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CHARACTERISATION OF ADJUVANTS FOR USE IN DROMEDARY IMMUNISATION

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ABSTRACT

Although camelids are an important domestic species with more than 25 million members, little is known about vaccine adjuvant efficacy, safety and mechanism of action in this species. This presents a major problem for design of effective camelid vaccines. This is of more than theoretical interest given the recent emergence of camels as vectors of transmission to humans of lethal viral diseases such as Middle Eastern Respiratory Syndrome (MERS) coronavirus. Hence availability of well-validated camelid vaccine adjuvants may be important not just for vaccines to prevent diseases of camels but also to block their ability to transmit disease to humans. In this study, we used dromedaries to test the safety and efficacy of four different adjuvant formulations (Advax™ HCXL, Advax AF-1, Advax AF-2 or alum) together with four different antigen formulations (*B. mallei, C. pseudotuberculosis, C. perfringens*, Rhinovirus) administered by subcutaneous injection in the neck region of adult animals. All the Advax delta inulin-based adjuvants and the alum adjuvant were well tolerated, with no severe lesions such as the draining granulomas that are caused by oil emulsion adjuvants. There was no trend for increased vaccine reactogenicity in camels that had existing immunity to the vaccine antigens. Overall, the vaccines had modest immunogenicity in these adult animals indicating the need for further research to identify the optimal adjuvant formulation, dose and immunisation route for camelid vaccines.

Key words: Adjuvant, camelid, immunogenicity, safety, vaccine

According to the European Committee for Veterinary Medicinal Products, an ideal adjuvanted vaccine should be free of local or systemic inflammatory reactions, or allergic reactions and should be safe for consumers of food produced from the immunised animals (CVMP, 1997). The most commonly used adjuvant systems used for human and veterinary vaccines are aluminum salts followed by oil emulsions. Unfortunately these adjuvants do not always generate the desired immune response needed for a particular vaccine application and can suffer from excess toxicity, hence there is a need for new adjuvants to be developed. A major problem besetting the veterinary vaccine field is that most new adjuvant compounds have never been tested in relevant animal species. To set up studies to test each of these compounds in all relevant animal species would be an enormous task, and this likely explains why for each animal species only a very limited number of adjuvants have been tested and confirmed to work.

Although camelids are an important domestic species with more than 25 million members in 2000, little is known about the mechanisms, efficacy and safety of more than a limited number of vaccine adjuvants in camelids (Wernery *et al,* 1999; Wernery *et al,* 2014). Immunological research has highlighted the potential of camelids as an important source of nanobody production (Abbas and Agab, 2002). Camel antibodies are unique because they are devoid of light chains and have a single domain antibody fragment (nanobody) capable of binding to specific antigen. Camelid nanobody technology has become an efficient tool in research, diagnostic and therapeutic fields. In a recent search for a better camel adjuvant, we reported that a new polysaccharide particulate adjuvant based on delta inulin Advax Horse and Camel XL (Advax HCXL) adjuvant when formulated with the relevant killed antigens induced high antibody titres against *Burkholderia mallei* and African horse sickness virus (AHSV) in dromedaries without inducing any significant inflammatory reactions (Eckesley *et al,* 2011). In this paper we report the results of a second immunisation trial conducted in dromedaries with four different adjuvants together with four different antigens. The aim of the study

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was to identify optimal well-tolerated adjuvant formulations for camel immunisation.

Materials and Methods

Animals

Sixteen dromedaries of different age and gender were selected for this experiment. These were housed at the Central Veterinary Research Laboratory in Dubai, UAE. The animals were kept in outdoor pens with shaded areas and received good quality hay *ad libitum* and 2 kg of mixed concentrate (wheat bran, alfalfa pellets, mixed seed pellets) daily as well as access to automatic drinkers.

Study Adjuvants

1. Advax HCXL (Delta inulin-based adjuvant) was developed by Vaxine Pty Ltd, Australia especially for use in horses and camels. It is an immune-stimulatory particulate adjuvant made from microcrystalline delta inulin, a natural plant derived polysaccharide consisting of linear chains of fructose with a terminal glucose together with a proprietary synthetic immuno-stimulatory oligonucleotide (400µg/ml).
2. VAX-SPL-1311-06 (Adjuvant Formulation 1)– This novel adjuvant formulation was developed by Vaxine Pty Ltd, Australia and contains a suspension of delta inulin (50mg/ml) plus purified Quillaja Saponaria Molina extract (200µg/ml) (Vetsap™, Desert King International, San Diego, USA).
3. VAX-SPL-1311-07 (Adjuvant Formulation 2) – This novel adjuvant formulation was developed by Vaxine Pty Ltd, Australia and contains purified polysaccharide suspension of delta inulin (50mg/ml), Quillaja Saponaria Molina extract (200µg/ml) plus synthetic immune-stimulatory oligonucleotide (400µg/ml).
4. Imject alum –was purchased from Thermo Scientific, USA and contains an aqueous solution of aluminum hydroxide and magnesium hydroxide.

All the adjuvants were stored at conditions recommended by the manufacturers. The first three adjuvants were stored at 4-8°C and Imject alum was kept at room temperature.

Antigens selected for the study

The bacterial strains used for antigen preparation included 3 formalin killed bacterial strains: *Burkholderia mallei (B. mallei), Corynebacterium pseudotuberculosis (C. pseudotuberculosis)* and *Clostridium perfringens (C. perfringens)* and an inactivated viral strain: *Rhinovirus* (Equine rhinitis A virus (ERA)) (Wernery *et al,* 2008). *B. mallei and C. pseudotuberculosis* antigens contained bacterial cell wall protein units of lipopolysaccharide (LPS) and phospholipase D (PLD) (Walter, 2007), respectively. *C. perfringens* antigen, a bacterial toxoid antigen, contained formalin-killed bacteria and filter sterilised active *C. perfringens* alpha toxin. The antigen preparation for the bacterial strains was done based on standard protocols. Inactivation of *Rhinovirus* was carried out with 0.001M binary ethyleneimine and the antigen used in this trial had a titre of 1.0×10^6 TCID/ml. *B. mallei* and Rhinovirus were given as pooled antigen (antigen 1). *C. pseudotuberculosis* (antigen 2) and *C. perfringens* (antigen 3) were administered individually.

All antigens were stored at 4-8°C and checked for sterility prior to use.

Vaccine preparation

Adjuvants and antigens were removed from their respective storage conditions for vaccine preparation. *B. mallei* and *Rhinovirus* (antigen 1) were mixed in the ratio 1:2 and given as pooled antigen. This ratio was selected to counteract the weaker immunogenicity of *Rhinovirus. C. pseudotuberculosis* (antigen 2) and *C. perfringens* (antigen 3) were administered individually. The antigens and adjuvants were mixed in the ratio 1.5 : 0.5ml, respectively. The mixtures were prepared in sterile Greiner tubes and then thoroughly mixed by vortexing. An injection volume of 2ml was drawn into 5ml injection syringes and left overnight at 4°C before use the following day.

Camel immunisation protocol

Prior to immunisation, blood samples were collected from all 16 dromedaries and analysed for antibody titres against *B. mallei, C. pseudotuberculosis, C. perfringens* and *Rhinovirus*. Based on the results of antibody titre, the 16 camels were allocated into four groups, so that each group contained 1 or 2 camels with negative or weak antibody titres against all the antigens included in the trial. The camel selections for this trial and their pre-study antibody titres are outlined in Table 1. Each group (4 camels) received one adjuvant and all 16 camels received all 3 antigens (Table 2). The antigen- adjuvant mix were administered on different sites on either side of the neck or shoulder region.

The dromedaries were immunised at weekly intervals with each antigen-adjuvant mix. At the start

of the trial period camels received antigen 1 followed by antigen 2 and antigen 3 on the successive weeks. At each immunisation, the respective antigen was mixed with one of the 4 adjuvants. The camels also received one booster dose 3 weeks post-primary immunisation with the respective antigens. The reactogenicity of each antigen alone and adjuvant alone formulation was also investigated. For this reason four camels in each group received the respective adjuvants alone (0.5 ml each) on the day of booster dose with last antigen of *C. perfringens*. A week later 5 camels (Group 1 camels and one camel from Group 4) were injected with *B. mallei* antigen (1.5 ml each) alone. Similarly, Group 2 camels and a second camel from Group 4 were injected with *C. pseudotuberculosis* antigen (1.5ml each) alone and Group 3 camels and the third camel from Group 4 were injected with *C. perfringens* antigen alone (1.5 ml each) (Table 2).

For vaccination, individual camels had their heads restrained and tied to a fence. A side of the neck was shaved to obtain an injection window. Near to the injection window, another area was shaved to obtain a control window (Fig 1). Before injection, temperature of the injection window and control window was measured using the infrared camera. A control window was included for temperature

Table 1. Separation of camels into groups according to their pre-study antibody titres and adjuvant administered.

Group 1: Adjuvant-Advax					**Group 2: Adjuvant-Formulation 1**				
Camel ID (gender)	***B. mallei***	***C. pseudotuberculosis***	***C. perfringens***	**Rhino-virus**	**Camel ID (gender)**	***B. mallei***	***C. pseudotuberculosis***	***C. perfringens***	**Rhino-virus**
91F (f)	neg	pos 4+	Neg	neg	A44 (f)	neg	pos 4+	neg	neg
54A (m)	neg	pos 3+	pos 3+	neg	DBO (m)	neg	pos 3+	pos 3+	Neg
O5E (f)	neg	pos 3+	pos 4+	pos 1:>128	CDE (f)	neg	pos 3+	pos 4+	pos 1:>128
610 (f)	neg	neg	pos 1+	neg	Max (m)	neg	pos 1+	pos 2+	neg
Group 3: Adjuvant -Formulation 2					**Group 4: Adjuvant - Imject Alum**				
Camel ID (gender)	***B. mallei***	***C. pseudotuberculosis***	***C. perfringens***	**Rhino-virus**	**Camel ID (gender)**	***B. mallei***	***C. pseudotuberculosis***	***C. perfringens***	**Rhino-virus**
973 (f)	neg	pos 4+	pos 1+	neg	782 (f)	neg	pos 2+	pos 4+	Neg
6A5 (m)	neg	pos 3+	pos 3+	pos 1:128	E2A (f)	pos 2+	pos 3+	pos 1+	Neg
Roy (m)	neg	pos 2+	Neg	neg	355 (m)	neg	pos 3+	neg	Neg
F7B (f)	neg	pos 1+	pos 3+	pos 1:64	OCF (m)	neg	pos 2+	pos 2+	Neg

Table 2. Immunisation protocol.

Camel groups	**Immunisation inoculum**		**Control inoculum**	
	Adjuvant 0.5ml	**Antigen 1.5ml**	**Adjuvant only (0.5ml)**	**Antigen only (0.5ml)**
Group 1 camels				
91F 54A O5E 610	Advax	Antigen 1, 2 and 3	Advax	*B. mallei*
Group 2 camels				
A44 DBO CDE MAX	Adjuvant Formulation 1	Antigen 1, 2 and 3	Formulation 1	*C. pseudotuberculosis*
Group 3 camels				
973 6A5 ROY F7B	Adjuvant Formulation 2	Antigen 1, 2 and 3	Formulation 3	*C. perfringens*
Group 4 camels				
782 E2A 355 OCF	Imject Alum	Antigen 1, 2 and 3	Imject Alum	*B. mallei* *C. pseudotuberculosis* *C. perfringens* *None*

measurement to compensate for daily fluctuation in skin temperature of each camel. The skin thickness of each injection window was measured using calipers and recorded. After the measurements, the centre of the injection window was cleaned with 70% alcohol and circled with a marker pen to show the injection site. Camels were inoculated subcutaneously within the circled area at the centre of the shaved area. All injections were carried out at different areas on either side of neck and shoulder region following the same procedure mentioned above.

Measurements of inflammatory responses

Camels were assessed for inflammatory responses daily for 6 days post primary and booster vaccination. Following the manufacturer's instructions, skin temperature of camels was measured using InfRec InfraRed Camera, Thermo Gear, G100/G120, Nec Avio Infrared Technologies Company, Ltd. The infrared pictures were analysed using the software programme InfRec Thermography Studio (Fig 2). Skin thickness of injection site was measured for 6 days post primary and booster dose of inoculants using a digital caliper (manufactured by Hauptner, Herberholz, Germany) capable of measuring skin thickness less than 1mm to maximum 35 mm. The increased skin thickness of the injection site as compared to thickness of skin before injection was measured using caliper and recorded. Presence or absence of any lumps, swollen areas, hardened or tight skin at injection sites were monitored throughout this period and recorded. Lump size was measured with a simple mathematical ruler and expressed in cm^2 area. Fig 3 shows one of the lumps developed by a camel in this trial. After 6 days all camels were re-checked weekly for a total of 1 month to evaluate severity of skin lumps or whether the lumps had receded.

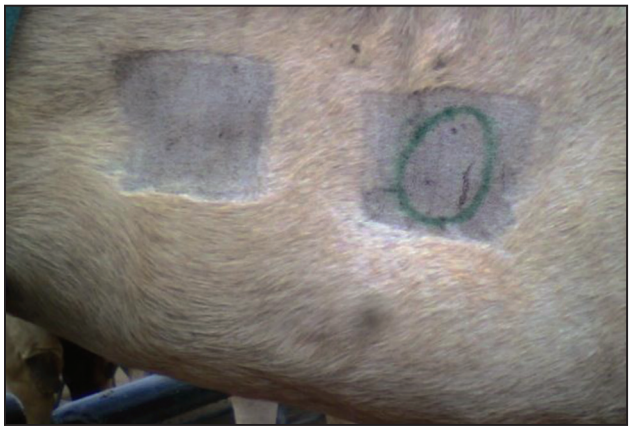

Fig 1. Control window and injection window(circled in green).

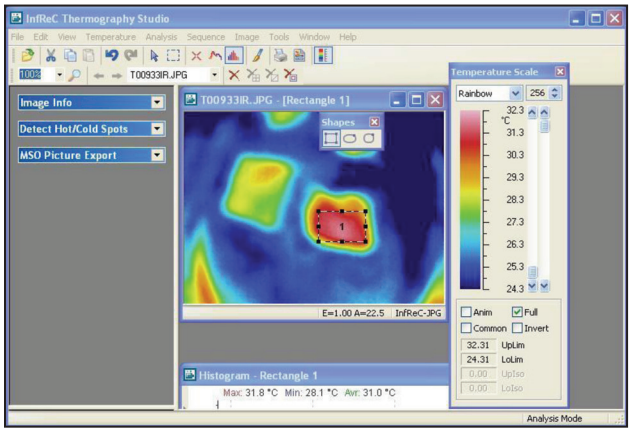

Fig 2. Screen shot of InfRec Thermography studio software programme. The infrared picture shows the control window (coloured yellow) and the injection window (coloured red). On right hand side is temperature scale. The area to be measured is selected and the software gives the temperature average of the area.

Blood collection and antibody detection

Following immunisation blood samples were collected weekly for a total of 8 weeks from the jugular vein into Vacutainer tubes. Blood samples were analysed for rise in antibody production against injected antigens using standard commercially available and validated test kits. Antibodies for *B. mallei* were tested by complement fixation test (CFT) and *Rhinovirus* by virus neutralisation test (VNT). Antibodies to *C. pseudotuberculosis* were tested by ELISA kit, ELITEST CLA, Hyphen Biomed, France and *C. perfringens* by Bio-X Alpha Toxin (C. perf) ELISA Kit Sero, Belgium.

Results

Mean values for increase in skin thickness and rise in skin temperature caused by each vaccine formulation were recorded for 6 days. Figs 4a, b – 8a, b show the skin thickness and temperature after

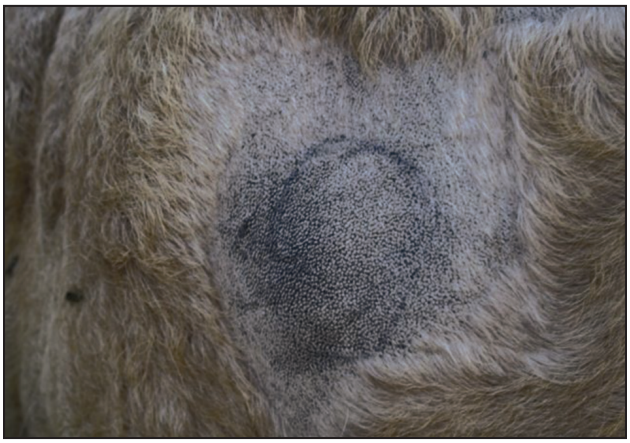

Fig 3. A significant lump at injection site.

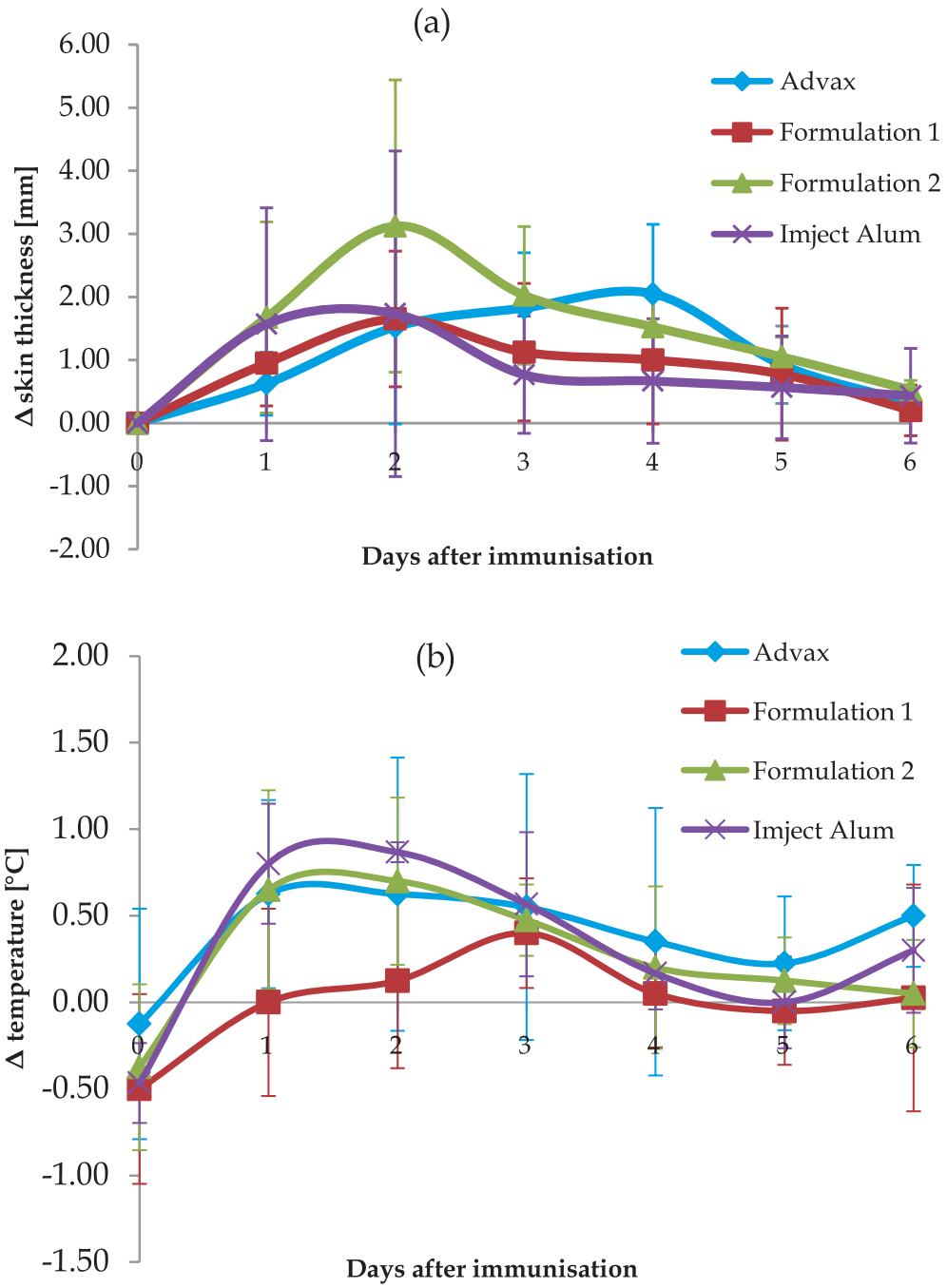

Fig 4a, b. Mean results of changes in injection site skin thickness (a) and temperature (b) after subcutaneous injection of different adjuvants.

administration of adjuvants alone, antigens alone and primary and booster immunisation of antigens with adjuvants. For the primary and booster immunisation, 4 different adjuvants were tested with 3 different antigens (2 bacterial antigens and one a mix of viral and bacterial antigens). The maximum lump size developed by each adjuvant after primary and booster immunisation with the 3 antigens, measured over a period of 4 weeks are shown in Figs 6c–8c.

Fig 4a shows the change in skin thickness after subcutaneous injection of adjuvants alone without antigen. There was a high degree of variability between animals and hence no significant difference was seen between the different adjuvants in terms of skin reactions, with all adjuvants being well tolerated. Maximal skin thickness was seen 2 to 4 days after injection and largely had returned to normal in the majority of animals by day 6.

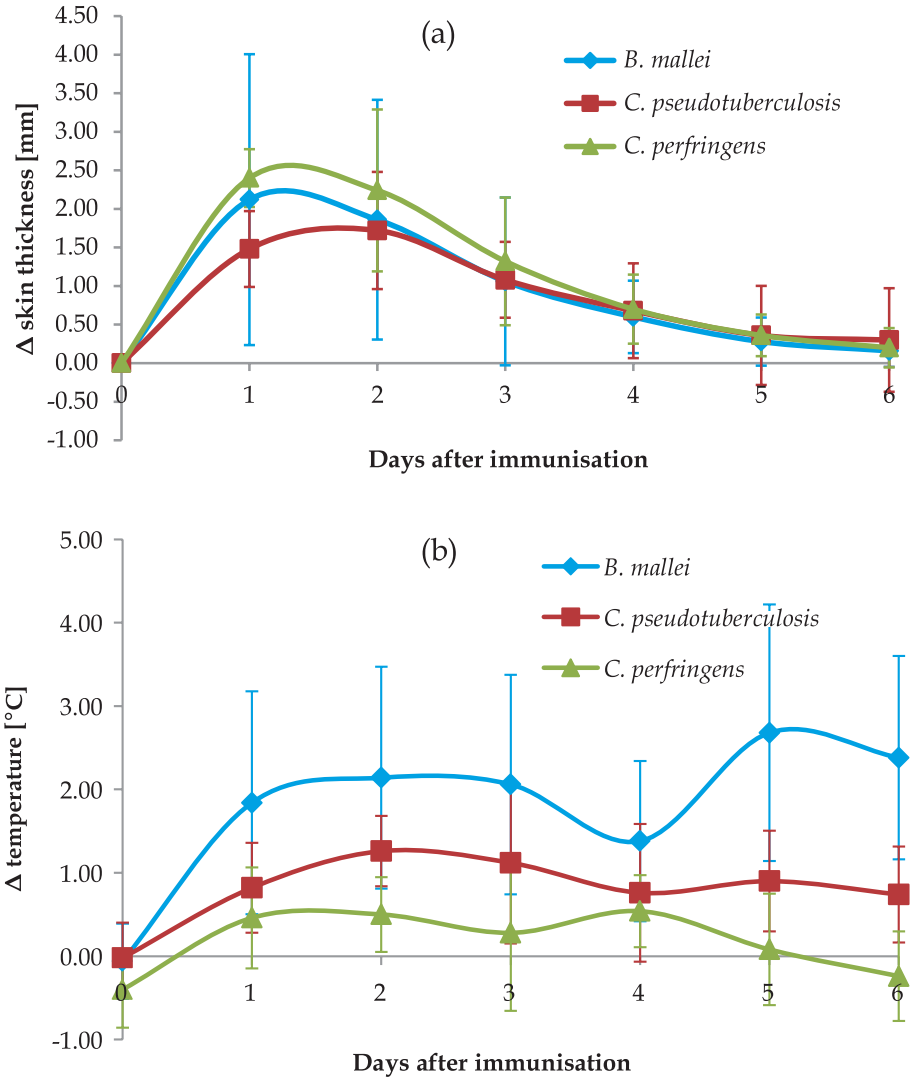

Fig 5a, b. Mean results of skin thickness (a) and temperature (b) after subcutaneous injection of antigens alone.

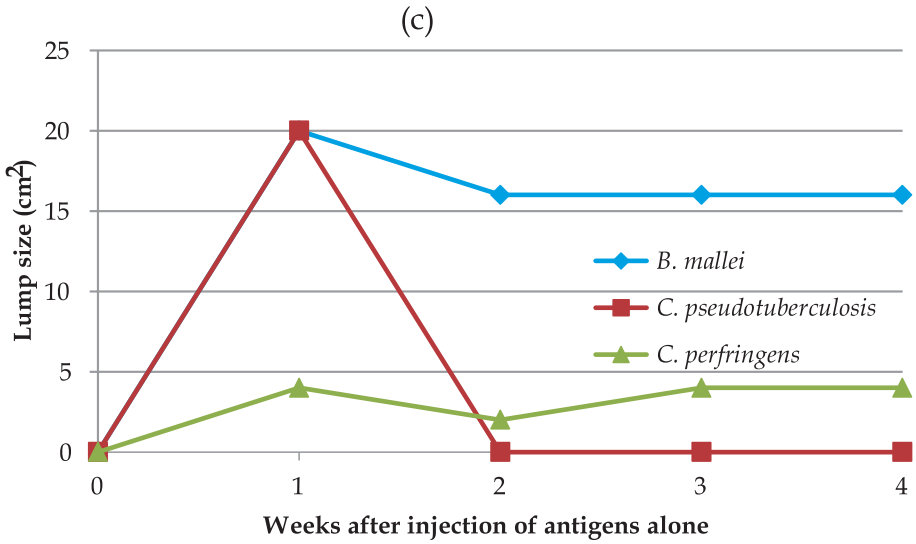

Fig 5c. Maximum lump size in cm^2 after subcutaneous injection of antigens alone.

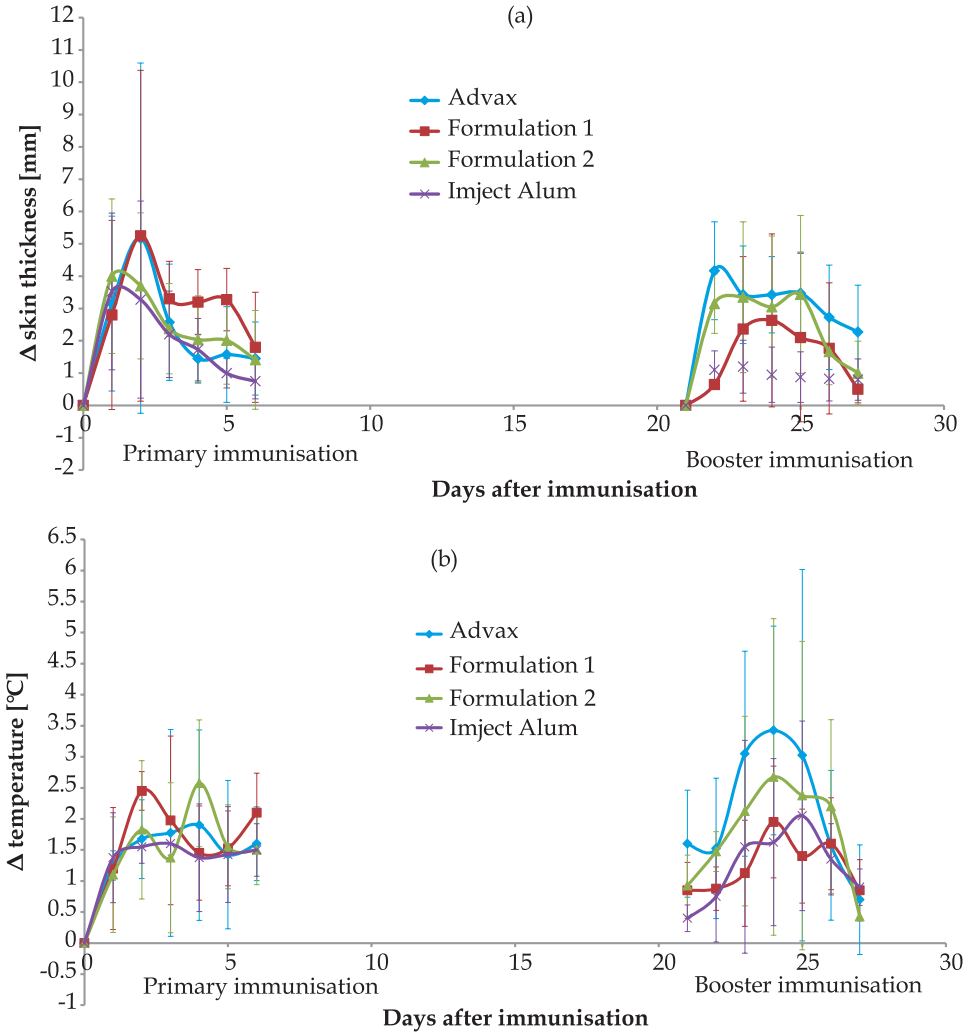

Fig 6a, b. Mean results of skin thickness (a) temperature (b) after pooled *B. mallei* and *Rhinovirus* subcutaneous injection (primary and booster dose).

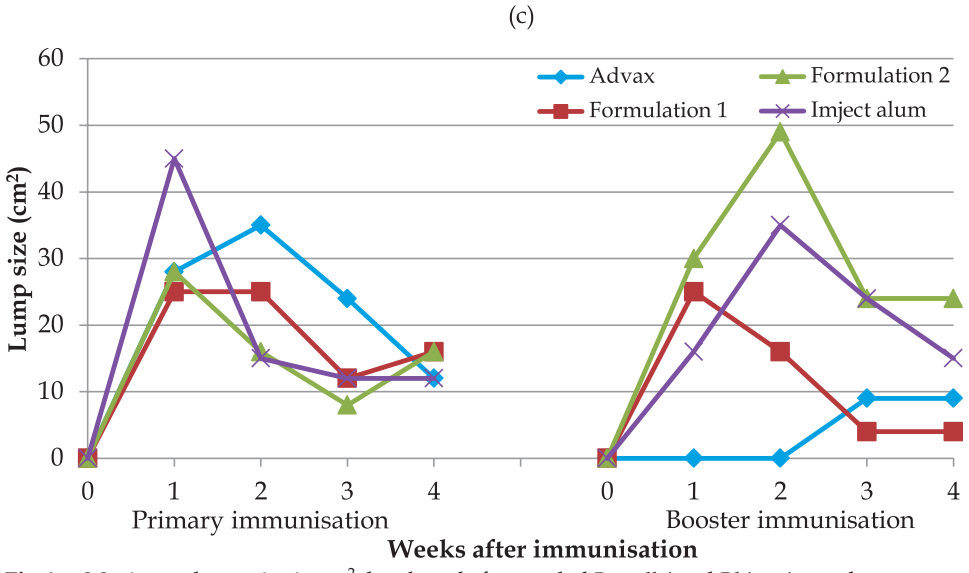

Fig 6c. Maximum lump size in cm^2 developed after pooled *B. mallei* and *Rhinovirus* subcutaneous injection (primary and booster dose).

Fig 4b shows the change of skin temperature after subcutaneous injection of adjuvants alone without antigen. A minor rise in skin temperature to a maximum of 0.87±0.05°C (mean±SD) was seen at 24-48 hrs in all adjuvant groups except for the animals receiving Adjuvant Formulation 1, which overall had the least elevation in skin temperature across all time points. The skin reactions largely reverted to near normal by day 5 in all groups.

No lumps, hardening or swelling of the injected area was observed in at any of the sites of adjuvant injection, consistent with all tested adjuvant formulations being well tolerated when injected subcutaneously in camels.

Fig 5a, b shows the mean result of skin reaction after subcutaneous injection of the different antigen formulations without adjuvant. A similar pattern of increased skin thickness maximal at 24 hrs post-immunisation was seen for all antigen formulations, which gradually reverted to normal over 6 days. Minor differences in post-immunisation skin thickness were seen between different antigens. Maximally increased skin thickness of 2.40±0.37mm (mean±SD) was caused by *C. perfringens* antigen at 24 hrs followed closely by *B. mallei* and *C. pseudotuberculosis* (Fig 5a).

However, significant differences in skin temperature changes post-immunisation occurred between the 3 antigen formulations (Fig 5b). *B. mallei* [2.68±1.53°C (mean±SD)] was the most reactogenic antigen whereas *C. perfringens* [0.54±0.43°C (mean±SD)] was the least reactogenic. The temperature of the injection sites reverted to normal within 6 days for the *C. pseudotuberculosis* and *C. perfringens* antigens, but not with *B. mallei* antigen where the skin temperature at the injection site remained elevated 2.38±1.21°C (mean±SD) even after 6 days (Fig 5b).

Fig 5c shows the maximum lump size developed in camels after subcutaneous injection of antigens alone for a period of 4 weeks. Notably, of the 5 camels injected with *B. mallei* antigen, 4 camels produced significant lumps varying in size between 9 cm^2 to 20 cm^2 and 1 camel had a hard and swollen injection site. Two of the camels injected with *C. pseudotuberculosis* developed lumps of sizes 9 cm^2 and 20 cm^2 respectively, on week 1, but receded by week 4. No skin lumps were observed with *C. perfringens* antigen in 3 camels; however, 2 camels developed swollen injection sites varying in size between 2 cm^2 to 4 cm^2. These skin conditions remained unchanged over the 4 weeks of observation.

Fig 6a, b shows skin reactions after injecting pooled *B. mallei* and *Rhinovirus* with 4 different adjuvants (primary and booster dose). Significant increase in skin reactions were observed within 48hrs after primary and booster immunisation of pooled antigen with all the adjuvants. There were only minor non-significant differences in skin thickness between the different adjuvants mixed with pooled antigen after primary immunisation and booster dose except for imject alum which showed low increase in skin thickness after booster dose (Fig 6a).

There was not much variation in temperature increase between adjuvants after primary immunisation and a non-significant trend for less temperature rise with Imject alum and Adjuvant Formulation 1 after the booster dose was seen (Fig 6b).

Fig 6c shows the maximum lump size developed with each adjuvant injected with pooled *B. mallei* and *Rhinovirus* antigen over a period of 4 weeks after primary and booster immunisation. Lumps of varying size were observed with pooled *B. mallei* and *Rhinovirus* antigen regardless of adjuvant, suggesting this was more a property of the pooled antigen than the adjuvant.

Fig 7a, b shows the skin reactions after injecting *C. pseudotuberculosis* antigen with the different adjuvants. After primary and booster immunisation there was significant variation in skin thickness between the different adjuvants, although due to high variation between animals none of these differences were statistically significant. However, there was a trend for Imject alum to have slightly lower skin thickness than the other adjuvant formulations after primary and booster immunisations (Fig 7a). There was not much variation in temperature increase between adjuvant combinations after the primary immunisation but after the booster dose adjuvant Adjuvant Formulation 2 gave the higher increase in skin temperature with Imject alum tending to be associated with the least increase in skin reactions although this was not statistically significant (Fig 7b).

Interestingly, although it tended to be associated with the greatest skin thickness and temperature, none of the four camels injected with *C. pseudotuberculosis* with Adjuvant Formulation 1 developed lumps at the injection site. One camel in each other group of Advax HCXL, Adjuvant Formulation 2 and Imject alum developed a lump after primary immunisation. After the booster dose, 2 camels developed lumps with Advax HCXL and 3

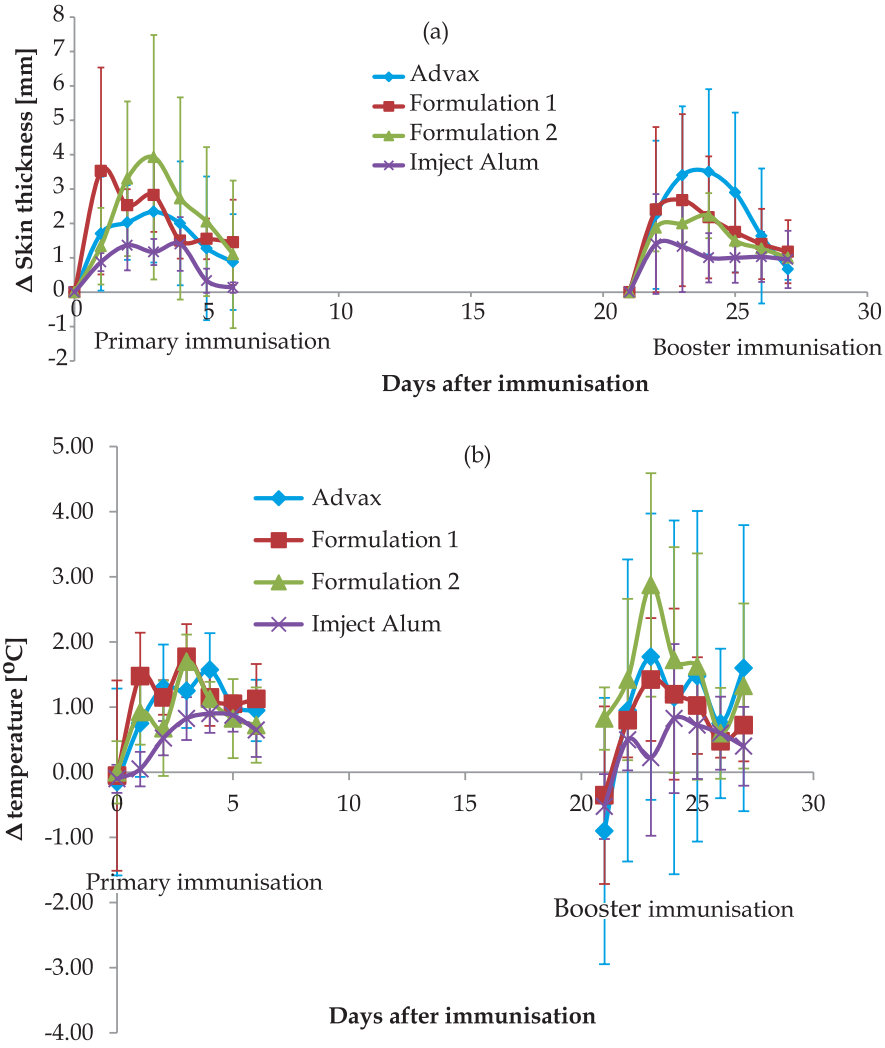

Fig 7a, b. Mean results of skin thickness (a) and temperature (b) after *C. pseudotuberculosis* subcutaneous injection (primary and booster dose).

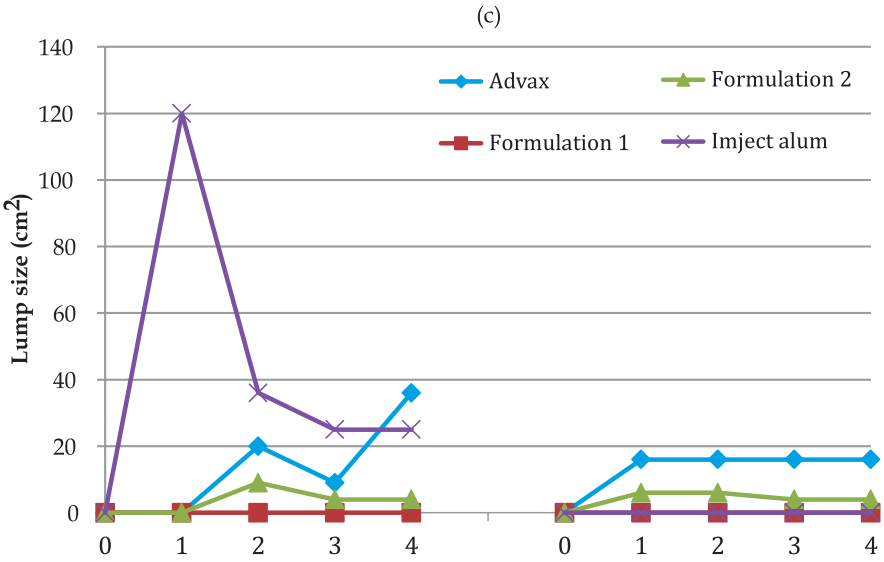

Fig 7c. Maximum lump size in cm2 developed after *C. pseudotuberculosis* subcutaneous injection (primary and booster dose).

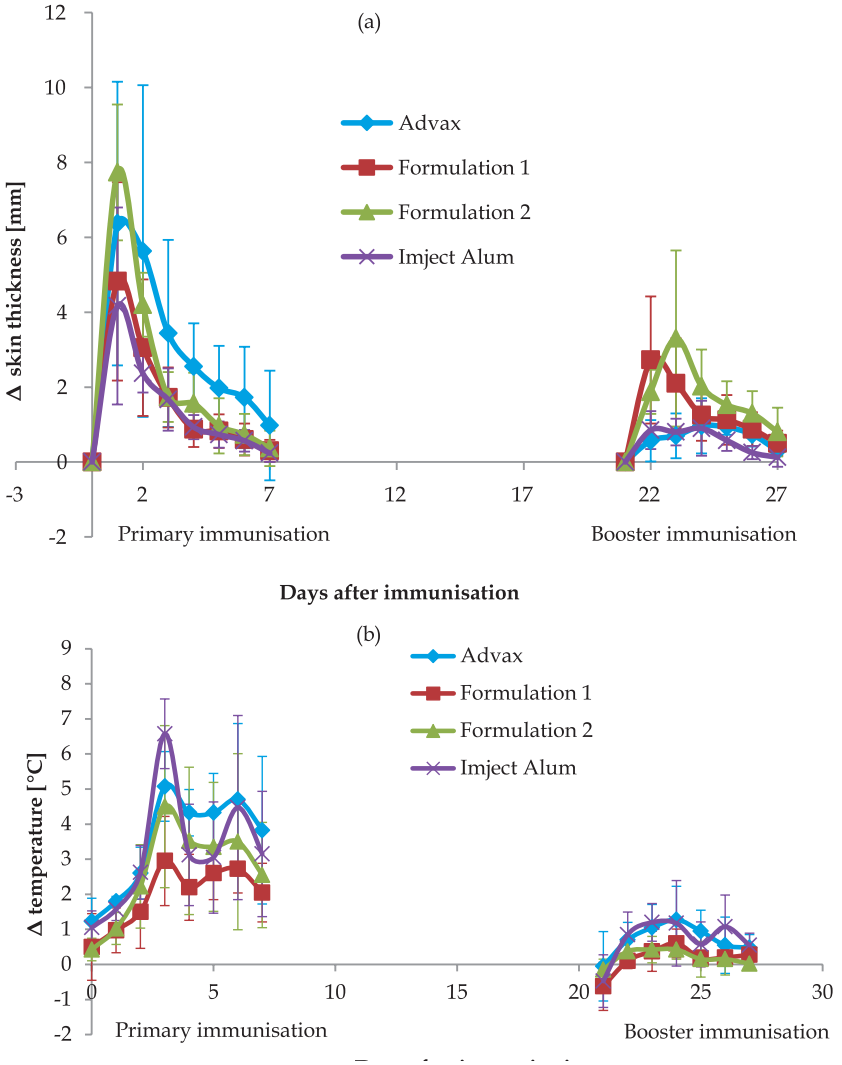

Fig 8a, b. Mean results of skin thickness (a) and temperature (b) after *C. perfringens* subcutaneous injection (primary and booster dose)

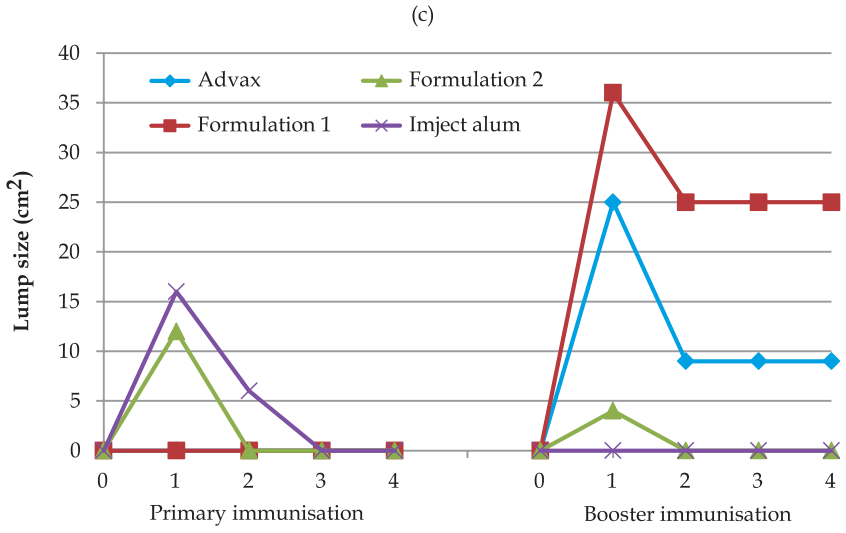

Fig 8c. Maximum lump size in cm^2 developed after *C. perfringens* subcutaneous injection (primary and booster dose)

camels with Adjuvant Formulation 2. Fig 7c shows the maximum lump size developed by each adjuvant. Lump size was greater for camels injected with Advax HCXL and Imject alum (48 cm^2 and 36 cm^2, respectively) as compared to the smaller lump size (9 cm^2) with Adjuvant Formulation 2.

Fig 8a, b shows skin reactions after injecting *C. perfringens* with the different adjuvants. Following primary vaccination with *C. perfringens*, there was significant increase in skin thickness to a maximum of 7.73±1.81mm (mean±SD) within 24 hrs and a significant rise in temperature to a maximum of 6.57±0.990C (mean±SD) of injection site within 72 hrs. This local rise in skin thickness was greatest for Adjuvant Formulation 2 and least for Alum (Fig 8a) and rise in skin temperature was greatest for Alum and least for Adjuvant Formulation 1 (Fig 8b). Unlike the pattern seen with the other antigens, the skin reaction to *C. perfringens* vaccine was much lower after the booster dose in all groups with a maximum increase in skin thickness of 3.30±2.34mm (mean±SD) and a maximum increase in skin temperature of 1.28±0.95°C (mean±SD).

Fig 8c shows the maximum lump size developed after *C. perfringens* subcutaneous injection. None of the camels developed lumps after primary immunisation with Advax HCXL or Adjuvant Formulation 1. However, after the booster dose, 1 camel in each group developed a lump of size 25 cm^2 and 36 cm^2, respectively at week 1 and sizes 9 cm^2 and 25 cm^2, respectively at week 2, 3 and 4. The lump, developed by 1 camel after primary and booster dose with Adjuvant Formulation 2, receded after 14 days. While Imject alum caused lumps after primary immunisation, it did not cause any lumps after the booster dose.

Rise in antibody titres after immunisation

The results of rise in serum antibody level after immunisation are shown in Tables 3a, b, c, d - 6a, b, c, d. Serum samples from each camel were checked weekly for 4 weeks after primary and booster immunisation.

Pooled antigen (*B. mallei* and Rhinovirus) administered with Advax HCXL (Table 3a, 3b): Only 1 camel (ID 610) showed weak antibody production against *B. mallei*, 1 week after booster dose. Camel (ID 05E) with an existing titre of 1:256 for *Rhinovirus* before immunisation, showed an increase in titre 1:1024 in 2 weeks but dropped back to 1:256, 4 weeks after booster immunisation. Weak antibody production (1:8) against *Rhinovirus* was shown by a second camel (ID 91F) 3 - 6 weeks after immunisation but became negative on week 7.

***Corynebacterium pseudotuberculosis* administered with Advax HCXL (Table 3c):** Except for 1 camel (ID 610), 3 camels immunised with *C. pseudotuberculosis* antigen had high titre of antibodies against *C. pseudotuberculosis* before immunisation and hence it was not possible to measure a further rise in titre. However, serum samples have been analysed and results showed maximum detection limit of the ELISA test method. The camel (ID 610) which had no antibodies to *C. pseudotuberculosis* before immunisation showed a gradual increase in antibody titre from a low titre in week 1 to a high titre in week 6.

Clostridium perfringens administered with Advax HCXL (Table 3d): Of the 4 camels immunised with *C. perfringens*, 2 of them had high titre of antibodies against *C. perfringens* before immunisation, 1 was negative (ID 91F) and the last one had low titre for *C. perfringens* antibodies (ID 610). The serum samples analysed from the strong positive cases gave results with the maximum detection limit of the ELISA test method. The sample from the camel without antibody before immunisation (ID 91F) became positive for antibodies after immunisation, but only a low rise in titre was observed. The weak antibody titre seen in one camel (ID 610) did not increase significantly after immunisation.

Pooled antigen (*B. mallei* and Rhinovirus) administered with Adjuvant Formulation 1 (Table 4a, 4b): Of the 4 camels, only 1 camel (ID CDE) developed antibodies against *B. mallei* after 3 weeks. None of the camels produced antibody to *Rhinovirus* or showed an increase in titre after immunisation with Rhinovirus.

***Corynebacterium pseudotuberculosis* administered with Adjuvant Formulation 1 (Table 4c):** 3 camels were strong positive for *C. pseudotuberculosis* antibodies and their serum analysis showed maximum detection limit of the ELISA test method. The fourth camel (Max) had weak antibody titre and did not increase after immunisation.

***C. perfringens* administered with Adjuvant Formulation 1 (Table 4d):** 2 camels were strong positive for *C. perfringens* antibodies and their serum analysis showed maximum detection limit of the ELISA test method. The camel with weak antibody titre (Max) showed a very good increase in antibody titre 2 weeks after immunisation. The camel which was negative for antibody titre (A44) also developed antibodies in just 2 weeks after immunisation.

Table 3a. *B. mallei* antibody titres in Group 1 camels (Advax HCXL group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
91F (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
54A (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
O5E (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
610 (titre)	Neg.	Neg.	Neg.	Neg.	Pos. 1:5 +++	Pos. 1:20 +++	Pos. 1:20 +++	Pos. 1:10 ++

Table 3b. *Rhinovirus* antibody titres in Group 1 camels (Advax HCXL group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
91F (titre)	Neg.	Neg.	Neg.	Pos 1:8	Pos 1:8	Pos 1:4	Pos 1:8	Neg.
54A (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
O5E (titre)	Pos 1:256	Pos 1:256	Pos 1:1024	Pos 1:1024	Pos 1:512	Pos 1:512	Pos 1:256	Pos 1:256
610 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Table 3c. *C. psedotuberculosis* antibody titres in Group 1 camels (Advax HCXL group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
91F (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	nd
54A (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	nd
O5E (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	nd
610 (titre)	Neg	Pos	Pos	Pos	Pos	Pos	Pos	nd

Table 3d. *C. perfringens* antibody titres in Group 1 camels (Advax HCXL group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
91F (titre)	Neg	Neg	Pos++	Pos++	Pos++	Pos+	nd	nd
54A(titre)	Pos +++	Pos +++	Pos +++	Pos +++	Pos +++	Pos +++	nd	nd
O5E(titre)	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos +++	nd	nd
610(titre)	Pos +	Neg	Pos ++	Pos ++	Pos ++	Pos +	nd	nd

Table 4a. *B. mallei* antibody titres in Group 2 camels (Adjuvant Formulation 1 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
A44 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
DBO (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
CDE (titre)	Neg.	Neg.	Neg.	POS 1:10 ++++	POS 1:10 ++++	POS 1:40 ++++	POS 1:40 ++++	POS 1:20 ++++
MAX (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg

Table 4b. *Rhinovirus* antibody titres in Group 2 camels (Adjuvant Formulation 1 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
A44 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
DBO (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
CDE (titre)	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256
MAX (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Table 4c. *C. psedotuberculosis* antibody titres in Group 2 camels (Adjuvant Formulation 1 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
A44 (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos
DBO(titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos
CDE(titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos
MAX(titre)	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Table 4d. *C. perfringens* antibody titres in Group 2 camels (Adjuvant Formulation 1 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
A44 (titre)	Neg.	Neg.	Pos +	Pos +	Pos ++	Pos +	nd	nd
DBO(titre)	Pos +++	Pos +++	Pos +++	Pos ++++	Pos ++++	Pos +++	nd	nd
CDE(titre)	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos ++++	nd	nd
MAX(titre)	Pos ++	Pos ++	Pos +++	Pos +++	Pos +++	Pos +++	nd	nd

Table 5a. *B. mallei* antibody titres in Group 3 camels (Adjuvant Formulation 2 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
973 (titre)	Neg.	Neg.	Neg.	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos +++
6A5 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
ROY (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
F7B (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Table 5b. *Rhinovirus* antibody titres in Group 3 camels (Adjuvant Formulation 2 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
973 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
6A5 (titre)	Pos 1:128	Pos 1:128	Pos 1:512	Pos 1:512	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256
ROY (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
F7B (titre)	Pos 1: 64	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256	Pos 1:256

Table 5c. *C. psedotuberculosis* antibody titres in Group 3 camels (Adjuvant Formulation 2 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
973 (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos
6A5 (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos
ROY (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos
F7B (titre)	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Table 5d. *C. perfringens* antibody titres in Group 3 camels (Adjuvant Formulation 2 group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
973 (titre)	Pos +	Neg.	Pos +	Pos +	Pos ++	Neg.	nd	nd
6A5 (titre)	Pos +++	Pos +++	Pos +++	Pos ++++	Pos ++++	Pos +++	nd	nd
ROY (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	nd	nd
F7B (titre)	Pos+++	Pos++	Pos +++	Pos +++	Pos +++	Pos +++	nd	nd

Pooled antigen (*B. mallei* and *Rhinovirus*) administered with Adjuvant Formulation 2 (Table 5a, 5b): Only 1 camel (ID 973) became positive for *B. mallei* antibodies after immunisation. The other 3 camels remained negative. The 2 camels (ID 6A5, F7B) with pre-existing antibodies against Rhinovirus showed rise in titre. The camels which were negative for Rhinovirus antibodies remained negative throughout the trial.

***Corynebacterium pseudotuberculosis* administered with Adjuvant Formulation 2 (Table 5c):** No change in *C. psedotuberculosis* antibodies was observed after immunisation. However, 3 camels developed lumps, 1 after primary immunisation and 2 after booster dose.

***C. perfringens* administered with Adjuvant Formulation 2 (Table 5d):** No change in *C. perfringens* antibodies was observed after immunisation.

Pooled antigen (*B. mallei* and *Rhinovirus*) administered with Imject Alum(Table 6a): From the 3 camels which were negative for *B. mallei*, 2 developed antibodies against *B. mallei* and 1 remained negative (ID 355, OCF). The one camel with weak titre (ID 782) showed a rise in antibody production. All camels were negative for Rhinovirus antibody and remained negative after immunisation.

***Corynebacterium pseudotuberculosis* administered with Imject Alum (Table 6c):** No change in *C. pseudotuberculosis* antibodies was observed after immunisation.

***C. perfringens* administered with Imject alum (Table 6d):** No change in *C. perfringens* antibodies was observed after immunisation in camels which had a low titre before immunisation. But, the camel which was negative (ID 355) developed antibodies 2 weeks after immunisation.

Discussion

There continues to be a paucity of data on suitable adjuvants for use with particular antigens for immunisation of camelids. We previously demonstrated that a novel polysaccharide adjuvant, Advax-HCXL, was effective in inducing high antibody titres against an inactivated African Horse sickness vaccine in camels, without the reactogenicity of other adjuvants (Eckersley *et al,* 2011). In this study we sought to extend the previous findings to see what would be the impact of Advax-HCXL on a variety of different bacterial and viral antigens, to see if its positive effects are generalisable across all antigens. At the same time we took the opportunity to test a

Table 6a. *B. mallei* antibody titres in Group 4 camels (Imject alum group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
782 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
E2A (titre)	Pos ++	in-conclusive	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos +	Pos +
355 (titre)	Neg.	Neg.	Neg.	Pos +++++	Pos ++++	Neg.	Pos +	dubious +++
OCF (titre)	Neg.	Neg.	Neg.	Pos ++++	Pos +++	Pos +	Pos +	Pos ++++

Table 6b. *Rhinovirus* antibody titres in Group 4 camels (Imject alum group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
782 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
E2A (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
355 (titre)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
OCF (titre)	Neg.	Neg.	Neg	Pos 1:8	Pos 1:8	Neg.	Neg.	Neg.

Table 6c. *C. psedotuberculosis* antibody titres in Group 4 camels (Imject alum group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
782 (titre)	Strong Pos	Pos	Pos	Pos	Pos	Pos	Pos	nd
E2A (titre)	Strong Pos	Pos	Pos	Pos	Pos	Pos	Pos	nd
355 (titre)	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	Strong Pos	nd
OCF (titre)	Strong Pos	Pos	Pos	Pos	Pos	Pos	Pos	nd

Table 6d. *C. perfringens* antibody titres in Group 4 camels (Imject alum group).

Camel ID	Before immunisation	Post-Primary Immunisation			Post-Booster Immunisation			
		1 week	2 week	3 week	1 week	2 weeks	3 weeks	4 weeks
782 (titre)	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos ++++	Pos ++++	nd	nd
E2A (titre)	Pos +	Pos+	Pos +	Pos +	Pos ++	Pos +	nd	nd
355 (titre)	Neg.	Neg.	Pos ++	Pos +++	Pos +++	Pos +	nd	nd
OCF (titre)	Pos++	Pos++	Pos++	Pos ++	Pos ++	Pos ++	nd	nd

number of alternative adjuvant formulations where additional immune-modulators notably a Quillaria saponin or a synthetic oligonucleotide or both were formulated with delta inulin microparticles. As a comparator adjuvant we used Imject alum. The results show the complexity of undertaking such studies, particularly in older camels that already have a high level of pre-existing immunity to the pathogens being immunised against. However, a number of conclusions can be made.

Firstly, all the adjuvant formulations tested were well tolerated, none causing severe lesions such as draining granulomas seen with other adjuvants such as oil emulsions (Eckersley *et al,* 2011). This relative lack of reactogenicity of all the adjuvants made it hard to separate them, and in fact when the adjuvants were tested by themselves without antigen, they all had negligible skin reactogenicity, suggesting most of the skin reactogenicity seen with the combined antigen and adjuvant formulations were in fact driven by the antigen component. In this study to better objectively measure the skin reactions we used an infrared camera to measure the temperature of the injection site, a technique to our knowledge has not been previously used in a camel vaccine study. This showed that there was a reasonable although not perfect concurrence between the increase in skin thickness post vaccination and the rise in skin temperature, consistent with both measuring a local increase in blood flow and oedema secondary to an immune reaction to the injected vaccine. Notably in almost all cases the increase in skin thickness and temperature subsided almost completely over 3-6 days post-immunisation, consistent with these adjuvant formulations being well tolerated and safe. Although some camels did develop non-tender lumps after vaccination these were sporadic with no consistent pattern after primary or booster immunisation or antigen or adjuvant group, suggesting these might be influenced by other factors, such as individual camel sensitivity to irritation and possible inoculation of skin bacteria etc. at the time of immunisation.

As previously noted, this study was primarily undertaken to test adjuvant safety and tolerability, rather than to assess the immunogenicity of specific vaccine antigens. However, as immune-pathology may occur where an excess immune reaction occurs to a specific vaccine, it was useful that some of the camels already had high antibody titres against some of the antigens being administered. This allowed us to assess whether this existing background immunity might be associated with increased vaccine reactogenicity. In fact, the data did not show any trends for increased vaccine reactogenicity in camels that had existing immunity to the vaccine antigen, suggesting this is not likely to be a major problem with camel vaccines.

The importance of this work is highlighted by the recent findings that young camels shedding virus may be a major vector transmitting MERS coronavirus to humans (Adney *et al,* 2014), leading to intensive efforts to develop an effective vaccine that could potentially be administered to young camels to prevent them becoming infective and transmitting the virus to humans who come in close contact. Such a camel vaccine will require an effective adjuvant.

Although in this and previous work, we have again confirmed the Advax™ delta inulin adjuvant system as amongst the safest and best tolerated adjuvant platforms available for camel (and horse) use, additional work is needed to better understand the camel immune system. This would allow further optimisation of adjuvant platforms for camels, thereby allowing development of more effective camel vaccines.

References

Abbas B and Agab H (2002). A review of camel brucellosis. Preventive Veterinary Medicine 55(1):47-56.

Adney DR, van Doremalen N, Brown VR, Bushmaker T, Scott D, de Wit E, Bowen RA and Munster VJ (2014). Replication and Shedding of MERS-CoV in Upper Respiratory Tract of Inoculated Dromedary Camels. Emerging Infectious Diseases 20(12):1999-2005. doi:10.3201/eid2012.141280.

Committee for Veterinary Medicinal Products (1997). The European Agency for the Evaluation of Medicinal

Products.Veterinary Medicines Evaluation Unit. VMP/IWP/043/97-Final.

Eckersley AM, Petrovsky N, Kinne J, Wernery R and Wernery U (2011). Improving the dromedary antibody response: The hunt for the ideal camel adjuvant. Journal of Camel Practice and Research 18(1):35-46

Walter U (2007). Protection in alpacas against *Corynebacterium pseudotuberculosis* using different bacterial components. Veterinary Microbiology 119(2-4):297-303.

Wernery U, Kinne J and Schuster RK (2014). Camelid Infectious Disorders, OIE Book.

Wernery U, Knowles NJ, Hamblin C, Wernery R, Joseph S, Kinne J and Nagy P (2008). Abortions in dromedaries (*Camelus dromedarius*) caused by equine rhinitis A virus. Journal of General Virology 89:660-666.

Wernery U, Fowler ME and Wernery R (1999). Color Atlas of Camelid Hematology. Blackwell Wissenschafts-Verlag Berlin, Germany.