P4-Mediated Antibody Therapy in an Acute Model of Invasive Pneumococcal Disease

Mathieu Bangert,1,3 Laura Bricio-Moreno,3 Suzanna Gore,3 Gowrisankar Rajam,2 Edwin W. Ades,2 Stephen B. Gordon,1,a and Aras Kadioglu3,a

1Respiratory Infection Group, Liverpool School of Tropical Medicine, and 2Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; and 3Department of Clinical Infection, Microbiology, and Immunology, Institute of Infection and Global Health, University of Liverpool, United Kingdom

New treatments against severe bacterial infections are needed because the response to antibiotic treatment is slow in acute settings and is becoming less effective owing to the emergence of antibiotic-resistant pathogens. P4-mediated antibody therapy offers a unique treatment strategy that combines exogenous immunoglobulin with the immunoactivating peptide P4. In an acute model of pneumococcal disease, mice were infected with Streptococcus pneumoniae and treated intravenously or intranasally with P4 and intravenous immunoglobulin (IVIG). Survival of P4-IVIG–treated mice increased from 0% to 60% among those that received intravenous treatment and from 0% to 100% among those that received intranasal treatment. Importantly, intranasal administration of P4 at an early stage of infection prevented the onset of bacteremia and sepsis. Increased survival was associated with reduced bacterial burden in affected tissues and with recruitment and activation of professional phagocytes, as manifested by increased expression of Fc-γ receptors. In vitro studies involving P4-stimulated alveolar, peritoneal, and J774.2 murine macrophages showed an increased ability of these immune cells to phagocytose pneumococci independent of capsule. The use of adjunct antibody therapies to treat infectious diseases shows promise.

Worldwide, pneumonia is the leading infectious cause of mortality in children and adults, with Streptococcus pneumoniae being the most commonly recovered isolate [1]. In developing countries, respiratory tract infections frequently progress to fatal sepsis and meningitis, particularly in immunocompromised, young, and elderly individuals [2, 3]. Approximately 50% of deaths occur during the first 48 hours of treatment, when antibiotic therapy does little to affect the risk of mortality [4–6]. Furthermore, rates of antibiotic resistance are rising, with over one-third of isolates in the United States and parts of Europe showing reduced susceptibility to penicillins [7, 8]. While pneumococcal conjugate vaccines have resulted in a decline in invasive disease caused by pneumococcal serotypes included in the vaccine [9], nonvaccine serotypes have been shown to cause replacement disease [10]. To address these clinical challenges, attention is shifting back to a century-old technique to treat infectious diseases: passive immunity. The transfer of pooled or pathogen-specific antibodies was initially developed in the pre-antibiotic era but became uncommon because of the advent of antibiotics and the emergence of frequent side effects [11]. There is now a renewed interest in passive immunity, particularly in cancer therapy and in the treatment of drug-resistant infections, because safe human monoclonal and polyclonal antibodies with low rates of side effects can be relatively easily synthesized [12, 13]. The slow response of the host immune system to exogenous antibodies, however, limits the use of passive immunity in acute infections and in immunocompromised individuals [14]. Adjunctive therapies with immunoactivating agents such as the P4 peptide offer a solution to overcome this.
P4 is a surface-expressed moiety of the pneumococcal virulence factor PsaA [15]. Initial studies indicated that P4 is an immunoactivating peptide that can enhance systemic defense against pneumococcal infection [16, 17]. Further work showed that P4 can augment the ability of human-derived and mouse-derived neutrophils to phagocytose opsonized pneumococci in vitro [18] and that P4 therapy is also effective in a coinfection model of influenza and S. pneumoniae in vivo [19]. Here we assess P4 therapy in an acute setting of invasive pneumococcal pneumonia and the targeted administration of the peptide. Furthermore, we investigate the cellular and bacterial responses to P4 therapy in the presence and absence of infection and evaluate P4-mediated opsonophagocytosis in alveolar, peritoneal, and J774.2 murine macrophages.

**MATERIALS AND METHODS**

**Mouse Strains**

Outbred, female MF1 mice (Charles River; United Kingdom) at 8–12 weeks of age were used to model acute invasive pneumococcal disease [20–24]. All mouse experiments were conducted following guidelines from the ethical review and animal welfare committee and under authority of UK Home Office license.

**Bacterial Strains**

Animal-passaged S. pneumoniae ST2-D39 (NCTC 7466) strains were used for in vivo experiments. ST2-D39 (NCTC 7879), ST14 (NCTC 11902), and unencapsulated ST2-D39 strains were used for all other experiments. Bacteria were grown overnight at 37°C (5% CO₂) on blood agar base containing 5% (v/v) horse blood (Oxoid) and confirmed to be pneumococci by Gram stain, detection of catalase and α-hemolysis, and sensitivity to optochin.

**Acute Pneumonia Model**

Mice were mildly anesthetized with 2.5% (v/v) isoflurane USP (Isocare) over oxygen (1.4–1.6 L/min), and 50 μL sterile phosphate-buffered saline (PBS) containing 1 × 10⁶ S. pneumoniae was equally distributed between both nostrils. Groups of 5 animals per time point (specified below in figure legends) were terminally anesthetized, and blood was collected by cardiac puncture before cervical dislocation. Lung tissue was dissected as described elsewhere [23], placed into 10 mL sterile PBS, and disrupted with an Ultra-Turrax T8 homogenizer (IKA). Colony-forming units (CFU) were enumerated from lung homogenate and blood samples by the Miles and Misra method [23].

**Intranasal and Intravenous P4 Therapy**

P4 peptide (28 amino acids: LFVESSVKRRPMKTVSQDT-NIPIYAQIF) was synthesized and purified at the Centers for Disease Control and Prevention (Atlanta, GA) and solubilized in DEPC-treated water (9922; Ambion) as previously described [16, 17, 25]. Gamunex (intravenous immunoglobulin [IVIG]; Talecris) was used as a source of pneumococcal serotype-specific polysaccharide antibodies [26, 27]. For studies of intranasal administration, P4 solution (100 μg/40 μL DEPC per mouse) was equally administered in both nostrils 12 and 18 hours after infection, 20 minutes following intraperitoneal administration of IVIG (100 μL). For studies of intravenous administration, P4 solution (100 μg/100 μL DEPC per mouse) was administered into tail veins 24 and 30 hours after infection, 20 minutes after IVIG administration (performed as described above). Control mice received DEPC-treated water, P4 solution, or IVIG alone.

**Flow Cytometry**

Lung tissue was weighed, manually disrupted, enzymatically digested (Collagenase D; Roche), and passed through a cell sieve (BD Biosciences) to yield cell suspensions. Blood samples were subjected to red blood cell lysis (BD Pharm Lyse; BD Biosciences) for 10 minutes at room temperature as recommended by the manufacturer’s instructions. Labeled anti-CD antibodies purchased from Biolegend were used. Cell suspensions were stained for 30 minutes at 4°C, using optimal concentrations of CD45 FITC (hematopoietic cells marker, 30-F11), F4/80 APC (macrophage/monocyte marker, BM8), GR1 PE/CY7 (neutrophil marker, RB6-865), and CD16/32-FITC (Fc-γ RII/III marker, 93). Appropriate isotype controls were used to determine baseline levels of fluorescence during analysis. Acquisition was carried out using a FACSCalibur (BD Biosciences) flow cytometer, and analysis was performed using FlowJo 8.7 for Macintosh (Tree Star).

**J774.2, Peritoneal, and Alveolar Macrophages**

All macrophage cell cultures were performed using Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. The J774.2 murine macrophage cell line was maintained as previously described [28]. Following euthanasia by pentobarbital overdose, alveolar macrophages were obtained by exposing and cannulating the trachea of mice with a 24-gauge plastic catheter (BD Insty; Becton Dickinson) and lavaging lungs twice with 1.5 mL medium. Peritoneal macrophages were obtained by exposing the peritoneum of mice euthanized by cervical dislocation and washing the peritoneal cavity twice, using 2.5 mL medium and a 14-gauge syringe. Macrophages were purified by adherence to plastic for 3 hours at 37°C (5% CO₂).

**Immunogold Labeling of Fc-γ Receptors**

P4 solution (100 μg/100 μL DEPC per mouse) was administered into tail veins, and blood was collected by cardiac puncture 24 hours later, before cervical dislocation. Blood samples were subjected to red blood cell lysis (BD Pharm Lyse; BD Biosciences) as recommended by the manufacturer’s instructions. The cell pellet was washed and stained for 30 minutes at 4°C, using an anti-mouse CD16/32 (Fc-γ RII/III marker, 93) antibody, followed by exposure to a secondary
probe, using a 10-nm colloidal gold-labeled antibody (G7035; Sigma). Cells were then washed, fixed, and submitted for transmission electron microscopy at the University of Leicester. Randomly selected cells were blindly assessed by 2 independent observers for the presence of gold particles.

**P4-Mediated Opsonophagocytosis**

A reference opsonophagocytic assay (OPA) described elsewhere [26] was used with minor modifications. Briefly, \(5 \times 10^4\) macrophages were incubated with \(5 \times 10^5\) opsonized *S. pneumoniae*, complement, and P4 for 1 hour at 37°C (5% CO2). CFU were determined following incubation. IVIG at a final dilution of 1:16 was used as the source for pathogen-specific antibody for opsonization. P4 peptide solution (10 µg/well) was added to the OPA mixture following the pre-opsonization stage, while the control wells received DEPC-treated water instead. Wells containing nonopsonized pneumococci and heat-inactivated complement were used as further controls.

**Statistical Analysis**

In vivo experiments were performed in duplicates and in vitro/ex vivo experiments in triplicates on separate assay days. Data were designated significant if \(P < .05\) by use of analysis of variance, using Graphpad Prism 5 for Macintosh.

**RESULTS**

**Intranasal Administration of P4 During Pneumococcal Pneumonia**

**Survival**

Mice intranasally treated with P4 (at 12 and 18 hours after infection) showed no mortality by 48 hours, compared with 100% moribundity in the control group (Figure 1A). There were no significant differences in groups that received IVIG and P4 only (data not shown).

**Infection**

At 24 hours following infection, there was no significant difference in bacterial lung CFU in both control and P4-IVIG–treated groups (Figure 1B). By 48 hours, however, bacterial loads in control mice significantly increased, compared with 24-hour levels, while pneumococcal loads in P4-IVIG–treated mice remained the same as those at 24 hours and were significantly less than those in control groups (\(P = .002\)). The presence of bacteria in the blood of control mice at 24 hours significantly increased by 48 hours, while none of the P4-IVIG–treated mice had any bacteria in blood at either 24 or 48 hours (Figure 1C).

**Leukocytes**

An influx of neutrophils by 24 hours was detected in both control and P4-IVIG–treated groups. However, by 48 hours, P4-IVIG–treated mice had significantly lower numbers of neutrophils in their lungs (\(P < .05\)), while neutrophil numbers remained constant in control mice (Figure 1D). Macrophage numbers in lungs remained similar between all groups throughout infection, except at 48 hours, when a substantial (albeit nonsignificant) decrease in macrophages was detected in P4-IVIG–treated mice (Figure 1E). Fc-\(\gamma\) RII/III expression on neutrophils was significantly higher at 48 hours in P4-IVIG–treated mice (\(P < .05\)) (Figure 1F), while Fc-\(\gamma\) RII/III expression on macrophages remained similar (Figure 1G).

**Intravenous Administration of P4 During Pneumococcal Septicemia**

**Survival**

Mice intravenously treated with P4 (at 24 and 30 hours after infection) exhibited 60% survival, compared with 0% survival in the control group (Figure 2A). There were no significant differences in groups that received IVIG or P4 only (data not shown).

**Infection**

P4-IVIG–treated mice had significantly fewer bacteria in their lungs at 36 hours (\(P = .0412\)) and 48 hours (\(P = .004\)) following infection (Figure 2B), compared with IVIG-only and DEPC-only control groups (data not shown). A significantly decreased amount of bacteria in the blood of P4-treated mice at 48 hours was found (\(P = .01\)) (Figure 2C).

**Leukocytes**

Neutrophil numbers remained similar between groups throughout the duration of infection, following the same pattern of an early increase, a peak at 36 hours, and a decline by 48 hours (Figure 2D). However, neutrophil Fc-\(\gamma\) expression levels increased sharply by 48 hours in P4-IVIG–treated mice, compared with controls (Figure 2F). Macrophages in lungs of P4-IVIG–treated mice were significantly higher (\(P = .001\)) at 36 hours, compared with those in nontreated controls (Figure 2E). However, unlike the changes observed for neutrophils, there was no significant difference in Fc-\(\gamma\) expression levels (Figure 2G).

**Intravenous Administration of P4 in the Absence of Infection**

Twenty-four hours following intravenous injection of P4 solution or DEPC-treated water, a significant increase in systemic neutrophil numbers was detected (\(P = .02\)), compared with the DEPC control (Figure 3A). In comparison, significant differences in the proportion of circulating monocytes were seen at 4 hours (\(P = .0005\)) and 24 hours (\(P = .0005\)), compared with proportions in controls (Figure 3B). Fc-\(\gamma\) RII and III expression in both neutrophils and macrophages increased within the first hour and was significantly increased, compared with expression control mice, at 4 hours (neutrophils, \(P = .02\); macrophages, \(P = .02\)) and 24 hours (neutrophils, \(P < .0001\); macrophages, \(P = .002\)) (Figure 3C–D).

**Immungold Labeling of Fc-\(\gamma\) RII/III Receptors**

Twenty-four hours following intravenous injection of P4 solution or DEPC-treated water, peripheral blood leukocytes were stained and assessed for the presence of gold particles (Figure 4A). A significant increase (\(P = .0009\)) in gold was observed in...
monocytes (n = 23 per group), while no significant difference was seen in neutrophils (data not shown). Unstimulated monocytes showed an average of 10 gold particles per TEM section, whereas P4-stimulated cells showed an average of 22 gold particles per TEM section (Figure 4B). Significant differences were also seen in the localization of gold particles; 35% of the particles in unstimulated cells and 52% in P4-stimulated cells were internalized into phagosomes (P < .005).

---

Figure 1. Intranasal P4 therapy to treat invasive pneumococcal disease. A, Survival of MF1 mice intranasally infected with 10^6 *Streptococcus pneumoniae* (D39) and treated at 12 and 18 hours. Treatment groups: P4—intravenous immunoglobulin (IVIG; 100 µL IVIG, 100 µg P4) and DEPC-treated water (100 µL) control (n = 10 per group). B and C, Log_{10} *S. pneumoniae* colony forming units (CFU) recovered per milligram of lung tissue and per milliliter of blood 0, 24, and 48 hours following infection in treated and nontreated mice. D and E, Neutrophils and macrophages detected per milligram of lung tissue 0, 24, and 48 hours following infection in treated and nontreated mice. F and G, Fc-y RI/II expression in neutrophils and macrophages detected in lungs 0, 24, and 48 hours following infection in treated and nontreated mice. *P < .05, by analysis of variance.
Opsonophagocytosis of *S. pneumoniae* by Macrophages

Following opsonization of bacteria, J774.2 murine macrophages showed a significantly increased ability (20%) to kill pneumococcal ST2 and unencapsulated pneumococcal ST2 when treated with P4 (Figure 5A). While nonstimulated cells were unable to induce detectable killing of ST14, P4-stimulated cells killed 11% of the ST14 inoculum. Macrophages isolated from both peritoneal specimens (*P* < .05) and bronchiolar lavage specimens (*P* = .01) were able to significantly increase their phagocytosis of ST2 *S. pneumoniae* by 25% following P4 stimulation (Figure 5B). Nonopsonized *S. pneumoniae* and inactivated complement-incubated cells had minimal levels of

---

**Figure 2.** Intravenous P4 therapy to treat invasive pneumococcal disease. 

A, Survival of MF1 mice intranasally infected with $10^6$ *Streptococcus pneumoniae* (D39) and treated at 24 and 30 hours. Treatment groups: P4—intravenous immunoglobulin (IVIG; 100 µL IVIG, 100 µg P4) and DEPC-treated water (100 µL) control (*n* = 10 per group). 

B and C, Log$_{10}$ *S. pneumoniae* colony forming units (CFU) recovered per milligram of lung tissue and per milliliter of blood 0, 24, 36, and 48 hours following infection in treated and nontreated mice. 

D and E, Neutrophils and macrophages detected per milligram of lung tissue 0, 24, 36, and 48 hours following infection in treated and nontreated mice. 

F and G, Fc-γ RII/III expression in neutrophils and macrophages detected in lungs 0, 24, 36, and 48 hours following infection in treated and nontreated mice. *P* < .05, by analysis of variance.
killing (<5%), with no significant differences between P4-stimulated and unstimulated groups (data not shown).

**DISCUSSION**

In this study, we demonstrated that combined treatment with P4 peptide and IVIG is synergistically able to rescue mice in an acute model of invasive bacterial pneumonia. Furthermore, we showed that intranasal administration of P4 as preemptive treatment for invasive pneumonia prevents the development of fatal sepsis.

Even in a well-resourced setting and with effective antibiotic therapy, mortality from invasive pneumococcal disease is >20% [5, 29] and in low-income countries can be >60% [30]. This is most likely due to the complexity of the immune response to infection. On one hand, a powerful response is needed to kill bacteria, while on the other, an uncontrolled host immune response itself is often responsible for the majority of injury caused during bacterial infection [31]. The ideal immune response specifically targets the infecting organism without damaging host cells through nonspecific innate inflammatory responses, and antibodies are the naturally occurring components of the immune system that meet these criteria. However, the body takes several days to produce sufficient quantities of antibodies, and vaccine-induced antibodies become less effective as a result of aging [32].

Antibody therapy is increasingly being used to treat infectious diseases. While IVIG preparations are currently not tailored to treat infectious diseases, titrated pooled immunoglobulin can be effectively used against pathogens and has several advantages over traditional treatments. First, because immunoglobulin G found in IVIG is specific to a wide range of pathogens [33–35], preemptive administration is likely to be effective when the pathogen is unknown. Second, IVIG has anti-inflammatory properties [36, 37] resulting from the sialylation of the Fc portion of immunoglobulin [38, 39]. This is particularly interesting in P4 therapy as it enables activated phagocytes to ingest opsonized pathogens while maintaining a reduced inflammatory response. Finally, because antibodies in IVIG products are specific to various antigens on a pathogen, the pressure to mutate is reduced, making IVIG treatment a feasible option for the long term. Supplying immunoglobulin to an immune system facing a rapidly progressing bacterial infection will lead to the formation of immune complexes, but in an acute setting, the host immune cells may not be able to clear these effectively. Administering P4 alongside IVIG results in the enhanced phagocytosis of these complexes, making it an

![Figure 3](image-url)
ideal adjunctive therapy. Previous work has shown that this combination is able to rescue young and aged mice of varying genetic backgrounds [16, 17, 19, 25] but has not addressed the use of P4 therapy in acute and severe infections, a major cause of mortality worldwide. MF1 mice are highly susceptible to S. pneumoniae [23, 40], with infection leading to mortality in 48 hours, a much shorter time than that for other strains used to model pneumococcal diseases (72–96 hours in BALB/c or C57 mice [40]). In the MF1 model, pneumonia is rapidly established following infection, and by 12 hours after infection pneumococci seed into the blood, causing bacteremia in a consistent and reproducible manner. The MF1 model is therefore a suitable model for testing the efficacy of P4 therapy because it shares the similarly high susceptibility to acute pneumococcal infections found in immunocompromised, young, and aged populations. Furthermore, in treating mice at a late stage in this model, we are addressing the most severe cases of acute infection, in which the efficacy of antibiotic treatment is low and mortality rates are high even in immunocompetent individuals [41]. To demonstrate recovery and survival, as well as their associated immunological mechanism(s), among mice with acute invasive pneumonia, standard Kaplan-Meier survival plots for these mice were compared with those for mice from the control group.

Intranasal administration of P4 results in exposure of the peptide to all sites of pulmonary immunity, including resident alveolar macrophages. P4 treatment of the respiratory tract as a preemptive measure against invasive pneumonia halted the exacerbation of disease and prevented pneumococcal seeding into blood, leading to complete host survival (Figure 1A–C). Importantly, though, this study has shown that P4 immunomodulation is effective in both the mucosal and systemic immune system and works against invasive pneumonia and sepsis, both of which are major causes of death worldwide. To our knowledge, this is the first time intranasal therapy has led to host protection after the onset of acute pneumonia, suggesting the potential for targeted treatment against bacterial respiratory infection and septicemia secondary to such infections.
Intravenous administration of P4 has the advantage that the peptide will circulate systemically and thereby rapidly activate a wide range of cells. This route of administration has successfully been used in previous studies [17, 25] and was also able to increase survival in this acute model (Figure 2A). Survival in this model is correlated with significant increases in lung macrophages ≤12 hours after P4 administration (Figure 2E) and with infiltration of neutrophils expressing higher levels of Fc receptors, compared with findings for control mice (Figure 2F). Although surviving mice were still infected, the progression of pneumonia and bacteremia was halted ≤12 hours after P4 and IVIG administration, leading to no increases in bacterial loads. In a clinical setting, this allows antibiotic therapy to take effect, such as the combination of P4, immunoglobulin, and ceftriaxone shown elsewhere to be effective against invasive pneumococcal disease [16].

The increased survival in both intravenous and intranasal studies can be attributed to reduced bacterial burden in affected tissues, which was most likely due to the enhanced ability of macrophages and neutrophils to engulf and eliminate opsonized pathogens. The immune response to P4 therapy, however, varied between the routes of administration, probably because of the site (mucosal vs systemic) and timing (acute pneumonia vs sepsis) of treatment. Although in both studies neutrophils were expressing higher levels of Fc-γ receptors, intranasal studies showed significantly reduced levels of neutrophils in lung tissue. This can be attributed to the absence of sepsis and associated cytokines and to decreased pneumonia severity in the intranasal model, which reduced the need for neutrophil infiltration [42]. The major difference, however, involved the response of macrophages to P4. Although a sharp increase in macrophages was observed in lung tissue during the intravenous studies, intranasal studies showed no increases, compared with controls. Instead, intranasal administration of P4 led to activation of resident pulmonary macrophages, as manifested by their increased expression of Fc-γ receptors, which was not seen in intravenous studies. The activation of pulmonary macrophages in the intranasal route is most likely due to the direct contact of P4 with alveolar macrophages, while the recruited macrophages in intravenous studies may have been released from a splenic monocyte reservoir in response to peripheral blood neutrophil or monocyte activation [43–45]. Similar to the intravenous infection studies, monocytes were the first immune cell population to increase in numbers in peripheral blood when P4 was administered intravenously in the absence of infection. An increase in neutrophil numbers was not detected before 24 hours, but cell numbers were significantly higher (Figure 3). An in-depth study of the differences in P4-mediated mucosal and systemic immunomodulation would contribute to a better understanding of the implicated mechanisms.

Fc-γ receptors are crucial for effective phagocytosis. When the receptor comes into contact with the Fc fragment of antigen-bound immunoglobulin G, both the receptor and the immune complex are internalized [46]. In this study, we showed involvement of these receptors in P4-mediated antibody therapy. By use of flow cytometry, we showed enhanced expression of Fc-γ II/III receptors in the presence and absence of infection, suggesting that this effect is P4 mediated and not a side effect of infection or IVIG administration. In addition to receptor expression, we wished to determine Fc-γ receptor localization on membranes and in phagosomes, as well as relative numbers per cell. By use of immunogold staining and TEM imaging, we showed that P4 treatment leads to an increase of monocyte Fc-γ receptors and to enhanced internalization of these receptors into phagosomes (Figure 4). This correlates with the ex vivo and in vitro opsonophagocytosis assays in this study, in which we assessed phagocytosis by murine J774.2 macrophages and ex vivo–derived peritoneal and alveolar macrophages in response to P4 stimulation (Figure 5). In the presence of IVIG, complement, and P4, macrophages were more effective at phagocytosing pneumococci, compared with macrophages from unstimulated controls. Nonopsonized bacteria are poorly phagocytosed because of the lack of Fc–Fc-γR interactions, and in this study no difference in killing between stimulated and unstimulated groups was detected in the absence of antibody. Similarly, the use of immunoglobulin or peptide-alone controls in our study and in previous in vivo studies did not significantly alter survival. This suggests that P4-mediated antibody therapy is specific by being effective only in the presence of opsonized pathogen and phagocytic immune cells.

The discovery of this immunoactivating peptide is only a few years old, but on the basis of our study the potential of using it in combination with IVIG to treat acute infections is apparent. P4 therapy can rapidly and effectively augment an immune response in patients with acute sepsis, before the effect of antibiotic treatment would be apparent. The success of the intranasal administration further adds to the versatility of this peptide, and with advances in commercial antibody production on the way, the use of adjunct antibody therapies to treat infectious diseases shows promise.

Notes

Acknowledgments. We thank Natalie Allcock from the TEM suite for her valuable help and expertise.

Financial support. This work was funded by the Crossley Barnes Fellowship and the NIHR Biomedical Research Centre in Microbial Diseases, with infrastructural support from the NWDA.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References
