Fig. 5A). By comparison with unformulated stock F1 and V (lanes 3 and 9) and thermo-stressed F1 stock (lane 5) and V (not shown) it is clear that these unformulated thermo-stressed proteins showed significant breakdown products. F1 and V proteins formulated on CaP PCMC (lanes 7 and 11) were significantly more conserved after 29 weeks at 40 °C, with heavy bands at the expected positions (15 and 37 kDa respectively). CaP PCMC formulated V and F1 were remarkably conserved after storage for 6 weeks at both elevated temperature (40 °C) and RH (75%) lanes 6 (Fig. 5B, left and right panels) compared with fresh stocks (lanes 3) and with PCMC formulations held for 6 weeks at 4 °C (lanes 5).

Oral formulations which had been held at 4 °C for 1 month, also showed preservation of structure, when analysed by SEC-HPLC (Supplemental Fig. 5C). The retention time of the F1 peak at (3.11–3.16 min) remained consistent for consecutive samples taken after 1, 2 or 4 weeks of storage. Similar data were obtained for the V protein (not shown).

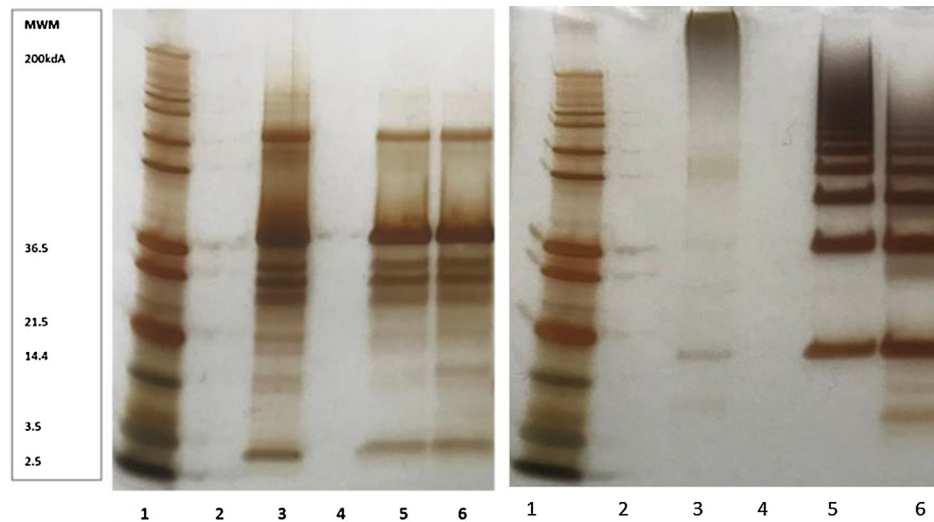
## 4. Discussion

We have successfully developed compatible formulations of F1 and V, for injected priming and oral boosting. This has involved formulation optimisation, initially for individual vaccination routes. For the primary vaccination, CaP-PCMC incorporating core glutamine or histidine provide a matrix for presentation of the F1 and V proteins. For the oral booster, the F1 and V proteins were incorporated into stable reverse micelles suspended in MO.

Used in a 2-dose dual route regimen, the oral formulation significantly boosted F1- and V-specific serum IgG titres, achieving values equivalent to those achieved initially with 3 injected doses. This dual route approach induced both strong systemic and mucosal immune responses, which were rapid in onset. F1 and V-specific circulating IgG was detectable at 14 days after the priming dose, with specific IgA present in blood at 14 days after a single



**Fig. 5A.** Stability of CaP-PCMC samples after storage at 40 °C for 29 weeks and analysis by SDS-PAGE. Lane 1 Mark 12 MWM, Lane 2 blank, Lane 3 fresh F1, Lane 4 blank, Lane 5 F1 stock 29 weeks at 40 °C, Lane 6 blank, Lane 7 F1/Gln PCMC 29 weeks at 40 °C, Lane 8 blank, Lane 9 fresh V, Lane 10 blank, Lane 11 V/His PCMC 29 weeks at 40 °C.



**Fig. 5B.** Stability of CaP-PCMC samples after storage at 40 °C/75%RH for 6 weeks and analysis by SDS-PAGE. Left panel: Lane 1 Mark 12 MWM, Lane 2 blank, Lane 3 fresh V stock, Lane 4 blank, Lane 5 V/His PCMC 6 weeks at 4 °C, Lane 6 V/His PCMC 6 weeks at 40 °C/75%RH. Right panel: Lane 1 Mark 12 MWM, Lane 2 blank, Lane 3 fresh F1, Lane 4 Blank, Lane 5 F1/Gln PCMC 6 weeks at 4 °C, Lane 6 F1/Gln PCMC 6 weeks at 40 °C/75% RH.

oral booster. Additional evidence for mucosal immunity induction was gained from the detection of IgA in faecal extracts at day 42, 11 days after a second oral booster dose in mice immunised in the interim 3-dose dual route regimen. It was important here to determine the induction of mucosal as well as systemic immunity, to justify subsequent testing of the vaccine in a pneumonic plague model.

The systemic IgG response to the dual route vaccination, was predominantly Th2- biased with high circulating levels of IgG1. The inclusion of MF59 in the priming formulation reduced this Th2 bias by inducing some IgG2a, reduced intra-group variability but unexpectedly, also limited the response to the oral booster. This differed from the adjuvantising effect of MF59 in the injected 3-dose regimen, where it significantly enhanced serum IgG titres. These paradoxical effects of MF59 may be partly attributable to the Th2-polarising effect of the oral booster curtailing the influence of MF59 to induce Th1 responses, but we cannot rule out other influences e.g. on antibody maturation and affinity. However, because of its constraining effect on the response to the oral booster, MF59 was omitted from the final injected formulation.

The CaP PCMC is exceptionally stable, so that protein integrity was maintained even after 29 weeks at 40 °C and 75%RH. No deterioration of protein in the oral formulation was observed over 1 month, albeit at 4 °C. Once encapsulated, the anhydrous oral formulation is also expected to withstand high ambient temperatures and humidity, so that neither formulation will require cold chain storage or distribution. In conclusion, we have developed a 2-dose, dual route vaccination regimen for plague and demonstrated that this approach rapidly induces systemic and mucosal immunity with high titres of specific IgG and detectable IgA in blood and faecal extracts. We have demonstrated that this approach is efficacious in a murine bubonic plague model at 25 d. after the booster dose and that survivors had no residual bacteria. Work is ongoing to determine the limits of protective efficacy for VypVaxDuo in pneumonic plague models.

Outbreaks of plague in endemic regions call for the approval of an effective vaccine for EUA and a number of candidates including VypVax Duo are in the research to clinical pipeline [9–11]. VypVax-Duo represents a practical solution to vaccination in LMIC, since it would require only one clinic visit to receive a priming dose and a capsule, for self-administered oral boosting. This dual- route dosing regimen provides a safe, stable, highly efficacious sub-unit

vaccine for plague which promises to have significant impact on disease burden in endemic areas.

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