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In situ hybridization to detect and identify *Burkholderia pseudomallei* in human melioidosis

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Burkholderia pseudomallei **causes a potentially fatal infection called melioidosis. We have developed a nonfluorescent, colorimetric** ***in situ*** **hybridization assay using a specific probe to target 16s rRNA of** ***B. pseudomallei*** **in formalin-fixed, paraffin-embedded infected tissues for diagnostic purposes and to study infectious disease pathology. A 63-base pair DNA probe was synthesized and labeled with digoxigenin by PCR. Probe specificity was confirmed by BLAST analysis and by testing on appropriate microbial controls. The** ***in situ*** **hybridization assay was specifically and consistently positive for** ***B. pseudomallei*****, showing strongly and crisply stained, single bacillus and bacilli clusters in mainly inflamed tissues in seven human acute melioidosis cases and experimentally infected mouse tissues. Intravascular and extravascular bacilli were detected in both intracellular and extracellular locations in various human organs, including lung, spleen, kidney, liver, bone marrow, and aortic mycotic aneurysm, particularly in the inflamed areas. Intravascular, intracellular bacteria in melioidosis have not been previously reported. Although the identity of infected intravascular leukocytes has to be confirmed, extravascular, intracellular bacilli appear to be found mainly within macrophages and neutrophils. Rarely, large intravascular, extracellular bacillary clusters/emboli could be detected in both human and mouse tissues.** ***B. cepacia*** **and non-*****Burkholderia*** **pathogens (16 microbial species) all tested negative. Nonpathogenic** ***B. thailandensis*** **showed some cross-hybridization but signals were less intense. This** ***in situ*** **hybridization assay could be usefully adapted for** ***B. pseudomallei*** **identification in other clinical specimens such as pus and sputum.**
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Burkholderia pseudomallei is a motile, Gram-negative, non-spore-forming soil bacterium that is readily recovered from water and wet soils in endemic areas of Southeast Asia and Northern Australia.[1] It causes melioidosis, an infectious disease of major public health importance. *B. pseudomallei* is responsible for 20% of community-acquired septicemia and 40% of sepsis-related deaths in Northern Thailand,[2] whereas in northern Australia, it accounts for 6% of all bacteremias and 32% of community-acquired bacteremic pneumonia.[3] In Malaysia, hundreds of cases have been reported in the past decade.[4] *B. pseudomallei* has demonstrated high antibiotic resistance[5] and has been classified as a category B potential bioterrorism agent by the United States Centers for Disease Control and Prevention.

It is believed that melioidosis is greatly underdiagnosed, and the true incidence and epidemiology of the disease in endemic areas remains unknown.[4] This is partly because melioidosis, often described as a great mimicker, has protean clinical manifestations, including aortic aneurysm.[6,7] Needless to

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say in infectious diseases, identification of the causative agent and its timeliness is of paramount importance in determining treatment outcome and prognosis. Traditional laboratory diagnosis of melioidosis using the gold standard bacterial culture requires prolonged incubation and laborious biochemical methods such as the API 20NE for confirmation. Molecular techniques using conventional, multiplex, or real-time PCR[8–10] and DNA microarrays[11] have greatly improved the speed of diagnosis. However, these newer methods may suffer from easy contamination leading to false positive results.

The *in situ* hybridization technique, a molecular assay that uses labeled probes to hybridize with nucleotide targets, can be readily adapted for the detection and identification of microorganisms. We believe the ability to visualize bacilli with this technique could reduce the likelihood of false positive results inherent in other molecular assays. *In situ* hybridization has been successfully applied to detect various bacteria, including *Helicobacter pylori*,[12] *Haemophilus influenza*,[13] and *Klebsiella pneumoniae*.[14] Recently, Hagen *et al*[15] reported a fluorescence *in situ* hybridization technique using a set of oligonucleotide probes for the identification of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* in bacterial cell smears and experimentally infected mouse tissues. Although the oligonucleotide probes were not specific to *B. pseudomallei*, they were able to differentiate *B. pseudomallei*, *B. mallei*, and *B. thailandensis* using a multiple-probe exclusion approach.

We have developed a larger DNA probe that targets the 16s rRNA of *B. pseudomallei* for its identification in an *in situ* hybridization assay that uses a nonfluorescent, colorimetric detection method. The purpose is to improve the diagnosis of melioidosis and to develop a research tool to study the pathology and pathogenesis in infected tissues from human cases as well as animal models. As the clinical diagnosis of melioidosis is often not suspected initially, optimal fresh specimens for bacterial culture and molecular investigations are often not sent at the time of biopsy for histopathological examination. As the *in situ* hybridization technique can be done on standard formalin-fixed, paraffin-embedded tissues, it can be readily adapted for routine pathological diagnosis. In contrast to fluorescence *in situ* hybridization, a nonfluorescent, colorimetric *in situ* hybridization has the advantage of facilitating light microscopic assessment of the surrounding tissues in which the bacilli are found without the problems of poor background details and autofluorescence as previously reported.[15] Herein, we report the demonstration of whole bacilli in infected human and mouse tissues using this technique that is potentially useful for diagnosis and to further understand the spread and pathogenesis of *B. pseudomallei* in the host.

Materials and methods

Preparation of Probe Targeting 16s rRNA

The rRNA sequences of *B. pseudomallei* (strain K96243, reference number: S000406475) and various other bacteria were downloaded from the RNA database project website (http://rdp.cme.msu.edu/). By aligning and comparing these sequences, we identified a 63-nucleotide region between nucleotides 955 and 1017 that appeared to be sufficiently specific and unique for *B. pseudomallei* for probe hybridization. This region had $<50\%$ sequence similarity to non-*Burkholderia* bacteria but shared 88.9% and 90.5% similarity to *B. cepacia* and *B. thailandensis*, respectively.

A forward primer 5′-ACCCTTGACATGGTCGG AAG-3′ and a reverse primer 5′-CTGTGCGCCG GTTCTCTT-3′ that flank this region were designed and synthesized for PCR use. These primers were checked by a BLAST analysis to confirm their specificity. A digoxigenin-labeled DNA probe (Patent pending: PI 2012700008) was made from the reverse-transcriptase PCR (RT-PCR) product of this region. Briefly, RNA was extracted from a pure colony of *B. pseudomallei* using TRIzol reagent (InvitroGen, USA). The RT-PCR was performed using the AMV RT-PCR kit (Promega, USA) with the reverse primer and RNA template to obtain cDNA. The PCR step uses the cDNA template and the HotStarTaq DNA Polymerase kit (QIAGEN, USA) performed in the Veriti 96 Wells Thermal Cycler (Applied Biosystems, USA) using a protocol of 95 °C for 10 min, 35 cycles at 94 °C for 1 min, 56 °C for 45 s, 72 °C for 45 s, and finally 72 °C for 10 min. The products were purified using the QIAGEN Gel Purification kit (QIAGEN), sequence-confirmed (FirstBase, Malaysia) and cloned using the pGEM-T Easy Vector system (Promega, USA). Finally, a second PCR that incorporates digoxigenin-11-dUTP (Roche, Germany)[16] was done using the cloned RT-PCR products as template and the same PCR protocol to produce the probe.

In Situ Hybridization Protocol

The *in situ* hybridization was performed as described by Ong *et al*[17] with some modifications. Briefly, bacterial smears and deparaffinized, rehydrated 3 μm tissue sections were pretreated sequentially with 0.2N HCl (20 min, room temperature), Proteinase-K (100 μg, 20 min, 37 °C), and lysozyme (1 mg/ml, 30 min, room temperature). The sections were then incubated overnight at 42 °C in standard hybridization buffer together with $\sim$1 ng/ml of probes. After the washing and blocking steps, anti-digoxigenin antibody (Roche) conjugated with alkaline phosphatase was added followed by the Liquid Permanent Red (Dako, USA) as substrate. The tissues were counterstained with Mayer's hematoxylin and mounted with Faramount

Table 1 Results of *in situ* hybridization specificity testing performed on control microorganisms

Microorganisms	Results	
	Microorganism cell smears on glass slides	*Formalin-fixed paraffinized microorganisms*
Gram-positive bacteria		
Lactobacillus sp.	–	ND
Streptococcus aureus	–	–
Gram-negative bacteria		
Burkholderia pseudomallei (2 strains)	+	+
Burkholderia thailandensis	+	+
Burkholderia cepacia	–	ND
Aeromonas hydraphila	–	–
Acinetobacter calcoaciniticus	–	–
Escherichia coli	–	–
Klebsiella pneumoniae	–	–
Neisseria gonorrhoeae	–	ND
Pseudomonas aeruginosa	–	ND
Proteus sp.	–	–
Salmonella enteriditis	–	–
Salmonella paratyphi	–	–
Shigella sonnei	–	–
Actinomyces spp.[a]	ND	–
Helicobacter pylori[a]	ND	–
Mycobacterium tuberculosis[a]	ND	–
Fungus		
Candida albicans	–	ND

Abbreviations: ND, not done; +, positive; –, negative.
[a]*Actinomyces spp*, *H. pylori*, and *M. tuberculosis* were human surgical biopsies in which these infections were confirmed.

(Dako) and systematically viewed under a light microscope.

In Situ Hybridization Controls

Three *Burkholderia* species, *B. pseudomallei* (two strains: K96243 and CMS), *B. cepacia*, and *B. thailandensis*, and 16 non-*Burkholderia* microorganisms were used as controls to test the specificity of the probe (Table 1). *B. mallei* was not included in the controls as the isolate was not available. All the microorganisms were grown overnight in LB broth, washed, formalin fixed, and resuspended in phosphate-buffered saline (PBS) before spreading onto silane-coated glass slides as bacteria cell smears. The smears were heat-fixed for 5 min before use. To prepare formalin-fixed, paraffin-embedded sections of the same bacteria cells, fresh, normal mouse liver and lung tissues were first incubated together with the bacteria in LB broth for 2.5 h, and then washed, formalin fixed, and routinely processed into paraffin blocks. The mouse tissues provided scaffolds for bacteria adhesion and penetration to minimize bacterial cell loss during tissue processing. Formalin-fixed, paraffin-embedded human tissues known to be infected by *Mycobacterium tuberculosis*, *Actinomyces spp.*, and *Helicobacter pylori* were also used as additional negative controls.

Human Melioidosis Cases

A total of seven human autopsy cases of acute melioidosis were studied (Table 2). Four were collected from 1978 to 1996 and archived in the Department of Pathology, Faculty of Medicine, University of Malaya. Three cases were referred from Hospital Queen Elizabeth, Sabah, Malaysia, for diagnostic purposes. Ethical clearance was obtained from the Ethics Committee of the University of Malaya Medical Center. All available tissue samples were tested and all cases (Table 2) were culture proven as melioidosis. Tissue sections (3 μm thick) from all available paraffin blocks were stained with hematoxylin and eosin and Brown–Hopps Gram stains as previously described,[18] and examined under a light microscope.

Experimentally Infected Mouse Tissues

Healthy Balb/c mice ($n = 15$) (obtained from Monash University Sunway Campus, Malaysia) were infected with $\sim 10^7$ CFU/ml of *B. pseudomallei* via the intraperitoneal (IP) route. Mock-infected mice ($n = 3$) were injected with PBS buffer. The mice were kept in an individual ventilated cage system (ITS, Italy) and observed daily. Tissues from dead mice were harvested, formalin fixed, and routinely processed to prepare paraffin blocks including 15 blocks each of spleen, lung, and liver, and 3 blocks each of stomach and kidney. All the tissues were cut into 3 μm thick sections and placed onto silane-coated glass slides for *in situ* hybridization. All the work involving bacteria and infected animals was done inside a Level II biosafety cabinet, and in accordance with the ethics code set by the Animal House, University of Malaya.

Results

Probe Specificity

The probe specificity test results on *B. pseudomallei*, *B. cepacia*, and *B. thailandensis* and 16 non-*Burkholderia* microorganisms are shown in Table 1. B. *pseudomallei* and *B. thailandensis* were both *in situ* hybridization positive in control bacterial smears and formalin-fixed, paraffin-embedded

Table 2 Clinical features and *in situ* hybridization results in seven autopsy cases of acute melioidosis

Case no.	*Sex/age (years)*	*Clinical features and diagnosis*	*Positive culture obtained from*	*Histopathological features*	*In situ hybridization results*
1	M/68	Had epididymo-orchitis, pus surgically drained. Died of septicemia despite antibiotics.	Blood, pus	Necrotizing inflammation in the lung with macrophages, neutrophils, and lymphocytes.[a]	Some small clusters and individual bacilli in the lung.
2	F/18	Had fever, joint pains, severe headache, and night sweats for 2 weeks. Developed watery diarrhea and epigastric pain and started on crystalline penicillin after admission. Died of septicemia.	Blood	Necrotizing inflammation with macrophage dominance and neutrophils and numerous 'giant cells' in liver and lung.[a]	A few bacilli in liver and lung.
3	M/36	Chest wall abscess 4 weeks before admission, treated but did not heal. Relapsing fever with chills, drowsiness, tachycardia, tachypnea, generalized lung rhonchi, and crepitations and hepatomegaly on admission. Died of acute fulminant melioidosis.	Blood, pus (lung, spleen, kidney and chest wall)	Necrotizing inflammation comprising mainly macrophages and neutrophils in lung, liver, and kidney, with some 'giant cells'.[a]	Numerous intra- and extracellular bacilli in the lung. Some bacilli also detected in liver and kidney.
4	F/19	Known case of diabetes mellitus, presented with fever, chills, and knee joint pain. Died of septicemia and respiratory failure.	Blood, Pus (lung)	Large areas of necrotizing inflammation in lung, comprising mainly macrophages and neutrophils, with pulmonary edema and hyaline membranes in some focal areas.	A few small clusters and individual bacilli in lung.
5	M/33	Had pneumonia for a month then developed multiorgan failure.	Lung and splenic swabs	Mild inflammation in the lung, spleen, bone marrow, and kidney with some macrophages and neutrophils observed. Multiple small foci of necrotizing inflammation with neutrophils and macrophages in liver.	Individual and diffuse clusters of bacilli in lung, liver, spleen, bone marrow, and kidney.
6	M/49	Known case of diabetes mellitus. Admitted for pneumonia, then discharged but did not improve. Readmitted in critical condition, died soon after.	Blood, lung swab	Severe lobar pneumonia.	Small clusters and individual bacilli in lung.
7	M/58	Known case of diabetes mellitus. Had low backache for 2 weeks and admitted for right adrenal and paraaortic mass. Treated with intravenous Ceftazidime. Died from gastrointestinal bleeding.	Blood	Large necrotic abdominal aorta aneurysm with thrombus and necrotic aortic wall.	Individual and small clusters of bacilli in mycotic aneurysm of abdominal aorta.

[a]Cases 1–3 were previously described by Wong *et al*.[19]

sections, but the signal intensities for *B. pseudomallei* were relatively stronger compared with *B. thailandensis*. *B. pseudomallei* was crisply and strongly stained red as single whole bacillus or bacilli clusters (Figure 1a). All other microorganisms, including *B. cepacia*, gave negative results (Figure 1b). The areas of *in situ* hybridization positivity generally corresponded very well with the areas in which Gram-negative bacilli were stained by the Brown–Hopps method.

In Situ Hybridization on Infected Human Tissues

Tissues from at least one organ tested positive in all the seven human cases studied and positive staining results were mainly associated with inflammatory lesions (Table 2). The lung was the most likely organ to be positive (cases 1–6). Bacilli were demonstrated in alveolar walls either within capillaries or intracellularly (Figure 2b). In more severe lung parenchymal inflammatory lesions, individual or clusters/chains of bacilli were often numerous (Figure 2a). Within blood vessels in the lungs and other organs, individual 'free-floating' bacillus or intracellular bacilli were observed (Figure 2d). Rarely, large 'free-floating' bacillary clusters or emboli measuring nearly 20 μm in diameter could be found (Figure 2c). Bacilli could be detected in kidney glomeruli, possibly within capillaries (Figure 2e), in bone marrow (Figure 2f), and in the wall of the aortic mycotic aneurysm (Table 2, case 7). Intracellular bacteria were found in cells that morphologically resemble macrophages and neutrophils.

In Situ Hybridization on Infected Mouse Tissues

Overall, bacteria found in tissues from mice with acute melioidosis (all mice died within 3 days after infection) showed very crisp and strong positivity.

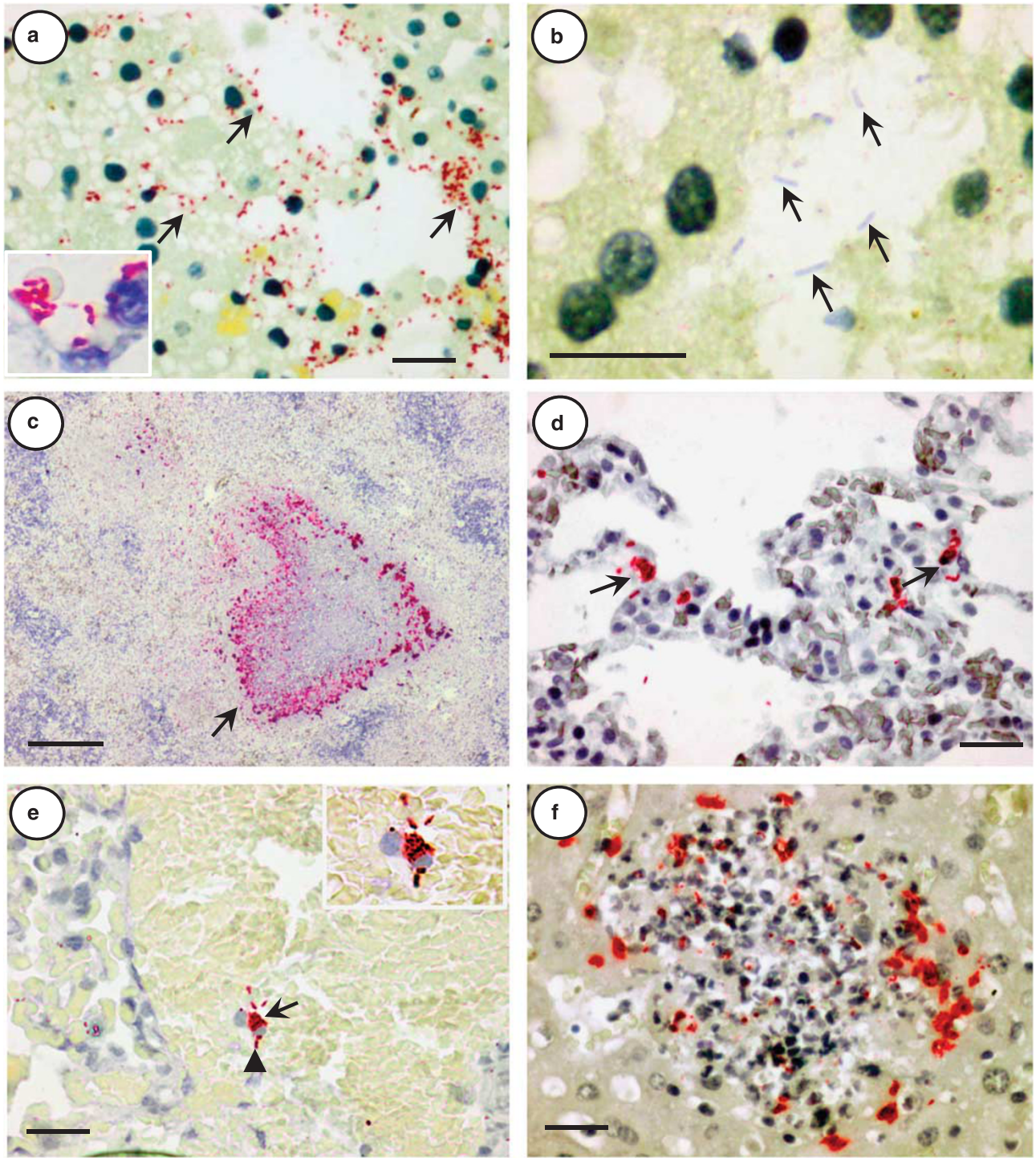

Figure 1 *In situ* hybridization to detect *Burkholderia pseudomallei* in control and infected mouse tissues with positive staining shown in red. (**a**) Formalin-fixed, paraffin-embedded *B. pseudomallei* bacilli in liver tissue scaffold positively stained as crisp single whole bacillus or bacilli clusters (arrows). The inset shows bacilli in higher magnification. (**b**) No cross-hybridization was observed in similar tissues containing *Acinetobacter spp*. bacilli as a negative control (arrows). (**c**) Numerous positively stained bacteria surrounding a large area of necrosis and inflammation in infected murine spleen (arrow). (**d**) Single bacillus and small bacilli clusters (arrows) in the alveolar wall of the murine lung. (**e**) Intracellular (arrow) and extracellular bacilli (arrowhead) found within a blood vessel in the murine lung. Inset shows a higher magnification. (**f**) Clusters of bacilli around a small abscess in the murine liver. Liquid Permanent Red substrate with Mayer hematoxylin counterstain. Magnification: (**a**, **d**, **e**, and **f**): $\times 40$ objective; (**c**): $\times 10$ objective; (**b** and inset): $\times 100$ objective. Scale bars $= 20\,\mu$m (**a**, **b**, and **d**–**f**); $100\,\mu$m (**c**).

Infection was most severe in the spleen (Figure 1c) and liver (Figure 1f) where large clusters of bacilli with or without surrounding inflammation were observed. Individual intracellular or extracellular bacilli were also well demonstrated. Compared with the spleen and liver, bacilli in the lungs were sparse,

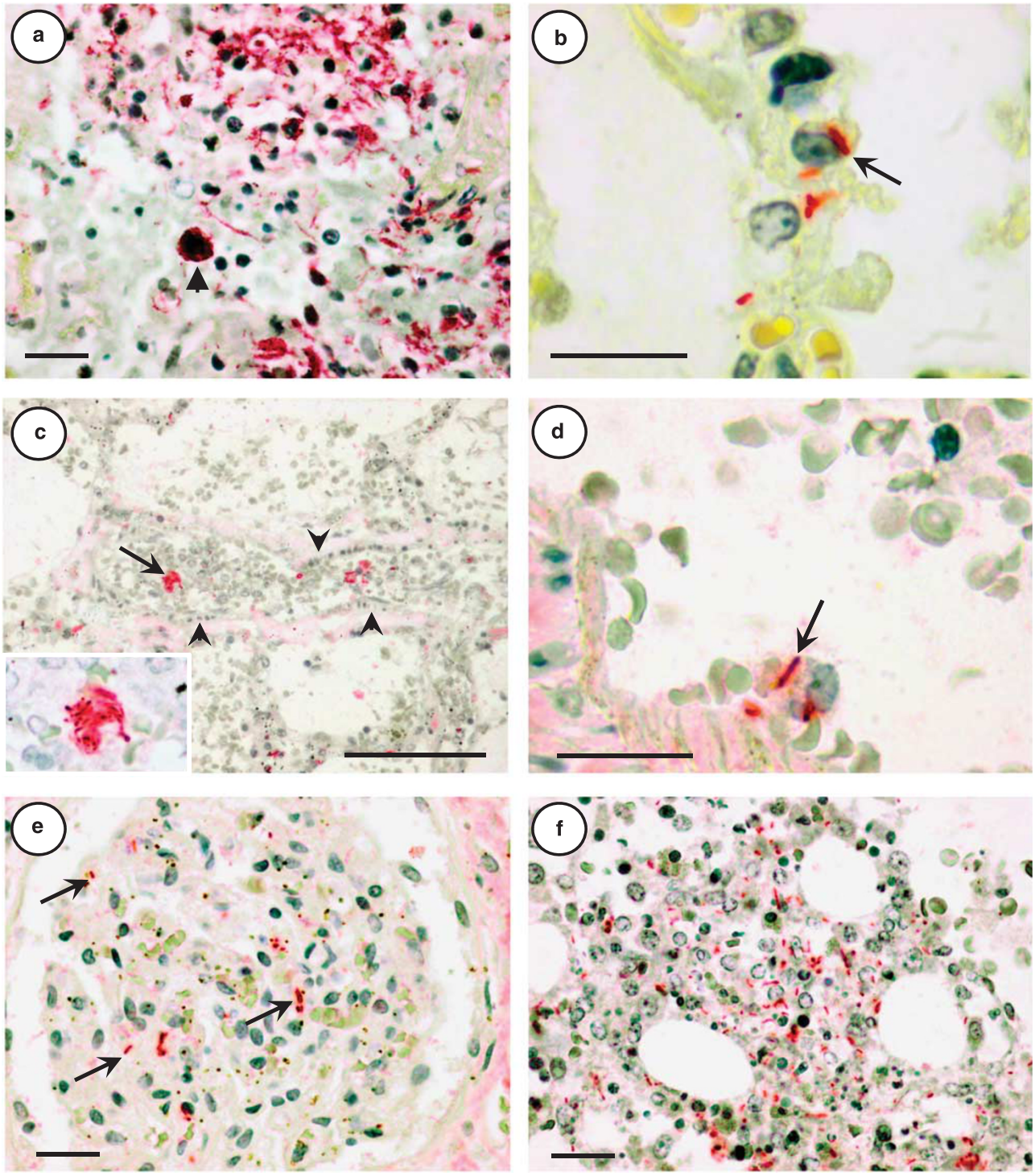

Figure 2 *In situ* hybridization to detect *Burkholderia pseudomallei* in human melioidosis cases with positive staining shown in red. (**a**) In the lung, extracellular and intracellular (arrowhead) bacilli were observed in and around inflammatory parenchymal lesions. (**b**) Intracellular bacillus at the alveolar wall (arrow). (**c**) Intravascular bacillary clusters and embolus (arrow and inset) in a pulmonary arteriole (arrowheads showing the vascular endothelium/smooth muscle layer). (**d**) Intravascular, intracellular bacillus (arrow) in a pulmonary blood vessel. (**e**) Scattered individual bacilli (arrows) in the kidney glomerulus. (**f**) Small clusters of bacilli in the bone marrow. Liquid Permanent Red substrate with Mayer hematoxylin counterstain. Magnification: (**a**, **e**, and **f**): ×40 objective; (**c**): ×20 objective; (**b** and **d** and inset): ×100 objective. Scale bars = 20 μm (**a**, **b**, and **d–f**), 100 μm (**c**); (**a**): case 3; (**b–f**): case 5.

and found mainly in alveolar walls (Figure 1d) or spaces. Within blood vessels in the lung, liver, and heart, single bacillus or bacillary clusters were either 'free floating' or found within leukocytes (Figure 1e). No bacteria were detected in the stomach and kidney.

Discussion

The *in situ* hybridization assay to detect *B. pseudomallei* is useful and sensitive enough to stain whole bacilli to facilitate localization and identification in infected tissues. As expected, in the human autopsy tissues, both intra- and extracellular bacteria were detected in the organs commonly reported to be involved, such as lung, spleen, liver, and kidney.[19,20] In the lung and other organs, bacilli were detected in or around parenchymal inflammatory lesions, and occasionally also in alveolar walls and other tissues that were not obviously inflamed. Within some blood vessels, intracellular intact single bacillus (Figure 2d) and 'free-floating' extracellular single bacillus or small bacillary clusters could be unequivocally identified. Although it is well known that *B. pseudomallei* is able to survive and proliferate intracellularly,[21] we believe this is the first time that bacteria have been demonstrated within circulating leukocytes *in vivo*. The overall morphological appearance of intact bacilli suggests that the bacteria were viable but further studies are needed to confirm this, and to identity which type of leukocyte could be infected intravascularly. It is possible that the intravascular, intracellular location could contribute to survival by helping bacteria evade the host immune response. Moreover, appropriate antibiotics may be less effective against intracellular bacteria.[21] The rare, large bacillary cluster or embolus in *B. pseudomallei* septicemia bear some resemblance to the septic emboli described in hamsters experimentally infected with *B. mallei*.[22] We speculate that bacillary emboli (Figure 2c) or smaller clusters may be held together and protected against antibiotics and host immune response by a surrounding biofilm, a well-known phenomenon in *B. pseudomallei* infection.[23]

Bone marrow infection, shown in one of our cases (case 5, Table 2), is consistent with bone marrow inflammation found in another series,[24,25] and the observation that bacteria could be cultured from bone marrow.[26] Morphologically intact bacteria were demonstrated in the wall of the mycotic aneurysm confirming *B pseudomallei* infection (case 7, Table 2). Although generally mycotic aneurysm is rare in melioidosis,[6] it has been reported that *B. pseudomallei* is the most common etiology of mycotic aneurysm in Thailand.[7]

The histology findings in the acute melioidosis mouse model appear to parallel the observations in the human tissues, although the mouse model was infected by the IP route whereas humans are usually infected via inhalation or skin inoculation. In particular, we were able to confirm intravascular, intracellular localization of bacteria in the mouse model (Figure 1e). Generally, bacilli stained more crisply, most likely because mouse tissues were formalin fixed immediately, whereas there is invariably some autolysis in human tissues because of autopsy delays. It may be possible to use *in situ* hybridization combined with other studies, for example ultrastructural studies, to investigate whether intravascular, infected leukocytes may act as 'trojan horses' to penetrate vascular walls to cause parenchymal inflammation in the mouse model. As bacterial spread in lymphatics is unknown,[27] our *in situ* hybridization technique could also be used to study this.

As the probes target 16s rRNA sequences, in addition to genomic DNA, theoretically it should be able to hybridize with the rRNA found in the thousands of free or membrane-bound ribosomes within a single bacteria cell. In practice, this strategy worked as single whole bacillus could be intensely and crisply stained. Our nonfluorescent colorimetric *in situ* hybridization assay has the advantage of allowing for direct visualization of bacilli and assessment of reactive changes in surrounding tissues. Our probe did not hybridize with the non-*Burkholderia* microorganisms and *B. cepacia* but cross-hybridized with *B. thailandensis*, although to a lesser extent. This is most likely because *B. pseudomallei* and *B. thailandensis* shared >90% homology in the targeted rRNA region. Fortunately, *B. thailandensis* is an avirulent, nonpathogenic *Burkholderia* species[28,29] and is an extremely rare infection in humans and animals, hence unlikely to be encountered in clinical materials/tissues. Higher stringency washes in the posthybridization step of the *in situ* hybridization procedure to minimize cross-hybridization were unsuccessful. A further drawback is that our *in situ* hybridization assay was not tested on *B. mallei*, although we predict our probe should cross-hybridize with *B. mallei* as the nucleotide homology in the targeted region is 100%. Fortunately, like *B. thailandensis*, human *B. mallei* infection is rare compared with *B. pseudomallei* infections in endemic areas. Confirmation of *B. pseudomallei* infection in infected tissues can be done by performing a PCR on nucleic acid extracted from formalin-fixed, paraffin-embedded tissues.[10] Using immunohistochemistry to detect *B. pseudomallei* is possible but it is very difficult to visualize individually stained bacteria,[20] and there is often a high background of positively stained bacterial debris. The *in situ* hybridization assay is far better to visualize intact bacilli and there is relatively little background staining. We believe this is essential for diagnostic confidence.

We speculate that this method could be applied directly on smears for bacterial identification using bodily fluids or pus as was demonstrated for *M. tuberculosis*[30] in sputum and *Legionella spp.* in bronchioalveolar lavages.[31] Further tests and more extensive studies should be carried out to validate the usefulness of the probe for various clinical diagnostic purposes.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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