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Peptide immunisation of guinea pigs against *Chlamydia psittaci* (GPIC agent) infection induces good vaginal secretion antibody response, *in vitro* neutralisation and partial protection against live challenge

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Running Title: Immunisation against *Chlamydia psittaci* GPIC agent (now termed *Chlamydophila caviae*) with synthetic peptide

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Abstract

Immunisation of female guinea pigs with a chimeric peptide consisting of variable domain IV (VDIV) and a region known as GP8 from the major outer membrane protein (MOMP) of *Chlamydomphila caviae*, formerly *Chlamydia psittaci* Guinea Pig Inclusion Conjunctivitis (GPIC) strain, was performed to assess whether humoral immune responses could be elicited in the reproductive tracts of immunised animals. The *C.caviae* strain is able to cause a sexually transmitted infection in the guinea pig that closely parallels *C. trachomatis* infections in humans. The best anti-VDIV antibody response in vaginal secretions was achieved by intraperitoneal priming with subsequent intravaginal boosting ($P<0.001$). Dot-blot analyses of vaginal secretions confirmed that these anti-VDIV antibodies, produced against a linear peptide, were able to recognise and bind to whole conformational *C. caviae* elementary bodies. Following live intravaginal challenge with *C. caviae*, a significant reduction in the intensity ($P=0.01$) and an apparent reduction in the duration of the infection was evident between the guinea pigs immunised with VDIV-GP8 and non-immunised controls.

Key Words: Peptide immunisation, *C.psittaci* (GPIC), vaginal secretion antibody, partial protection

Introduction

Chlamydia trachomatis is an obligate, intracellular, gram negative pathogen which causes infections at the mucosal surfaces of humans. Chlamydial infection of the human reproductive tract is of particular concern, as *C. trachomatis* is implicated in the development of pelvic inflammatory disease (PID) and salpingitis with subsequent infertility in females¹. Such damage is thought to occur as a result of deleterious host (hypersensitivity) responses against, as-yet, undefined chlamydial epitopes².

The development of a vaccine for sexually transmitted disease caused by *C. trachomatis* presents unique challenges. Delivery of whole *C. trachomatis* to humans has been shown to induce partial immunity against genital infection³. Unfortunately, whole chlamydial vaccines have also been shown to cause deleterious hypersensitivity responses in the host, resulting in more severe pathology compared to the natural disease⁴. As a result of these findings, research into chlamydial vaccines has centred on the development of subunit vaccines which contain protective epitopes, but exclude epitopes which produce pathological responses in the host.

The guinea pig model of genital infection with GPIC (Guinea Pig Inclusion Conjunctivitis strain of *C. psittaci*; recently re-named *Chlamydophila caviae*⁵) was chosen since this organism naturally infects the reproductive tract of guinea pigs and causes a disease which closely resembles *C. trachomatis* infections in humans. The similarities between the guinea pig and human chlamydial reproductive diseases include the mode of transmission⁶, vaginal target tissues⁷, histopathology,

immunology and infection sequelae, including the occurrence of salpingitis and infertility ⁸.

The chlamydial antigen chosen for this investigation was a chimeric linear peptide representing two distinct regions (variable domain IV - VDIV and a region known as GP8) of the major outer membrane protein (MOMP) of *C.psittaci* GPIC. The immunogenicity of these MOMP regions in GPIC has not previously been investigated, however, corresponding regions in *C. trachomatis* are known to elicit both B- and T- cell immune responses, with VDIV the target of antibody production, and GP8 providing T-cell help ⁹.

By using the guinea pig model of genital infection with the synthetic antigenic peptide, VDIV-GP8, we were able to examine both the immunogenicity of the VDIV and GP8 antigens and also to determine whether or not immunisation provides protective immunity *in vivo* against infection with the naturally occurring chlamydial agent.

Materials and methods

Experimental Animals

Sexually mature (600-700g), female, random outbred English short-haired (ROES) guinea pigs (Institute of Medical and Veterinary Sciences, Adelaide, SA, Australia), were housed individually in filter-top mini-isolators in a room kept at 21°C on a 12hour light and 12 hour dark cycle. Animals were fed standard guinea pig pellets (Norco Co-operative, Lismore, NSW) and tap water supplemented with 4mg/20L ascorbic acid (Melrose Laboratories, VIC) *ad libitum*. All work undertaken complied with relevant Queensland University of Technology Biomedical Ethics Committee guidelines (BEC # 875/1A).

Peptides

The oligopeptides (synthesised and conjugated by Chiron Mimotopes, Melbourne, VIC, Australia) were provided at 70% purity and were quantitated by Mass Spectral analysis (Perkin-Elmer Sciex API III) and also confirmed as containing the target peptide by MS analysis.

The synthesised peptide GP8 contained a predicted T_H-cell epitope (Roger Rank, pers.comm.) and corresponded to GPIC MOMP residues 106-130 (WDRFDVFCTLGASNGYKANAFAFN). Peptide VDIV corresponded to GPIC MOMP residues 285-315 (PTAILNLTWNPTLLGEATTINTGAKYADQL). The VDIV and GP8 oligopeptides were synthesised individually and a mixture of the two peptides was conjugated at both termini (GP8 at the amino terminus and VDIV at the carboxy terminus) to the carrier molecule Keyhole Limpet Haemocyanin (KLH). The synthetic, conjugated oligopeptide (i.e. VDIV and GP8 peptides linked to KLH) was

used for all immunisations in this study and is, hereafter, referred to as VDIV-GP8 peptide.

Cell culture and detection of *Chlamydia*

C. psittaci GPIC was cultured in McCoy cell monolayers¹⁰ using Dulbecco's modified Eagle's medium (DMEM) (Commonwealth Serum Laboratories, Australia), supplemented with 10% fetal calf serum (Gibco/BRL). GPIC inoculum containing 2×10^6 inclusion forming units/mL (IFU/mL) was added onto 1×10^7 McCoy cell monolayers before centrifugation at 850g for 1 hour at room temperature. The infected cells were incubated (36°C/5%CO₂) for 2 hours before the addition of 5 mL DMEM with 1µg/mL cyclohexamide (Sigma Biochemicals). The media was replaced (DMEM with 1µg/mL cyclohexamide) at 24 hours post-infection and the level of infection analysed by fluorescent monoclonal antibody staining using an FITC-conjugated monoclonal antibody against chlamydial lipopolysaccharide (Chlamydia-cel LPS kit, Cellabs Diagnostics). Cultures were frozen at -80°C, diluted 1:1 in sucrose-phosphate-glutamate (SPG) buffer until use. *C. caviae* elementary bodies (EBs) were purified on a renografin gradient¹¹.

Specimen Collection

The guinea pigs were anaesthetised with 0.75-1.0 mL/kg Hypnorm by intraperitoneal injection prior to collection of serum and vaginal secretions. Vaginal smears were collected by insertion of a sterile cotton swab (Disposable Products, S.A) approximately 2cm into the vagina with vigorous rotation against the vaginal wall and cytological smears rolled onto glass before immediate fixation with Cytospray (SEA Trading Pty.Ltd., QLD, Australia). Smears taken for assessment of chlamydia infection were air dried and fixed in methanol for 10 minutes. Vaginal secretions were

collected using surgical sponges (Weck-cel, Xomed-Treace, Florida, USA), cut to form 'wicks', inserted into the guinea pig vagina for 0.5-2.0 hours¹² before removal and immediate storage at -20°C. Antibodies for immunoassays were eluted from the 'wicks' by the addition of 1mL PBS containing 1% Tween-20 and 5% sodium azide (PBS-T 5% NaN₃) followed by centrifugation at 15,000g for 5 minutes at 4°. The supernatants were analysed immediately, or stored at -80°C (for less than 4 weeks) before analysis. Antibodies for *in vitro* neutralisation were eluted from the 'wicks' in 1mL sterile PBS.

Blood was collected into lithium-heparin tubes (Becton Dickinson Vacutainer Systems) from the lateral saphenous leg vein of the guinea pigs using a 23G needle¹³ and serum recovered following centrifugation at 200g for 5 minutes at 4°C before storage at -20°C.

Peptide Immunisation and challenge with live GPIC

Five animals were immunised with VDIV-GP8 peptide by intraperitoneal priming and intravaginal boosting (test group), while another five were immunised with carrier and adjuvant only (control group). Each immunisation with the conjugate contained 500µg VDIV-GP8 peptide in phosphate buffered saline (PBS), pH 7.4. Freund's complete and incomplete adjuvants (Calbiochem Corporation) (50% v/v) were used for intraperitoneal priming and lysophosphatidyl glycerol (Sigma Biochemicals) (0.5% w/v) was used as the mucosal adjuvant for intravaginal immunisations¹⁴.

Intravaginal immunisations were given to guinea pigs under xylazine/ketamine anaesthesia (5-10mg/kg xylazine and 60mg/kg ketamine i.p.) at a non-oestrus stage of the guinea pig oestrus cycle, as determined by Papanicolaou staining of vaginal smears. Following intravaginal delivery of the peptide [and LPG adjuvant](#) with a

micropipette, the vaginal opening was plugged with cotton wool saturated in sterile PBS to prevent inoculum leakage and uptake into the plug.

Thirty-eight days following immunisation (either with VDIV-GP8 peptide or negative control preparation) all 10 animals were challenged intravaginally with live GPIC. Serum and vaginal secretions were collected from animals prior to, and weekly for nine weeks following, immunisation. Vaginal smears were collected twice weekly post infection. All individual secretion samples were assayed by ELISA (in duplicate, at two different dilutions) both for anti-VDIV IgG and for IgA levels.

For live challenge experiments, the animals were inoculated intravaginally at day 38 post-immunisation with 0.05mL of 1×10^7 GPIC inclusion forming units (IFU)/mL, according to the method used for intravaginal peptide delivery. Vaginal smears were stained using fluorescent monoclonal antibodies (described above). Vaginal smears were assessed for percentage of infection and size of inclusions.

Dot Blot Antibody Analyses

For the analysis of antibodies in sera, unconjugated VDIV peptide, purified GPIC elementary bodies (EBs) and whole McCoy cells were diluted in **PBS** and applied independently to nitrocellulose membranes using a vacuum-blot apparatus (Biodot, Biorad) to deliver 2 μ g per dot for each antigen used. Membranes were blocked overnight in blocking solution (3% skim milk in Tris buffered saline, pH 7.4 TBS) before washing 3 times with TBS and once with TBS/ 0.5% Tween-20 (TBS-T). For the analysis of antibodies in vaginal secretions, secretions were diluted to 0.125mg/ml of protein (DC Protein assay, Biorad) and control sera diluted 1:5 in blocking solution before incubation with the membrane and subsequent washing (as above). Horse radish peroxidase (HRP) conjugate (rabbit anti-guinea pig IgG-HRP) (ICN Biomedicals) was diluted 1:1000 in TBS before incubation with dot blots for 1

hour and washing (as above) for detection of IgG antibodies. Analysis of IgA antibodies in vaginal secretions was achieved using rabbit anti-guinea pig IgA primary antibody and goat anti-rabbit HRP conjugate as secondary antibody. The blots were visualised using solubilised 10mg 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate tablets (Sigma Immunochemicals).

ELISA Analysis

Briefly, 96 well microplates (Maxisorp, Nalge-Nunc International) were coated at 4°C overnight with 0.75µg/well unconjugated VDIV peptide (diluted in PBS) before blocking overnight at 4°C with 100µl of 3% skim milk in PBS. Vaginal secretions were tested at dilutions of 1:25 and 1:250, with each plate also incorporating standard dilutions (1:10 - 1:5000) of untitrated positive anti-VDIV secretions, the absorbances of which were used to generate standard curves. The antibodies used for detection of IgG and IgA were identical to those used in the dot blots. Plates were washed 5 times in PBS containing 0.5% Tween-20 (PBS-T) on an ELISA plate washer (Murex Diagnostics) following each incubation. The HRP substrate used for colorimetric detection of antibodies was K-blue (ELISA technologies). Reactions were stopped at 3 minutes with an equal volume of 3N HCl and absorbances read at 650nm against a blank of 450nm on an ELISA plate reader (Biomek, Beckman).

***In Vitro* Neutralisation Assays**

According to the method of Su and Caldwell⁹ 1×10^5 McCoy cells were seeded onto glass coverslips in 24 well microplates (Nalge-Nunc International) and incubated at 36°C/5%CO₂/24hr. Two-fold dilutions of vaginal secretions were made in DMEM, and incubated (36°C/5%CO₂/24hr) for 30 minutes with an equal volume of DMEM

containing 5×10^6 IFU/mL GPIC before 0.25 mL of each mixture was inoculated onto the McCoy cell monolayers. Standard culture methods as described previously were used, except that the cells were washed with PBS at 24 hours, before the addition of DMEM. The cultures were fixed in methanol at 42 hours post-infection and coverslips stained immediately for identification of chlamydothila inclusions. Approximately 100 cells per coverslip were counted and the percentages of infected cells calculated. Levels of chlamydothila infection were compared between two sets of samples: pooled pre-immune (control, day 0) and pooled post-immune (test, day 37) secretions. Neutralisation was defined as a 50% reduction in the percentage of infected cells present in the test assay when compared to the control assay at the same dilution⁹. The neutralising titre was defined as the reciprocal of the highest dilution of the post-immune sample that exhibited neutralisation.

Results

Intraperitoneal and intravaginal immunisation with VDIV-GP8 peptide induces anti-VDIV antibody responses in vaginal secretions

Preliminary trials to determine the best route for VDIV-GP8 peptide immunization investigated intravaginal (i.vag) immunisation for priming and boosting (i.vag/i.vag) and a combination of intraperitoneal(ip)/ intravaginal(i.vag) immunisation routes. Results showed that i.p immunisation followed 3 weeks later by i.vag antigen delivery produced the best anti-VDIV IgG and antibody responses in vaginal secretions of immunised guineapigs (**Figure 1**) ($P < 0.001$). The wide error bars in Figure 1 are explained by the fact that the animals in the study are Random outbred guinea pigs. Anti-VDIV IgG (low amounts) and IgA antibodies in secretions were evident at day 14, but increased dramatically following an intravaginal boost with VDIV-GP8 peptide

at day 21. The combination of intraperitoneal priming followed by intravaginal boosting with the synthetic peptide was, therefore, chosen as the route of immunisation for subsequent experiments.

Immunisation with linear VDIV-GP8 peptide induces antibodies able to bind the epitope on native MOMP and neutralise infection *in vitro*

Dot-blots were used to determine whether or not anti-VDIV antibodies in vaginal secretions (produced against linear peptide VDIV-GP8) were able to bind to the native MOMP on whole EB's (**Figure 2**). The test assays were done using pooled secretions of two animals that showed high amounts of vaginal anti-VDIV IgG and IgA antibodies following peptide immunisation but prior to live *C.caviae* challenge. The same amount of 'secretion' protein (wick eluate) was added for test and control specimens to allow the intensity of the dots to be used as a direct measure of antibody concentration. The reactivity of test sera compared to the negative control (the same animals pre-immunisation) indicates that IgG antibodies produced in response to immunisation with linear VDIV peptide bound to the conformational VDIV epitope on whole EBs. Similar results were obtained with IgA dot blots although reactions were much weaker than those seen on the IgG blots (data not shown).

In vitro neutralisation assays were undertaken to determine whether or not anti-VDIV antibodies produced in guinea pig vaginal secretions could prevent GPIC infection of McCoy cells in a cell culture system. [The assays were done using the same pooled secretions as those used in the western blot \(Fig. 2\)](#). Assays using the two sets of vaginal secretion samples (pooled pre-immune and pooled post-immune secretions) showed neutralisation defined as a 50% reduction in the percentage of infected cells present in the test assay, when compared to the control assay at the same dilution. At a 1:20 dilution of post-immune secretions there was at least 50% reduction in the

percentage of infected cells compared to the 1:20 dilution of the pre-immune secretions (**Figure 3**).

Figure 3 is a plot of the percentage of McCoy cells infected with *C. caviae* against dilutions (1/20-1/320) of secretions both for pre- and for post-immune samples. Antibodies in post-immune secretions show neutralising activity not only at the 1:20 dilution, but also at 1:40 and 1:80 dilutions, as evidenced by the separation of the two lines (pre-immune vs. post immune secretions) in Figure 3. The neutralising titre was found to be 20 which reflects the method used to extract antibodies from the vaginal wicks which dilutes the secretions. *It was also noted that the activity of the serum did not titrate until a dilution of 1/80 although the reasons for this are presently unclear and require further investigation.*

Protection of immunised guinea pigs to live challenge does not correlate directly to antibody levels

A challenge experiment was undertaken to determine whether i.p/i.vag immunisation with the linear VDIV-GP8 peptide could provide protection against GPIC infection of guinea pigs *in vivo*. On the day prior to challenge vaginal secretions were collected and analysed by ELISA for anti-VDIV IgG and IgA antibodies. Three out of five of the immunised animals showed anti-VDIV IgG responses, whilst only one guinea pig exhibited an IgA response. None of the control animals showed antibody responses against VDIV (data not shown).

When challenged i.vag with a moderate level of live GPIC, a degree of protection (as evidenced by a 15-20% reduced parasite load) was found in the immunised group (**Figure 4**). This observed reduction in parasite load was present immediately following challenge and persisted until the infection was cleared. While this reduction in parasite load was modest (5-15%) it was significant ($P=0.01$).

There was an apparent reduction in the intensity of infection in immunised when compared to the non-immunised animals over the full 26 days following challenge (**Figure 4**). This apparent reduction is confirmed by statistical analysis. Using a two factor analysis of variance test ¹⁵ with repeated measures on one factor (time) there was a significant ($P = 0.01$) decrease in the intensity of infection in the immunised group when compared with non-immunised controls. These results indicate that although immunisation of guinea pigs i.p./i.vag with the VDIV-GP8 peptide did not completely block initial intravaginal infection with *C. caviae*, partial protection, represented as a reduced level of infection over the course of the experiment, was evident in immunised animals.

Four out of five immunised guinea pigs cleared the infection within the course of the experiment, in comparison to only one of five non-immunised animals (data not shown). There was an apparent inverse correlation between pre-challenge anti-VDIV secretion antibody levels and the duration of infection. For example, the immunised animal which recovered most quickly from the challenge (day 18) showed no anti-VDIV antibody responses in the pre-challenge secretion collection (data not shown). Conversely, the immunised guinea pig that was unable to clear infection in the experimental period showed high levels of IgG and was the only animal tested that showed any anti-VDIV IgA response in pre-challenge vaginal secretions (data not shown).

Live GPIC challenge of immunised animals did result in moderate protection. There was, however, no correlation between pre-challenge anti-VDIV IgG and IgA levels in secretions and initial levels of infection (day 7 post infection) seen in the animals ($r^2=0.04$, $r^2.<0.01$ respectively).

Discussion

Since *C. trachomatis* enters the body via the reproductive tract mucosa, the ability of a vaccine to elicit strong immune responses in the vagina is an important indicator of protection against infection with this organism. The hypothesis that local (vaginal) antibody production against chlamydia is at least partially protective was derived from a study in which *C.caviae* infection of the guinea pig genital tract was prolonged by estradiol administration to the animals. The prolongation of infection correlated with a delay in the production of anti-GPIC antibodies in secretions that occurred in the presence of normal (non-delayed) serum antibody responses. Resolution of the infection was associated with the appearance of both IgG and IgA in genital secretions.¹⁶ GPIC infection of guinea pigs is, therefore, a good model to use for studying Pelvic Inflammatory Disease caused by *C.trachomatis* in humans.

Several investigators have measured serum antibody responses following parenteral delivery of various *C. trachomatis* MOMP subunits to mice⁹, were no studies on the ability of synthetic chlamydial peptides to elicit humoral responses in the vagina of immunised hosts, or of immune responses against chlamydial peptide delivered to animals by anything other than parenteral routes. The purpose of this study was, therefore, to investigate whether combination systemic/mucosal (i.p/i.vag) immunisation of female guinea pigs with a chimeric chlamydial peptide could elicit humoral immune responses in the reproductive tracts of immunised animals. Our finding that i.p./i.vag delivery of a chimeric peptide to guinea pigs can elicit specific anti-chlamydomydia responses in vaginal secretions of immunised animals is, therefore, important towards the progress of vaccination against this mucosal pathogen.

Two important questions must be addressed when attempting to immunise against pathogens with a synthetic linear peptide. Firstly, will the linear amino acid sequence be recognised as an immune epitope by the host and result in activation of B and T lymphocytes? Secondly, in the case of B-lymphocytes, will antibodies produced against the linear epitope be able to recognise and bind to the native epitope upon challenge?

Although the amino acid sequences for the variable domains the *C.caviae* agent were known, and various B- and T-cell epitopes had been predicted, the properties of antibody directed against the variable domains of *C. caviae* previously were unknown. It was an important finding of this study, therefore, that antibodies in vaginal secretions directed against the linear VDIV-GP8 peptide are able to bind to whole *C. caviae* elementary bodies (Figure 2). This is the first step in establishing the potential of antibody to protect against chlamydial infectivity.

Previous studies have shown that antibodies produced in *serum* against *C. trachomatis* VDIV are able to neutralise the infectivity of *C. trachomatis* in cell culture ¹¹. However, it has also been shown that high serum IgG antibody levels do not protect against chlamydial genital infections *in vivo*. In our investigation, we immunised animals that subsequently produced anti-VDIV antibodies *at the site of infection* and another new finding was that these antibodies provided neutralising activity against *C. caviae* EB's in a McCoy cell system.

Results of previous chlamydial challenge experiments using guinea pigs have indicated that parenteral and oral immunisation with whole inactivated EBs of *C.caviae* ¹⁵ and parenteral immunisation with the entire MOMP protein ²⁰ were able to induce partial protection against subsequent infection. Partial protection in these cases was seen as a statistically significant reduction in the duration and/or the

intensity of infection. Non-replicating antigens, including whole, inactivated *Chlamydia* have not, however, been reported to induce complete protection upon challenge in the guinea pig²⁰. Our results showed that a chimeric peptide antigen delivered ip/ivag to guinea pigs induced specific anti-chlamydial antibodies in the vagina of immunised animals and the antibodies were neutralising *in vitro*.

Interestingly, the partial protection seen did not correlate with pre-challenge antibody levels in vaginal secretions. The anomalous fact that the guinea pig with the highest anti-VDIV response cleared the challenge infection last, and that a guinea pig with no response at all recovered first indicates that the protection observed in this study may be cell mediated in nature.

Several studies in recent years have highlighted the importance of T-cells^{21, 22} and associated cytokines^{23,24} in resolution of infection and prevention of re-infection with chlamydial agents. The role of cellular immunity in response to the peptides in this study will be a focus of future research.

It is encouraging that we are able to induce partial protection against genital infection with the GPIC agent, *C. caviae*. Of particular importance is the fact that the protection in this study was induced by vaccination with a peptide antigen. Indeed, the partial reduction in parasite load that we have observed in our study may be useful for vaccination leading possibly to reduced transmission and /or reduced pathology in infected hosts.

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Figure 1

ELISA showing levels of anti-VDIV antibodies in vaginal secretions of guinea pigs after immunisation with VDIV-GP8 peptide either by: i.p (priming)/i.vag. (boosting) or by the i.vag/i.vag. routes (n=5). Anti-VDIV IgG (Fig 1a) and IgA (Fig 1b) levels are expressed as x-fold increases over background levels exhibited in the non-immunised (control) samples. The baseline OD value (negative cutoff) was an Absorbance value of 0.2 units. Individual results from each animal were grouped according to the immunisation route used. The mean anti-VDIV IgG and IgA levels in vaginal secretions and the standard deviations for each group were determined at each of the sample collection time points. Arrows indicate the time of priming and boosting with peptide (once for i.p delivery of peptide, three immunisations/two days apart for i.vag. delivery)

Figure 2

Dot blots showing reactions of IgG in vaginal secretions against GPIC EBs, McCoy cells and VDIV (in duplicate). Secretions used were pooled from two animals in the chlamydia challenge experiment and standardised with respect to total protein contained in the wicks. Samples used to probe the blots were (1) post-challenge (positive control), (2) pre-immune (negative control) and (3) post-immunisation (test) secretions.

Figure 3

In vitro neutralisation assays using pooled guinea pig vaginal secretions. The secretions used were taken from two animals and were collected either pre-immunisation (control) or at day 37 post-immunisation (test) with VDIV-GP8 peptide. Secretions were standardised with respect to total protein concentration.

Figure 4

Chlamydia challenge infection of animals immunised i.p.(priming)/i.vag.(boosting) with VDIV-GP8 peptide, compared with non-immunised controls (which received carrier and adjuvant only). Levels of infection were determined as follows: Individual data from each collection (pre-challenge, days 7, 11, 14, 18, 21, 26 post-challenge) was grouped (either immunised or non-immunised) and for each smear, three oil-immersion fields were examined and the percentage of infected cells per field calculated. Levels of infection are presented as the mean level of infection per group of 5 animals at each sample collection point. Error bars indicate one standard deviation from the mean.

Figure 1

Figure 3

Figure 4

Figure 1

Figure 3

Figure 4

Figure 1

Figure 3

Figure 4

Figure 1

Figure 3

Figure 4