Pathological findings and diagnostic implications of a rhesus macaque (Macaca mulatta) model of aerosol exposure to Burkholderia mallei (glanders)

Samuel L. Yingst, Paul Facemire, Lara Chuvala, David Norwood, Mark Wolcott and Louis Huzella

1US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA
2Ordway Research Institute, Albany, NY, USA
3Integrated Research Facility, Division of Clinical Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Frederick, MD, USA

Burkholderia mallei is a Gram-negative bacillus that causes a pneumonic disease known as glanders in equids and humans, and a lymphatic infection known as farcy, primarily in equids. With the potential to infect humans by the respiratory route, aerosol exposure can result in severe, occasionally fatal, pneumonia. Today, glanders infections in humans are rare, likely due to less frequent contact with infected equids than in the past. Acutely ill humans often have non-specific clinical signs and in order to diagnose cases, especially in scenarios of multiple cases in an unexpected setting, rapid diagnostics for B. mallei may be critical. The pathogenesis of acute glanders in the rhesus macaque (Macaca mulatta) was studied as an initial effort to improve diagnostic methods. In the study described here, the diagnostic techniques of PCR, culture and histopathology were compared. The results indicated that PCR may provide rapid, non-invasive diagnosis of glanders in some cases. As expected, PCR results were positive in lung tissue in 11/12 acutely infected rhesus macaques, but more importantly in terms of diagnostic algorithm development, PCR results were frequently positive in non-invasive samples such as broncho-alveolar lavage or nasal swabs (7/12) and occasionally in blood (3/12). However, conventional bacterial culture failed to recover bacteria in many of these samples. The study showed that the clinical presentation of aerosol-exposed rhesus macaques is similar to descriptions of human glanders and that PCR has potential for rapid diagnosis of outbreaks, if not individual cases.

INTRODUCTION

Glanders is a zoonotic disease caused by Burkholderia mallei, a Gram-negative, non-motile, facultative anaerobic bacillus that does not survive well in the environment. Humans and equids are primarily affected, with the latter serving as the reservoir for transmission. Small areas of endemicity exist in Asia, the Middle East, Africa and South America. A decrease in disease prevalence elsewhere in the world is often attributed to the reduced reliance on equids for transportation and work. B. mallei is closely related to Burkholderia pseudomallei, the cause of melioidosis; however, B. pseudomallei is a motile bacillus with the ability to survive more effectively in the environment (Brook et al., 1997; Carr & Waag, 2007; Khan et al., 2012; Larsen & Johnson, 2009).

Clinical glanders in humans exposed by the aerosol route typically exhibits a wide array of non-specific clinical signs that may initially include fever, myalgia, headache and fatigue. Later in the course of the disease, signs may progress to diarrhoea, weight loss, and pneumonia with pulmonary abscesses. Chronic glanders may involve dissemination of the organism via lymphatics to other organs, including the haematopoietic system, muscle and skin (as in farcy). Septicaemic glanders is a rare manifestation. In human glanders, complete blood count (CBC) aberrations do not always occur and, if present, typically reflect neutrophilia, but occasionally neutropenia is reported. Blood chemistries surprisingly often have no abnormal or diagnostically relevant values (Carr & Waag, 2007).

B. mallei is infectious in aerosol and therefore represents a possible threat for intentional exposure. As glanders is currently rare in western countries, diagnosis may be delayed simply because relevant assays are not widely available. When specific assays are conducted in an effort to diagnose
glanders, serology may be complicated by cross-reactivity between \( B. \) \textit{mallei} and \( B. \) \textit{pseudomallei} antisera. Although PCR assays for \( B. \) \textit{mallei} have been available since the late 1990s, a search of the medical literature reveals no documentation of their use in a comparative study (Brook \textit{et al.}, 1997; Merritt \textit{et al.}, 2006). In the study reported here, PCR, culture and histopathology were compared for diagnostic applicability.

Glanders has been extensively modelled in rodents, with hamsters demonstrating high susceptibility similar to equids (Fritz \textit{et al.}, 2000). Mice have proven to be valuable models for Burkholderia vaccine development (Fritz \textit{et al.}, 2000; Jeddeloh \textit{et al.}, 2003; Lever \textit{et al.}, 2003; Ulrich \textit{et al.}, 2005).

Studies of glanders in non-human primates for purposes of developing diagnostics and diagnostic algorithms for human cases have not been done formally or with regard to developing an animal model. There has been an initial observational assessment of the pathogenesis of glanders in rhesus macaques (Miller \textit{et al.}, 1948); however, the mode of exposure in this case was subcutaneous inoculation. This early study indicated that rhesus macaques, like humans, are not as susceptible as species such as hamsters and equids. There have been some studies of immunological effects of glanders in hamadryas baboons; however, the details of these studies are vague in terms of animal model development (Khomiakov \textit{et al.}, 1998; Manzeniuk \textit{et al.}, 1996, 1997, 1999). In these reports, subsequent to either subcutaneous or intravenous exposure (it is not entirely clear which), there was mention of lymph node oedema, purulent and/or haemorrhagic lymphadenitis, and lymphoid depletion, as well as possible indications of toxic shock, i.e. lung, liver and kidney oedema and hyperaemia. However, these clinical features or diagnostic indicators were not thoroughly detailed, whilst the focus appeared to be on other factors such as blood hormone and free major histocompatibility complex molecule levels (Manzeniuk \textit{et al.}, 1997). One item of note from the Russian reports is that \( B. \) \textit{mallei} ‘strain Ts5’ at \( 2.5 \times 10^6 \) c.f.u. was lethal for hamadryas baboons by 5 days post-exposure (p.e.) (Khomiakov \textit{et al.}, 1998). Thus, the study reported here is the first controlled study of the early pathogenesis of aerosol-exposure glanders in a non-human primate species that is commonly used in preclinical studies. The primary purpose of the study was to establish an archive of tissues and other biological samples for further diagnostic development, and as such, allowed for an initial assessment of selected, currently available diagnostics, as well as descriptive pathology that may potentially contribute to animal model development in general. Whilst serology is often valuable in diagnostics for both glanders and melioidosis, the focus of the work described here was rapid diagnostics in the acute phase of the disease, at which time serology would not be expected to be of value.

### METHODS

**B. mallei strain and culture.** \( B. \) \textit{mallei} strain ATCC 23344, China 7 strain was cultured on Difco tryptone with 4 % glycerol, 15 \( \mu \)g polymyxin B ml\(^{-1}\) and 32 \( \mu \)g ampicillin agar ml\(^{-1}\) for 18 h. A small number of colonies was picked from a plate and suspended in glycerol tryptone broth and this method provided sufficient c.f.u. to expose all animals.

**Animals, aerosol exposure, sampling, necropsy and tissue collection.** Twenty-four adult rhesus macaques (\textit{Macaca mulatta}) from various sources were utilized for this study after a quarantine and acclimation period of \( > 1 \) year. Rhesus macaques were chosen in light of the early reports of pathogenesis that appeared to indicate consistency with human disease (Miller \textit{et al.}, 1948); no information on an established non-human primate animal model could be found in the literature. Twelve rhesus macaques were assigned to this study and 12 were used as ‘historical controls’ from a previous study in which all handling, conditions and time points were identical, thus eliminating unnecessary animal use. All animals were surgically implanted with TA-D70 temperature and activity telemetry transmitters (Data Sciences International). Experimental animals were subjected to aerosol exposure of \( B. \) \textit{mallei} organisms diluted in glycerol tryptone broth. Historical controls were exposed to saline solution only. Historical control animals served as a baseline for PCR, haematology, serum chemistry and telemetry (body temperature and activity) data. Temperature and activity data were read every 15 min for 1 week before exposure and until euthanasia at study end. For direct assessment of body temperature spikes, fever was defined as a repeated body temperature measurement \( \geq 39.5 \, ^oC \), in accordance with the institute animal care standard operating procedure. Graphical comparisons of the diurnal rhythm were made with pre-exposure temperature measurements of individual monkeys as well as with historical controls. Unexposed rhesus macaques demonstrate a daily temperature swing of \( \sim 1.5–2 \, ^oC \), with the peak at approximately mid-day and the nadir during the night.

CBCs and blood chemistries were performed 7, 14 and 30 days before exposure and on the day of euthanasia (note that reference ranges were originally based on published data but were continually adjusted based on institute observations and as such represent local parameters that may differ from other laboratories). Swab samples were taken from the pharynx and nares, and broncho-alveolar lavage (BAL) was performed immediately before exposure to obtain animal-matched negative control samples for PCR analysis.

Experimental animals were exposed separately to \( B. \) \textit{mallei} in a dynamic head-only exposure chamber that was controlled by an automated bioaerosol exposure system (Hartings & Roy, 2004; Dabisch \textit{et al.}, 2010). Prior to exposure each animal was anaesthetized by intramuscular injection with 9 mg ketamine kg\(^{-1}\) and 0.1 mg acepromazine kg\(^{-1}\). Each animal was placed into a head-out plethysmography chamber where the minute volume (MV) was measured for 3 min (BioSystem XA; Buxco). The mean MV obtained was used to adjust the exposure time to achieve the same target dose for all animals; the exposure times ranged between 8 and 12 min. Small particle aerosols were generated from 10 ml \( B. \) \textit{mallei} in glycerol tryptone broth using a 3-Jet Collison nebulizer (BG1). A sample of the aerosol atmosphere was collected in 10 ml glycerol tryptone broth using an all-glass impinger (model 7541; Ace Glass); to reduce foaming during collection, 40 ml antifoam (Antifoam Y-30; Sigma-Aldrich) was added. Immediately following the exposure, all-glass impinger samples were titrated to determine the concentration of the aerosol atmosphere. The respiratory deposition fraction for the aerosol presented was assumed to be 100 %; therefore, individual presented doses were calculated as the product of the mean aerosol concentration, the MV and exposure duration.
Research was conducted under an Institutional Animal Care and Use Committee approved protocol in compliance with the Animal Welfare Act, federal statutes and regulations relating to animal-use experiments, and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted was fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

There were three non-human primates per group with one group euthanized per day on days 1, 3, 5 and 7 p.e. The time points were selected to represent an early snapshot of pathogenesis and organism dissemination for diagnostics development. On the day of euthanasia, CBCs and serum chemistries, swab sampling (pharynx and nares), and BAL were performed. During necropsy, organs and tissues were examined grossly, and representative samples (from the same site from each animal, regardless of any gross pathology) were collected for histopathological evaluation. Tissue samples collected for evaluation included: tongue, tonsil, salivary gland, nares, lip, larynx, trachea, oesophagus, thyroid gland, parathyroid gland, aorta, thymus, lungs, heart, liver, gallbladder, kidneys, urinary bladder, pituitary gland, adrenal glands, spleen, bone marrow, axillary lymph node, inguinal lymph node, tracheobronchial lymph node, mesenteric lymph node, mandibular lymph node, stomach, pylorus, duodenum, pancreas, oesophagus, thyroid gland, parathyroid gland, aorta, thymus, lungs, heart, liver, gallbladder, kidneys, urinary bladder, pituitary gland, adrenal glands, spleen, bone marrow, axillary lymph node, inguinal lymph node, tracheobronchial lymph node, mesenteric lymph node, mandibular lymph node, stomach, pylorus, duodenum, pancreas, jejunum, ileum, ileocaecal junction, caecum, colon, haired skin, mammary gland, reproductive organs, skeletal muscle, sciatic nerve, brachial plexus, eyes and brain.

Diagnostics. Various tests were conducted in order to assess tissue distribution of *B. mallei* and the ability to detect the bacteria or its DNA in various samples.

Bacterial culture. Roughly 1 g tissue sections (lung, liver and spleen) were ground with a manual tissue grinder and diluted to a 10% (w/v) concentration in PBS. Pharyngeal and nasal swabs were immersed in 1 ml PBS. EDTA anti-coagulated blood, serum and BAL wash fluid were evaluated without further manipulation before serial dilution. Serial 10-fold dilutions were cultured on Difco tryptone with 4% glycerol, 15 μg polymyxin B ml⁻¹ and 32 μg ampicillin agar ml⁻¹, and incubated in 5% CO₂ at 37 °C in a humid incubator overnight. Cultures were evaluated for colonies demonstrating the phenotypic characteristics of *B. mallei*, i.e. relatively small, but variably sized, whitish to yellow, round, entire colonies.

Histopathology. Tissue samples were immersion fixed in 10% neutral-buffered formalin for 21 days, then trimmed and processed routinely. Tissues were embedded in paraffin, sectioned at 5–6 μm thickness, mounted on glass slides, and then stained with haematoxylin, dehydrated and coverslipped with Permount.

Immunohistochemistry. Unstained tissue sections were deparaffinized, rehydrated, subjected to methanol hydrogen peroxide block for 30 min and then rinsed in PBS. A serum-free protein block plus 5% normal goat serum was applied for 30 min (EnVision Plus System; Dako). A rabbit polyclonal antibody was diluted 1:500 and incubated at room temperature for 60 min (*B. mallei* zB151-2 polyclonal rabbit antibody; courtesy Dr Dave Waag, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA). A polymer-labelled horseradish peroxidase anti-rabbit secondary antibody was applied for 30 min at room temperature (EnVision Plus System; Dako). All sections were exposed to 3,3'-diaminobenzidine for ~5 min, rinsed, counterstained with haematoxylin, dehydrated and coverslipped with Permount. Cross-reactivity and background staining were evaluated by testing the antibody against normal or uninfected tissues. Known tissues infected with *Yersinia pestis*, *Francisella tularensis*, *Bacillus anthracis* and monkey poxvirus were also evaluated. There was no cross-reactivity or non-specific staining identified.

PCR. DNA from the swab diluents, BAL wash fluid, tissue samples, whole blood and serum was extracted using a Qiagen DNA blood kit according to the manufacturer’s instructions. PCR was performed on a LightCycler 1.5 Real-Time PCR instrument (Roche) with an assay specific for *B. mallei*, as well as a more sensitive assay that detected both *B. mallei* and *B. pseudomallei* (IS407A insertion element) (Ulrich et al., 2006). Assays were carried out in 20 μl volumes. Each assay contained 1× PCR buffer (50 mM Tris, pH 8.3, 250 μg BSA ml⁻¹) (Idaho Technology), 0.2 mM dNTP mix, 1.0 U Platinum Taq DNA polymerase (Invitrogen) and 5 mM MgCl₂. Previously determined optimal concentrations of primers and probe were added, and 15 μl master mix was distributed to reaction tubes (Ulrich et al., 2006). An aliquot of 5 μl control/template/sample DNA was added just before analysis on the instrument. Thermal cycling conditions were standardized for both assays, and consisted of one cycle at 95 °C for 2 min, followed by 45 cycles of 95 °C, 5 s and 60 °C for 20 s. Fluorescence readings were taken at the end of each 60 °C step. Each reaction capillary tube was read in channel 1 (F1) with data analysed by LightCycler Data Analysis software version 4.0. Samples were run in triplicate and considered positive if they crossed the software-generated threshold by cycle 35 (Tables 1).

**RESULTS**

**Culture and clinical findings**

The mean dose of *B. mallei* was 8.91×10⁵ c.f.u. (range: 2.86×10⁵−1.59×10⁶ c.f.u.) (Table 2). *B. mallei* could only be detected by culture in very low numbers from a minor percentage of animals in pharyngeal

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**Table 1.** Real-time PCR primer and probe sequences for the *Burkholderia* genus screening assay (‘BPISO’/‘BMISO’) and *B. mallei*-specific assay (‘BMSEC’)

<table>
<thead>
<tr>
<th>Target</th>
<th>Amplicon size (bp)</th>
<th>Primer</th>
<th>Sequence</th>
<th>Final concentration (μM)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS407A (insertion element)</td>
<td>100</td>
<td>BPISO2F1</td>
<td>CTCGAGGTGGAGAATGCCTC</td>
<td>0.5</td>
<td>1 fg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BPISO2R1</td>
<td>CGCTCAGGAGATGTTGACCTTC</td>
<td>0.5</td>
<td>1 fg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BPISO2P2</td>
<td>TGGGCGAAGACATGCTGATATGG</td>
<td>0.1</td>
<td>1 pg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMSEC3-F9</td>
<td>CAGTTGATCTCCACC</td>
<td>0.5</td>
<td>1 pg</td>
</tr>
<tr>
<td>bimAlag (actin polymerization)</td>
<td>96</td>
<td>BMSEC3-B144</td>
<td>TGTCTTGGTTGAGGCTAGA</td>
<td>0.5</td>
<td>1 pg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMSEC3-P104R-MGB</td>
<td>CATAGGATGTAAGACCAAT</td>
<td>0.15</td>
<td>1 pg</td>
</tr>
</tbody>
</table>
(4/12) and nasal swabs (1/12) immediately post-exposure (i.e. as the animal was being removed from the exposure chamber), suggesting that the inoculum was almost entirely inhaled or cleared by other mechanisms. Culture results at the time of euthanasia varied, but generally indicated initial proliferation of the organism in the lungs, followed by dissemination to the spleen and liver (Table 3). At later time points during the study, it was possible to obtain lung samples in non-abscessed areas composed of healthy tissue from which organisms could not be recovered. That is, initially, the entire lung seemed uniformly affected, whereas, later, abscess formation began and intervening tissue appeared less affected. Also, at later time points, organisms were occasionally isolated from nasal and pharyngeal swabs, whereas this was not the case at earlier time points.

Clinically, within 2 days p.e., animals were generally depressed and showed signs of respiratory illness with laboured and rapid breathing, but never became moribund. Activity levels were not obviously different after exposure. One animal appeared to display meningism (nuchal rigidity and photophobia) without any other clinical signs. Body temperature aberrations began in most animals on day 3 p.e. and persisted thereafter (Table 2). In some cases, there was not fever according to the criteria used in this study; however, there was obvious disruption of the normal diurnal temperature swing of ~2 °C from a peak about mid-day to a nadir ~12 h later. Two of three of the animals euthanized on day 3 p.e. became intermittently febrile (temperatures >39.5 °C for repeated measurements) on day 3 p.e. One of three animals euthanized on days 3 and 5 p.e. maintained normal temperature and diurnal temperature patterns subsequent to exposure. Of the other two animals euthanized on day 5 p.e., one developed intermittent fever on day 3 p.e. and showed loss of the normal diurnal temperature variation. A second animal euthanized on day 5 p.e. did not have fever according to the criteria used in this study, but its

**Table 2. Clinical signs and haematology results of rhesus macaques aerosol exposed to B. mallei at days 1, 3, 5 and 7 p.e.**

<table>
<thead>
<tr>
<th>Euthanasia day p.e.</th>
<th>Dose (c.f.u.)</th>
<th>Clinical signs</th>
<th>CBC/chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.47 × 10⁵</td>
<td>Moderate neutrophilia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.53 × 10⁵</td>
<td>Moderate neutrophilia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.22 × 10⁵</td>
<td>Mild neutrophilia</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.83 × 10⁵</td>
<td>Fever day 3</td>
<td>Mild neutrophilia</td>
</tr>
<tr>
<td></td>
<td>1.49 × 10⁶</td>
<td>Neutropenia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.56 × 10⁶</td>
<td>Fever day 3</td>
<td>Mild neutrophilia</td>
</tr>
<tr>
<td>5</td>
<td>2.86 × 10⁵</td>
<td>Fever day 3 loss of diurnal rhythm</td>
<td>Neutropenia</td>
</tr>
<tr>
<td></td>
<td>6.63 × 10⁵</td>
<td>Persistently higher temperature</td>
<td>Mild neutrophilia</td>
</tr>
<tr>
<td></td>
<td>8.87 × 10⁵</td>
<td></td>
<td>Mild neutrophilia</td>
</tr>
<tr>
<td>7</td>
<td>4.70 × 10⁵</td>
<td>Fever day 3, meningism?</td>
<td>Mild neutrophilia</td>
</tr>
<tr>
<td></td>
<td>1.44 × 10⁶</td>
<td>Fever day 3</td>
<td>Mild neutrophilia</td>
</tr>
<tr>
<td></td>
<td>1.49 × 10⁶</td>
<td>Loss of diurnal rhythm progressing to fever by day 7</td>
<td>Moderate neutrophilia</td>
</tr>
</tbody>
</table>

**Table 3. PCR and conventional bacterial culture results, from various tissues of rhesus macaques aerosol-exposed to B. mallei, on day 1, 3, 5 or 7 p.e.**

PCR results are qualitative and include an assay specific for *B. mallei* (left column) and a *Burkholderia* genus screening assay (right column) under 'PCR'. PCR data represent the number of animals positive out of a total of three. Culture results are also number positive out of a total of three, with the mean bacterial load [(g tissue)⁻¹ or (ml fluid)⁻¹] from rhesus macaques in parentheses. See Methods. Numbers represent log₁₀c.f.u. *B. mallei* recovered [limit of detection: 100 organisms: log₁₀(100) = 2]; ‘0’ in a given cell represents no PCR detection or no organisms recovered.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>PCR</td>
<td>Culture</td>
<td>PCR</td>
<td>Culture</td>
</tr>
<tr>
<td>Lung</td>
<td>3</td>
<td>2 (3.52)</td>
<td>3</td>
<td>3 (4.58)</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>BAL</td>
<td>2</td>
<td>3</td>
<td>1 (2.00)</td>
<td>1</td>
</tr>
<tr>
<td>Blood</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Serum</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Pharyngeal swab</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nasal swab</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>
diurnal rhythm ceased and its temperature remained steady at ~38 °C. Two of the three animals euthanized on day 7 p.e. had intermittent fever on day 3 p.e., then temperatures dropped back to <39.5 °C, but diurnal rhythm variations were substantially reduced. The third animal had a total loss of diurnal rhythm with slowly increasing temperature finally rising to >39.5 °C on day 7 p.e. Control animals never became febrile, and never showed changes in diurnal rhythm.

**CBC results**

Whilst there was wide biological variation seen in the challenged animals, most responded with an overall increase in neutrophils, from day 1 p.e. and throughout the 7 day study period (Table 2). On day 1 p.e., all animals showed a mild-to-moderate mature neutrophilia (total white blood cells 12.9, 21.6 and 23.8 × 10³ μl⁻¹, reference range 4.5–10.5 × 10³ μl⁻¹, with manual differentials showing 80, 89 and 89 % segmented neutrophils, respectively). On both days 3 and 5 p.e., one of three animals showed absolute neutropenia (210 and 253 neutrophils μl⁻¹, respectively). Also on day 3 p.e., the other two animals showed mild mature neutrophilia (14.3 and 18 × 10³ μl⁻¹ total white blood cells with 78 and 86 % segmented neutrophils, respectively). On day 5 p.e., one animal showed mild mature neutrophilia (13 × 10³ μl⁻¹ total white blood cells, with 67 % segmented neutrophils), whilst the other had a normal total white blood cell count. On day 7 p.e., one animal had normal CBC parameters, whilst the others showed mild-to-moderate mature neutrophilia (17 and 24.8 × 10³ μl⁻¹ total white blood cells). There were no serum chemistry aberrations observed in experimental animals. Haematological and serum chemistry values for the control animals remained within the reference ranges or were consistent with previous measurements from days 7, 14 and 30 prior to mock exposure.

**PCR results**

In most cases, PCR results mirrored culture results, although with possible indication of greater sensitivity, perhaps due to the ability of PCR to detect DNA of dead organisms that would not produce a colony (Table 3). For instance, the *Burkholderia* genus screening assay was positive in all animals, whilst culture failed to detect organism from lung samples taken on day 7 p.e. PCR was positive in a subset of blood and serum samples on days 3, 5 and 7 p.e., and was more frequently positive in all sample types relative to culture results (day 7 p.e. PCR positive results from blood and serum were for the genus-level screening assay only).

**Gross findings**

No gross lesions were observed in control animals. In infected animals, gross lesions most consistently occurred in the respiratory tract and lymph nodes. The lesions in the respiratory tract varied, but included diffuse red mottling and congestion in the lungs in six of the nine animals euthanized on days 3, 5 and 7 p.e. The affected lungs usually did not collapse upon opening the thoracic cavity. These six animals had additional gross pulmonary lesions depending on the time of death that included fibrinous pleural adhesions (2/9), suppurative abscesses (3/9) and pulmonary necrosis along the dorsal surface of the lungs (1/9). Pleural adhesions were present, predominantly on days 3 and 5 p.e., whilst abscesses and necrosis did not develop until days 5 and 7 p.e. The three day 1 p.e. animals had pinpoint haemorrhages on the lungs as the only pulmonary change. The tracheobronchial and mesenteric lymph nodes were mildly enlarged and oedematous in most animals (10/12 and 9/12, respectively). One animal, euthanized on day 5 p.e., had haemorrhagic gastritis.

**Histological findings**

The most significant histopathologic findings in *B. mallei*-exposed animals (6/12) were present in the lungs on days 3, 5 and 7 p.e., and were composed of one or more of the following lesions: abscesses, necrosis and pleural adhesions. Lesions on day 1 p.e. consisted of acute inflammation in alveolar airspaces composed predominantly of neutrophils with rare macrophages and mild-to-moderate amounts of oedema. Inflammation was typically noted in alveoli adjacent to the terminal bronchioles or in the terminal bronchioles themselves. The foci extended no more than four to five alveolar spaces in diameter (Fig. 1a). There was no significant necrosis of alveolar septae at this stage. Mild bronchoalveolar-associated lymphoid tissue hyperplasia was noted in all the rhesus macaques. Bronchial and bronchiolar Airways were not affected at this stage.

The remaining nine animals (days 3, 5 and 7 p.e.) had variable and chronologically progressive foci of necrotizing to necrosuppurative bronchopneumonia, pleuritis and variable suppurative bronchitis (Fig. 1c). These foci were composed of numerous degenerate neutrophils with fewer, often necrotic leukocytes (most likely macrophages), and low numbers of lymphocytes with fibrin, oedema and obliterated (necrotic) alveoli. Areas of inflammation increased progressively with time; however, the actual foci of destruction and necrosis of the lungs were variable between individual rhesus macaques (ranging between 0.5 and 1.2 cm in diameter) and areas between the necrotizing foci were often unaffected. Interestingly, the animal exhibiting apparent meningism on day 7 p.e. had no pulmonary lesions, but was the only animal with focal neutrophilic leptomeningitis (in one section of the parietal lobe, not affecting the underlying neuropil). This animal had no other lesions. The meningitis was composed of degenerate neutrophils and fewer lymphocytes that minimally expanded the subarachnoid space.

Lymph node follicular depletion was noted in half of the animals (6/12) with the change noted in the day 3–7 p.e. range. Overall, there was a mild-to-moderate decrease in the number of lymphocytes in the lymphoid follicles, and a minor increase in paracortical and medullary
histiocytes with a concurrent oedematous expansion of the sinuses. On day 3 p.e. and on day 7 p.e., rhesus macaque had a mild depletion of lymphocytes in the splenic white pulp.

**Histochemical and immunohistochemical evaluation of B. mallei**

Tissues from four animals were selected to attempt to identify *B. mallei* using Giemsa, Lillie Twort Gram stain and immunohistochemistry. Two cases were chosen from day 1 p.e. on the basis of earliest possible detection and day 5 p.e. based on differing severities of pulmonary necrotic foci (noted grossly). Lungs, mediastinal lymph nodes and the liver were examined to attempt identification of the agent. The liver was examined due to minimal non-specific inflammation often considered an incidental or background lesion (Chamanza *et al.*, 2010).

Immunohistochemistry provided the earliest and best method to identify pathogen antigens by light microscopy. All four cases demonstrated strong immunoreactivity within the cytoplasm of neutrophils, macrophages and necrotic debris in the lungs. Small numbers of immunoreactive inflammatory cells were readily identifiable in the alveoli in day 1 p.e. animals, but not within alveolar septae (Fig. 1b). The abscesses containing suppurative material demonstrated strong immunoreactivity in the necrotic debris and inflammatory cells (Fig. 1d). Suppurative inflammation along the ciliated border of the bronchiolar epithelium was immunoreactive, but not within the ciliated epithelial cells (Fig. 1e).

Fig. 1. (a) Lung: multiple small foci of neutrophilic inflammation accumulated in the terminal bronchioles and alveoli (day 1 p.e.). Stain: haematoxylin and eosin; bar, 64 μm. (b) Lung: low numbers of immunoreactive inflammatory cells within alveolar airways (day 1 p.e.). Stain: immunohistochemistry; bar, 64 μm. (c) Lung: severe pulmonary necrosuppurative foci with necrosis of pulmonary architecture, oedema and fibrinous pleuritis (day 5 p.e.). Stain: haematoxylin and eosin; bar, 300 μm. (d) Lung: inflammatory cells and necrotic debris are immunopositive within the necrosuppurative pulmonary foci whilst surrounding tissue often has no positive immunoreactivity (day 5 p.e.). Stain: immunohistochemistry; bar, 125 μm. (e) Lung: the suppurative exudate in bronchi and cilia of bronchiolar epithelial cells is strongly immunoreactive, but underlying tissue is not (day 5 p.e.). Stain: immunohistochemistry; bar, 50 μm. (f) Lung: low numbers of intracellular and extracellular bacteria by Giemsa stain; inset has a higher magnification of these bacteria (arrows) (day 5 p.e.). Bar, 25 μm.
Giemsa stain can be an alternative diagnostic tool when immunohistochemistry is not available. Bacteria were rarely identified in the lungs of one of the two day 1 p.e. animals. Low numbers of bacteria were detectable in the lungs of the day 5 p.e. animals within the previously described inflammatory cells and extracellular necrotic debris (Fig. 1f). Gram stain proved to be of little diagnostic value in this study; even in the most severe lesions evaluated, bacteria were rarely discernible from necrotic debris.

**DISCUSSION**

The study reported here was intended to serve as a step toward diagnostics assay development and algorithm refinement for early, acute glanders, especially in a situation of multiple cases in unexpected circumstances. Aliquots of all samples were archived and may serve as a standardized resource for further assay development. Additionally, the effort resulted in an opportunity to serially study and report on early disease progression as assessed by dissemination of the organism, and gross and histopathology over the first 7 days p.e.

There was no obvious correlation between the presented dose and lesions observed. All rhesus macaques were affected, with moderate-to-severe, but variable, lesions by days 3, 5 and 7 p.e. Variability may have resulted in part from the varying genetic backgrounds of the animals as they were acquired from at least three different sources. There is also the slight possibility that some animals could have been previously exposed to *B. mallei*, which could have affected their immune response.

In general, the gross pathology was consistent with case reports of human glanders, which are also similar to clinical disease in donkeys and mules (Amemiya et al., 2006; Carr & Waag, 2007; Dvorak & Spickler, 2008). The primary initial manifestation in rhesus macaques in the study reported here was bronchopneumonia, followed by lung abscessation. If this is representative of human disease after exposure by this route, then it is probable that patients would seek medical care and would receive a chest radiograph. In such cases, swab samples as well as sputum might be analysed and lung fine-needle aspiration or even biopsy might rarely be done in order to achieve a definitive diagnosis. The results of this study support the possibility that culture of non-invasive samples could provide a diagnosis and PCR of such samples would provide a more rapid diagnosis.

Notably, one rhesus macaque in the study reported here did not develop acute pneumonic lesions; however, it was the only animal to demonstrate neutrophilic meningitis, illustrating and emphasizing the variable presentation that can occur in glanders cases. Meningitis has been previously described in a hamster study, but was accompanied by lesions in other organ systems (Fritz et al., 1999). It is possible that this was an ascending infection directly through the olfactory bulb, as no indication of pneumonia or other systemic infection was noted grossly in this animal and organism was only recovered by culture from the nasal swabs. However, there was evidence of dissemination based on PCR, so haematogenous infection of the meninges is another possibility, albeit less likely.

Interestingly, the organism may tend to proliferate in the lung regardless of the route of infection as pulmonary lesions were noted by day 3 p.e. in hamsters exposed by intraperitoneal injection (Fritz et al., 1999). Therefore, diagnostics based on respiratory tract samples may be diagnostic for glanders in general, rather than only in the case of aerosol exposure.

In addition to pulmonary pathology, there was occasional mild lymphadenopathy, both in lymph nodes associated with the respiratory tract as well as some mesenteric lymph nodes in rhesus macaques in the study reported here. Enlarged lymph nodes could also be a source of diagnostic material in human cases if human pathology is consistent with that noted in the study reported here; lymphadenopathy is commonly noted in the case reports available in the literature (Carr & Waag, 2007; Srinivasan et al., 2001). The observations in the rhesus macaque as reported here are more consistent with human case reports as opposed to models such as the hamster, in which pyogranulomatous lymphadenitis has been observed early in the course of infection (Fritz et al., 1999).

In terms of diagnostics involving histopathological samples, immunohistochemistry appears to be the most effective method to detect the presence of bacterial antigens within the first day post-exposure (during subclinical presentation and in the absence of gross lesions). *B. mallei* stains well with Giemsa, but must be in significant numbers in order to be accurately diagnosed, i.e. its presence was only observed with gross lesions (pulmonary abscesses). Gram stains were not diagnostic in this study, as in others (Fritz et al., 2000).

Although the CBC data in this study were broadly consistent with what might be expected in a bacterial infection, results were variable and would not have provided useful diagnostic information. Importantly, no serum chemistry abnormalities were noted – whilst consistent with human glanders case reports, this would not contribute to suspicion of bacterial infection. However, gross pathology and clinical signs of depression with occasional laboured breathing, if they occurred in humans, could lend credence to a suspicion of bacterial pneumonia. Therefore, taken together, the clinical signs and haematology parameters, if consistent in human cases with the data presented here, probably would result in bacterial culture attempts, to include blood culture in many settings. Based on the limited available case reports, it is expected that human cases would present similarly to the animals studied here (Carr & Waag, 2007; Srinivasan et al., 2001). However, culture of non-invasive samples (especially blood) was generally not useful for diagnosis early in the course of infection. PCR of BAL fluid, nasal and pharyngeal swabs, blood, and serum was positive in a substantial proportion of cases.
throughout the 7 day study (e.g. 7/12 BAL samples), and may therefore represent a valuable addition to the diagnostic repertoire for glanders when an aspirate or biopsy is not indicated. These results indicate that PCR could be especially useful as a tool to rapidly diagnose an outbreak, i.e. a group of cases with similar history and onset of illness, when an aerosol exposure is suspected. It is essential to note that during necropsy in the study reported here, the pathologist strictly adhered to protocol in terms of the sampling site within each organ. This is particularly important with regard to the lung, where obvious abscesses had developed at later time points in this study. Clearly, abscesses would be the target of any ante-mortem diagnostic aspirate in a human case as well as post-mortem sampling and would almost invariably have been positive both by culture and PCR in this study. Thus, negative results especially from lung tissue must be assessed as not necessarily suggesting that the organ as a whole did not contain viable or detectable organisms (as the course of glanders progresses, abscesses develop and intervening tissue may be normal).

In general terms, the results of the study reported here are consistent with human disease and call for further studies to more definitively establish the rhesus macaque as a model of human glanders (Carr & Waag, 2007; Srinivasan et al., 2001). A limitation of this study for model development was its short duration and one time point per animal haematology. As a result, whilst there was clearly disease progression within the time frame of this study, there was no basis to predict the final outcome, i.e. it is entirely possible that most if not all of these animals could have ultimately recovered.

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REFERENCES


