

An Improved Selective Culture Medium Enhances the Isolation of *Burkholderia pseudomallei* from Contaminated Specimens

Andrew Goodyear, Linda Strange, Drew A. Rholl, Joy Silisouk, David A. B. Dance, Herbert P. Schweizer, and Steven Dow*

Department of Microbiology, Immunology, and Pathology, Rocky Mountain Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research, Colorado State University, Fort Collins, Colorado; Department of Biology, North Park University, Chicago, Illinois; Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao People's Democratic Republic; Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford, United Kingdom

Abstract. *Burkholderia pseudomallei* is a Gram-negative environmental bacterium found in tropical climates that causes melioidosis. Culture remains the diagnostic gold standard, but isolation of *B. pseudomallei* from heavily contaminated sites, such as fecal specimens, can be difficult. We recently reported that *B. pseudomallei* is capable of infecting the gastrointestinal tract of mice and suggested that the same may be true in humans. Thus, there is a strong need for new culture techniques to allow for efficient detection of *B. pseudomallei* in fecal and other specimens. We found that the addition of norfloxacin, ampicillin, and polymyxin B to Ashdown's medium (NAP-A) resulted in increased specificity without affecting the growth of 25 *B. pseudomallei* strains. Furthermore, recovery of *B. pseudomallei* from human clinical specimens was not affected by the three additional antibiotics. Therefore, we conclude that NAP-A medium provides a new tool for more sensitive isolation of *B. pseudomallei* from heavily contaminated sites.

INTRODUCTION

Burkholderia pseudomallei is a saprophytic bacterium present in soil and surface water that causes melioidosis in humans and animals.¹ Major endemic areas include southeast Asia and northern Australia, although as diagnostic facilities improve, the bacterium has been isolated from additional countries in tropical and subtropical latitudes.^{1,2} Melioidosis can be an acutely fatal disease, with many patients succumbing shortly after clinical presentation, although long latency periods of up to 62 years have been reported.^{3,4} Infection is thought to occur after inoculation, inhalation, or ingestion, although the contribution of each route is not fully understood.¹ Symptoms are highly protean, and proper diagnosis is critical, because timely administration of appropriate antibiotics is important for clearance of *B. pseudomallei*.^{5–7} Culture remains the gold standard for diagnosis, and although *B. pseudomallei* can grow on most common laboratory media, isolation from non-sterile sites requires the use of selective media.

A number of selective media have been developed for *B. pseudomallei*, although the selective medium that has been shown provide the best combination of sensitivity and specificity for isolation of *B. pseudomallei* is Ashdown's medium (ASH).^{8–10} ASH contains crystal violet and gentamicin as selective components as well as glycerol and neutral red to allow for differentiation based on colony morphology.⁸ Multiple modifications of ASH and other selective media have been developed using different antibiotics, including colistin (polymyxin E), polymyxin B, chloramphenicol, vancomycin, penicillin, ampicillin, streptomycin, amikacin, and increased gentamicin concentrations to improve specificity.^{9,11–17} Although some media formulations have included multiple antibiotics, most have used a single antibiotic to improve selectivity. Moreover, to our knowledge, fluoroquinolones have not yet been investigated as an additive to selective medium for *B. pseudomallei*.

Intrinsic antibiotic resistance is a hallmark of *B. pseudomallei*. For instance, numerous antibiotics belonging to different classes are extruded from the cell by resistance nodulating cell division (RND) efflux pumps. For example, AmrAB-OprA is responsible for the intrinsic aminoglycoside and macrolide resistance observed with most *B. pseudomallei* strains.^{18,19} In strain KHW, BpeAB-OprA was also shown to efflux aminoglycosides, but this finding was not corroborated by studies with strain 1026b, where this pump was shown to confer low-level chloramphenicol, fluoroquinolone, macrolide, and tetracycline resistance but not extrude aminoglycosides.^{20,21} Certain fluoroquinolones have also been shown to be effluxed (T. Mima and others, unpublished observations), and other studies have shown that alterations in DNA gyrase can lead to resistance.²² In addition, the *B. pseudomallei* genome encodes seven putative Ambler class A, B, and D β -lactamases.^{23–26} Of these β -lactamases, only the PenA Class A β -lactamase has been shown to confer clinically significant β -lactam resistance.²⁴ Finally, the cell envelope structure, notably lipopolysaccharide, is known to be responsible for resistance to cationic peptides, such as polymyxins.²⁷ Although significant levels of antibiotic resistance are a major challenge in treating patients with melioidosis, these *Burkholderia* traits provide options for development of selective media.

Selective media have improved *B. pseudomallei* isolation rates, although *B. pseudomallei* recovery from certain sites can still be challenging. For instance, although *B. pseudomallei* has been isolated from rectal swabs of melioidosis patients, overgrowth of enteric bacteria was also reported.²⁸ Therefore, we investigated the ability of the addition of norfloxacin, ampicillin, and polymyxin B to improve the specificity of ASH for recovery of *B. pseudomallei* from clinical specimens. These antibiotics were chosen because of their previous use in *B. pseudomallei*-selective media (ampicillin and polymyxins),^{12,16,17,29} intrinsic resistance among *B. pseudomallei* isolates (norfloxacin, ampicillin, and polymyxins),^{30–32} and previous use against enteric flora (norfloxacin and ampicillin).³³ This report describes the optimization of the new medium norfloxacin, ampicillin, and polymyxin B ASH medium (NAP-A medium). Specificity and sensitivity studies were performed using samples from infected mice. In addition,

* Address correspondence to Steven Dow, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80523. E-mail: sdow@colostate.edu

a side-by-side comparison of recovery rates using NAP-A and ASH medium was done using human clinical samples.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A detailed description of *B. pseudomallei* strains used in this study is provided in Table 1. Briefly, *B. pseudomallei* strains included 12 clinical strains (7 blood, 3 pus, and 2 lung isolates), 10 environmental isolates, and 4 clinical isolates commonly used in laboratory experiments. All *B. pseudomallei* strains were originally isolated in Thailand, and provided by Sharon Peacock (Mahidol University, Mahidol, Thailand and University of Cambridge, Cambridge, United Kingdom). Routine experiments involving *B. pseudomallei* were performed with strain 1026b, a strain isolated from the blood of a melioidosis patient in Thailand.³⁴ Other *B. pseudomallei* isolates, *B. thailandensis* strains, and additional *Burkholderia* spp. strains are described in Table 1.

Freezer stocks of all bacterial strains were prepared from stationary phase Luria-Bertani (LB) broth (BD Biosciences, San Jose, CA) cultures grown at 37°C with shaking at 250 rpm. Sterile glycerol (Fisher Scientific, Pittsburgh, PA) was added to each culture at a 20% (vol/vol) final concentration, and glycerol stocks were frozen at -80°C. Unless noted, plating of pure bacterial cultures was performed using bacteria thawed directly from freezer stocks. In experiments where overnight cultures were used, cultures were grown in LB broth at 37°C with shaking at 250 rpm, and stationary phase cultures were used for plating. All procedures involving *B. pseudomallei* performed at Colorado State University were performed in a Biosafety Level 3 (BSL3) facility in accordance with approved BSL3 and Select Agent protocols.

Mice and animal infections. Female BALB/c and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male and female Institute for Cancer Research (ICR) mice were purchased from Harlan Laboratories (Indianapolis, IN). All mice used in experiments were housed under pathogen-free conditions in microisolator cages, and

mice were 12–20 weeks of age at the time of infection. Oral (p.o.) and intranasal (i.n.) infection of mice with *B. pseudomallei* was performed as described previously.³⁵ Immediately before animal inoculation, bacterial stocks frozen in LB broth with 20% glycerol were thawed and diluted in sterile phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO). Infectious doses were determined by plating serial dilutions of each inoculum on LB agar (BD Biosciences, San Jose, CA). Oral inoculations were performed with a stainless steel 22-gauge gavage needle, and mice were inoculated in a total volume of 100 µL. For i.n. inoculation, mice were anesthetized with intraperitoneal (i.p.) injection of ketamine (100 mg/kg; Pfizer, New York, NY) and xylazine (10 mg/kg; Lloyd Laboratories, Shenandoah, IA), and inoculations were done using in a total volume of 20 µL (10 µL per nostril). Median lethal dose (LD₅₀) values for acute disease (i.e., euthanasia required on or before day 7) in BALB/c mice were previously determined to be 1.04×10^7 cfu for p.o. infection and 9×10^2 cfu for i.n. infection with *B. pseudomallei* strain 1026b.³⁵ All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Colorado State University and performed in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals.

Antibiotic preparation and storage. Gentamicin was purchased as a pre-made solution in deionized water (10 mg/mL). Norfloxacin powder was dissolved at a concentration of 100 mg/mL in glacial acetic acid and then diluted to a final concentration of 10 mg/mL with PBS. Based on previous studies showing that low pH buffers increase the stability of ampicillin, ampicillin powder was dissolved in 0.1 M acetate buffer (pH 6) at a concentration of 50 mg/mL.^{36,37} Polymyxin B powder was dissolved in deionized water at a concentration of 2.5×10^5 units/mL. Vancomycin powder was dissolved in deionized water at 0.5 mg/mL. Rifampicin powder was dissolved in dimethyl sulfoxide at 50 mg/mL. Fosfomycin powder was dissolved in deionized water at 50 mg/mL. All reagents were purchased from Sigma-Aldrich (St. Louis, MO).

TABLE 1
Bacterial strains

<i>B. pseudomallei</i>				<i>B. thailandensis</i>		<i>Burkholderia</i> spp.	
Clinical* and laboratory strains		Environmental strains		Strain	Source	Species	Source
Strain	Source	Strain	Source				
2613a	Blood	E0008	Thailand	E264	Thailand	<i>B. oklahomensis</i>	60
2650a	Blood	E0031	Thailand	E421	Thailand	<i>B. gladioli</i> pv. <i>cocovenenans</i>	61
2668a	Blood	E0037	Thailand	E426	Thailand	<i>B. dolosa</i>	62
2674a	Blood	E0237	Thailand			<i>B. ubonensis</i>	63
2692a	Blood	E0342	Thailand			<i>B. vietnamiensis</i>	A. Hoffmaster†
2704a	Blood	E0356	Thailand				
2671a	Blood	E0377	Thailand				
2617a	Pus	E0383	Thailand				
2618a	Pus	E0386	Thailand				
2685a	Pus	E0394	Thailand				
2670a	Lung						
2719a	Lung						
1026b	Blood ³⁴						
K96243	Human ²⁶						
1710b	Blood ⁵⁸						
1106b	Pus ⁵⁹						

* All Strain isolated from patients in Ubon Ratchatani in northeast Thailand.

† Centers for Disease Control and Prevention.

Gentamicin solutions were stored at 4°C according to the manufacturer's instructions. Stock solutions of norfloxacin, ampicillin, and polymyxin B were stored at -80°C and used within 6 months as determined by stability experiments performed in the current investigation (see below).

Selective media preparation. *Modified trypticase soy agar.* Modified trypticase soy agar (MTSA) was prepared as described previously.⁸ Briefly, 4% glycerol (Fisher Scientific, Pittsburgh, PA), 5 µg/mL crystal violet (EMD Science, Gibbstown, NJ), and 50 µg/mL neutral red (Sigma-Aldrich, St. Louis, MO) were added to trypticase soy agar (BBL 211043; BD Biosciences, San Jose, CA).

ASH. ASH was also prepared as described previously.⁸ Briefly, MTSA was prepared as described above, autoclaved, and cooled to 55°C, and gentamicin was added at a final concentration of 4 µg/mL.

NAPA-A. NAP-A was prepared by using ASH medium as the basal medium and incorporating three additional antibiotics. MTSA was prepared as described above, autoclaved, and cooled to 55°C, and gentamicin at 4 µg/mL, norfloxacin at 4 µg/mL, ampicillin at 10 µg/mL, and polymyxin B at 300 units/mL were added.

Additional selective media. Norfloxacin, ampicillin, and polymyxin B MTSA (NAP-MTSA) was prepared the same as the NAP-A medium described above, although gentamicin was not added. LB agar with gentamicin, norfloxacin, ampicillin, and polymyxin B was also prepared by autoclaving LB agar, cooling the media to 55°C, and adding gentamicin at 4 µg/mL, norfloxacin at 4 µg/mL, ampicillin at 10 µg/mL, and polymyxin B at 300 units/mL.

Fecal pellet collection and processing. Fecal pellets were collected from mice and processed as described previously.³⁵ Briefly, mice were transferred into small plastic containers, and fecal pellets were then collected from these containers. Fecal pellets were resuspended in PBS at a concentration of 0.1 g feces/mL PBS and homogenized using a Stomacher 80 Biomaster (Seward, Port Saint Lucie, FL). Serial dilutions of fecal homogenates were prepared in sterile PBS and plated on agar plates. The lower limit of bacterial detection in feces was 10–60 cfu/g feces.

Optimization of norfloxacin, ampicillin, and polymyxin B concentrations. Based on previous reports showing that ASH medium provides the best combination of specificity and sensitivity, the ability of additional antibiotics to improve the specificity of ASH was investigated.^{9,10} Optimized concentrations of norfloxacin, ampicillin, and polymyxin B were determined using 10 different enteric bacteria colony types that were routinely cultured from murine gastrointestinal (GI) tissues plated on ASH (isolates were not identified on a genus or species level). These 10 isolates were subcultured onto ASH medium supplemented with different concentrations of each antibiotic. Several different concentrations of all three antibiotics were evaluated for their ability to suppress endogenous flora. To determine if enteric bacteria capable of growing in the presence of gentamicin would be susceptible to a different antibiotic in the same class, streptomycin was investigated at 10 µg/mL.³⁸

Determination of organ bacterial burden. Bacterial burden in organ homogenates was quantitated as described previously.³⁵ Mice were euthanized, and organs were placed in 4 mL sterile PBS. Before homogenization, the stomach and cecum were cut into ~1- to 2-cm² sections, and small intestine

and colon tissues were cut open longitudinally and then cut into 2- to 3-cm lengths. Organs were homogenized using a Stomacher 80 Biomaster, and serial 10-fold dilutions of organ homogenates were prepared in sterile PBS. Organ homogenates and serial dilutions were plated on LB, ASH, or NAP-A agar plates. Unless noted, agar plates were incubated at 37°C, and colonies were counted at 48 hours. The limit of detection in organ homogenates was 20 cfu/organ.

Clinical evaluation. To assess the use of NAP-A medium to isolate *B. pseudomallei* from human clinical samples, the medium was compared with ASH in a small-scale evaluation in a diagnostic laboratory in an endemic area (Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao People's Democratic Republic). Clinical samples that were being cultured routinely for *B. pseudomallei* on ASH and in a selective broth based on ASH containing colistimethate (SBCT; 50 mg/L)¹⁷ that was subcultured onto ASH after 48 hours of incubation were also cultured on NAP-A plates in parallel. The formulations of media were similar to those formulations described above, except that the local recipe for ASH currently in use was followed. This recipe comprised 10 g Tryptone soya broth (Oxoid, Basingstoke, United Kingdom), 15 g Agar No. 1 (Oxoid, Basingstoke, United Kingdom), 40 mL glycerol (VWR, East Grinstead, United Kingdom), 5 mL 0.1% crystal violet (Merck, Beeston, United Kingdom), and 5 mL 1% neutral red (Rankem, Faridabad, India) per 1 L, to which gentamicin for injection (Bidiphar, Quy Non, Vietnam) was added to give a final concentration of 5 µg/mL. For NAP-A medium, norfloxacin, ampicillin, and polymyxin (Sigma-Aldrich, St. Louis, MO) were added to achieve the same final concentrations as listed above. Cultures on ASH were read as usual by the clinical laboratory staff, whereas NAP-A cultures were read by another staff member who was blind to the results of ASH cultures. The study was carried out in two phases. During the first phase, the presence or absence of *B. pseudomallei* was recorded qualitatively (i.e., present or absent). During the second phase, the growth of both *B. pseudomallei* and other competing flora on both media was recorded semiquantitatively according to the method by Nye and others.³⁹

Stability of antibiotic stock solutions. The Kirby-Bauer disk diffusion method was chosen to investigate the stability of antibiotic stock solutions used in NAP-A medium.^{36,40} The disk diffusion assay was performed according to the Clinical and Laboratory Standards Institute guidelines.⁴¹ Antibiotic stocks were prepared as described above, aliquoted, and stored at either 4°C or -80°C. At various time points after preparation, an aliquot from each antibiotic was tested for the ability to kill *Escherichia coli* strain DH5α.⁴² *E. coli* was grown overnight on Mueller Hinton (MH; BD Biosciences, San Jose, CA) agar plates, and bacterial colonies were resuspended in sterile PBS at a density equivalent to a 0.5 MacFarland standard. The bacterial suspension was then streaked on an MH agar plate using a sterile swab to create a lawn of bacteria; 6-mm disks (Whatman, Piscataway, NJ) were loaded with the desired amount of antibiotic (gentamicin [10 µg], norfloxacin [10 µg], ampicillin [10 µg], and polymyxin B [300 units]) in a total volume of 25 µL, and disks were allowed to air dry aseptically.⁴³ Antibiotic loaded disks were added to the MH agar plate containing the *E. coli* lawn, plates were incubated at 37°C, and zones of inhibition were measured after 16–18 hours of growth. At each time point, zone diameters were compared

with zones determined from the original antibiotic stocks before storage at 4°C or -80°C.

Statistical analysis. Statistical analyses were performed using Prism 5.0 software (Graph Pad, San Diego, CA). Differences in percentages of positive samples were compared using a two-tailed Fisher exact test. Analyses comparing two groups were done using a two-tailed Student *t* test, and analyses comparing more than two groups were performed using a one-way analysis of variance (ANOVA) followed by a Tukey multiple means comparison test. In experiments comparing two variables, data were analyzed by a two-way ANOVA followed by a Bonferroni post-test. Differences were considered statistically significant for $P < 0.05$.

RESULTS

While developing a murine model of chronic enteric melioidosis, we observed that enteric bacteria often overgrew *B. pseudomallei* when cultured on ASH.³⁵ The effects of norfloxacin, ampicillin, and polymyxin B on the growth of enteric bacteria subcultured from ASH are reported in Table 2. Norfloxacin selected against 9 of 10, 8 of 10, and 6 of 10 enteric colonies at concentrations of 10, 5, and 1 µg/mL, respectively (Table 2). We found that 10 µg/mL ampicillin prevented growth of all 10 enteric bacteria isolates. However, increasing the concentration of polymyxin B up to 600 units/mL did not significantly inhibit enteric isolate growth compared with ASH ($P = 0.06$). Only 3 of 10 enteric bacteria were inhibited by streptomycin, similar to the result with gentamicin.

In summary, these studies showed that 10 or 5 µg/mL norfloxacin or 10 µg/mL ampicillin significantly reduced the growth of enteric bacteria. Increasing polymyxin B provided only minor improvements in selectivity against enteric isolates. Therefore, studies were performed to investigate the ability of *B. pseudomallei* to grow in the presence of antibiotics capable of inhibiting enteric isolate growth.

B. pseudomallei strain 1026b was serially diluted in PBS and plated on ASH containing each individual antibiotic. We found that neither 10 µg/mL ampicillin nor 300 U/mL polymyxin B affected *B. pseudomallei* growth. Norfloxacin was tested at 1, 5, and 10 µg/mL, and it was further titrated over a 2–10 µg/mL range in 2-µg/mL increments (Table 2) (data not shown). From these studies, 4 µg/mL was consistently identified as the concentration that did not alter *B. pseudomallei* growth

(data not shown). We also evaluated several other antibiotics for use in selective medium, including fosfomycin, rifampicin, and vancomycin. However, we found that none of these antibiotics offered any particular advantage over the norfloxacin, ampicillin, and polymyxin B combination. Therefore, 10 µg/mL ampicillin, 4 µg/mL norfloxacin, and 300 units/mL polymyxin B were chosen for additional study.

To assess the ability of each individual antibiotic to select against enteric bacteria when added to MTSA, inhibition of bacterial growth from mouse fecal pellets was used as a screening method. Fecal pellets were collected from BALB/c, C57BL/6, and ICR mice and processed as described in *Materials and Methods*. Each dissolved fecal pellet sample was plated on LB, MTSA, and MTSA medium supplemented with a single antibiotic (Figure 1). MTSA medium alone significantly reduced the growth of enteric bacteria compared with growth on LB ($P < 0.001$). The addition of each individual antibiotic to MTSA medium also produced a significant reduction in enteric bacterial titers compared with MTSA medium alone ($P < 0.001$). Comparisons between the different antibiotics showed that norfloxacin was significantly more effective than gentamicin ($P < 0.05$). In addition, compared with polymyxin B, both norfloxacin ($P < 0.01$) and ampicillin ($P < 0.05$) provided significant reductions in enteric bacterial growth, although no differences were observed between polymyxin B and gentamicin. To summarize, all four antibiotics reduced the growth of enteric bacteria when added to MTSA, although ampicillin and norfloxacin seemed to be the most effective against fecal bacteria. However, none of the antibiotics

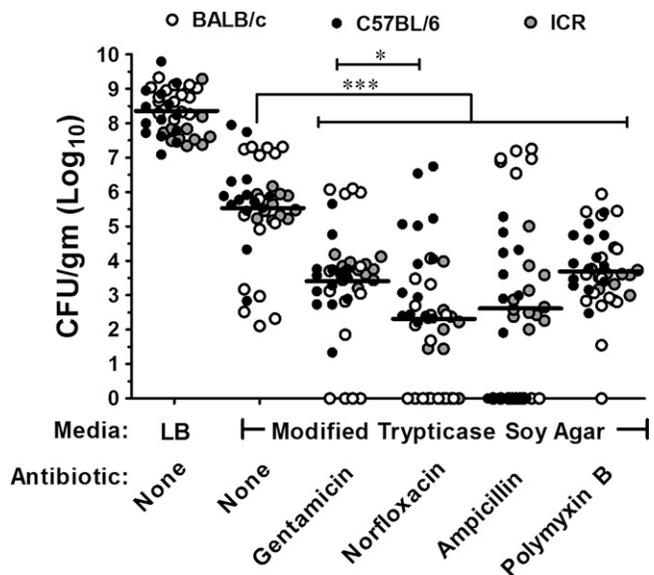


FIGURE 1. Effect of individual antibiotics on growth of fecal bacteria. Fecal pellets were homogenized in PBS, and serial dilutions were plated on various agar media. MTSA was supplemented with gentamicin (4 µg/mL), norfloxacin (4 µg/mL), ampicillin (10 µg/mL), or polymyxin B (300 U/mL). Fecal pellets were collected from cages of five mice. Data from BALB/c ($N = 15$ cages), C57BL/6 ($N = 14$ cages), and ICR ($N = 12$ cages) mice were pooled ($N = 41$ cages). Samples were processed as described in *Materials and Methods* and plated on each medium. Data are presented as individual log₁₀ cfu per 1 g feces, with bars representing the mean titer for each group. Statistical differences between antibiotics were determined by a one-way ANOVA followed by a Tukey multiple means test ($*P < 0.05$; $***P < 0.001$). Data were pooled from seven individual experiments.

TABLE 2

Titration of additional antibiotics used in NAP-A medium

Medium	Enteric bacteria		<i>B. pseudomallei</i> †
	Positive	<i>P</i> value*	
ASH	9/10	NA	8.85 ± 0.06
ASH + 10 µg/mL Nor	1/10	$P = 0.001$	8.62 ± 0.01
ASH + 5 µg/mL Nor	2/10	$P = 0.006$	8.88 ± 0.03
ASH + 1 µg/mL Nor	4/10	$P = 0.06$	8.69 ± 0.04
ASH + 10 µg/mL Amp	0/10	$P = 0.0001$	8.94 ± 0.08
ASH + 5 µg/mL Amp	4/10	$P = 0.06$	ND
ASH + 600 U/mL PxB	4/10	$P = 0.06$	ND
ASH + 500 U/mL PxB	7/10	$P = 0.58$	ND
ASH + 300 U/mL PxB	8/10	$P = 1.0$	8.80 ± 0.05
ASH + 10 µg/mL Str	7/10	$P = 0.58$	ND

Amp = ampicillin; NA = not applicable; ND = not determined; Nor = norfloxacin; PxB = polymyxin B; Str = streptomycin.

*Statistical differences compared with ASH were determined by a two-tailed Fisher exact test.

†*B. pseudomallei* 1026b titers reported as log₁₀ cfu per 1 mL ± SEM.

were sufficient to consistently inhibit growth of fecal bacteria when used alone. Therefore, we concluded that including all four antibiotics would likely be the most effective formulation for inhibiting the growth of contaminating flora.

NAP-A medium is more effective at inhibiting enteric bacterial growth. To assess the effectiveness of the new four-antibiotic NAP-A medium, growth of enteric bacteria in both GI tissues and fecal pellets was assessed (Figure 2). We found that use of NAP-A medium resulted in a significant reduction in enteric bacterial growth from BALB/c, C57BL/6, and ICR mice compared with ASH medium. For example, enteric bacteria from GI tissues of BALB/c mice grew on ASH medium, whereas enteric bacterial growth was not observed on the four-antibiotic NAP-A medium (Figure 2). The addition of these four antibiotics to LB media also prevented enteric bacterial growth from fecal pellets of BALB/c and C57BL/6 mice (data not shown). Therefore, the use of four-antibiotic NAP-A medium significantly reduced the growth of enteric bacteria from multiple strains of mice, which was assessed by both qualitative and quantitative assays.

NAP-A medium improves *B. pseudomallei* isolation from samples with low bacterial burdens. To determine whether *B. pseudomallei* could be isolated from infected GI tissues using NAP-A medium, tissues obtained from BALB/c mice infected p.o. or i.n. individually with each of four different *B. pseudomallei* strains (strain 1026b, 2671a, 2685a, or 2719a) were subjected to bacterial culture; 125 GI tissues (stomach,

small intestine (SI), cecum, or colon) were plated in parallel on both ASH and NAP-A media. No significant differences in *B. pseudomallei* titers were observed between samples plated on ASH versus NAP-A medium ($P = 0.35$; data not shown). These results showed that the three additional antibiotics in NAP-A medium did not affect the ability to isolate *B. pseudomallei* from tissues.

A major effect of NAP-A medium was observed when cultures of undiluted organ homogenates (Figure 2B) were compared with cultures on ASH. Therefore, we next assessed the effect of NAP-A medium on *B. pseudomallei* detection in tissues with very low levels of infection (titer $\leq 1,000$ cfu/organ). Of 125 GI samples plated on both NAP-A and ASH, 34 low-titer samples were identified. In the low-titer samples, a significant increase ($P = 0.03$) in *B. pseudomallei* titers was observed on NAP-A medium (Figure 3). This result is likely caused by the ability of NAP-A medium to prevent the growth of enteric bacteria, which was often observed to overgrow *B. pseudomallei*. Indeed, qualitative examples were also observed in GI tissues from mice infected with *B. pseudomallei* (Figure 3).

The four antibiotics in NAP-A medium did not have an effect on the growth of *B. pseudomallei* strain 1026b (Table 2) (data not shown). However, it was also important to assess the wider ability of NAP-A medium to be used as a general culture medium for multiple *B. pseudomallei* strains. Therefore, 25 additional *B. pseudomallei* strains (12 clinical,

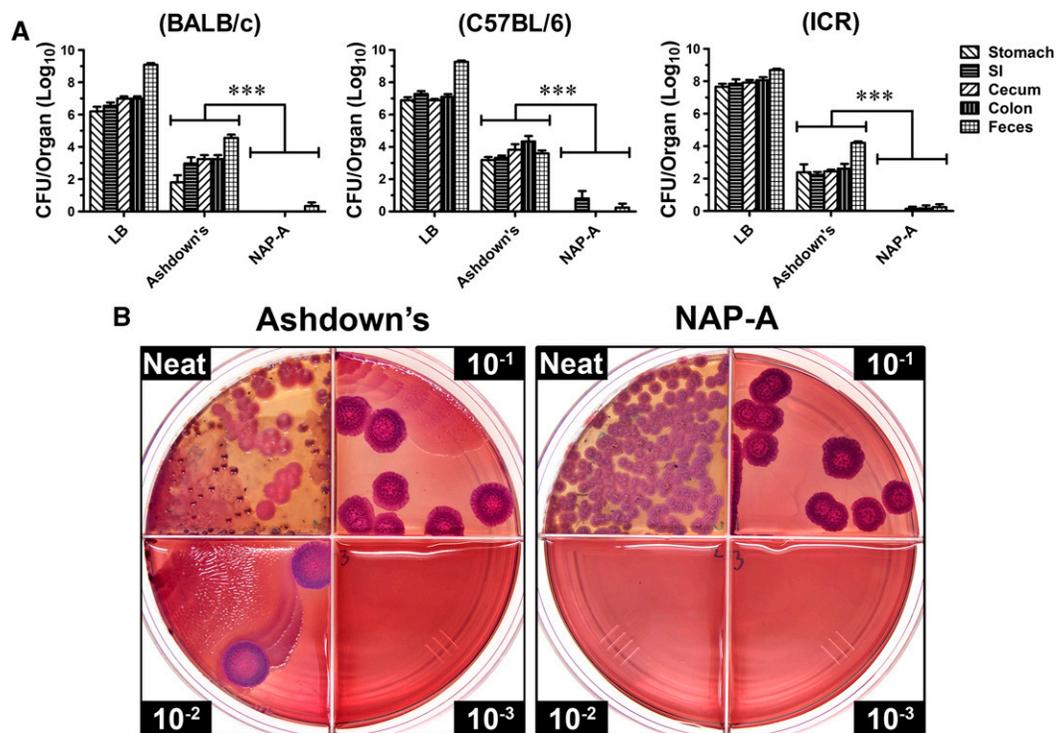


FIGURE 2. Increased selectivity of NAP-A medium compared with ASH. (A) Stomach, small intestine (SI), cecum, colon, and feces tissues from BALB/c ($N = 10$), C57BL/6 ($N = 11$), and ICR ($N = 11$) mice were homogenized, and serial dilutions were plated on LB, ASH, or NAP-A medium agar plates. Data are graphed as \log_{10} cfu per organ \pm SEM for stomach, SI, cecum, and colon and \log_{10} cfu per 1 g \pm SEM for feces. Differences in bacterial titers between different media in all organs were determined by a two-way ANOVA followed by a Bonferroni post-test ($***P < 0.001$). For each mouse strain, data were pooled from two individual experiments. (B) BALB/c mice were infected orally with 4.7×10^5 cfu *B. pseudomallei* strain 1026b and euthanized 3 days after infection. SI tissue was processed for determination of bacterial burden as described in *Materials and Methods*. Growth after culture of the same tissue sample on ASH or NAP-A media is presented. Agar plates were incubated at 37°C for 5 days. Quadrants where undiluted (neat) homogenate, and serial dilutions were plated and labeled accordingly.

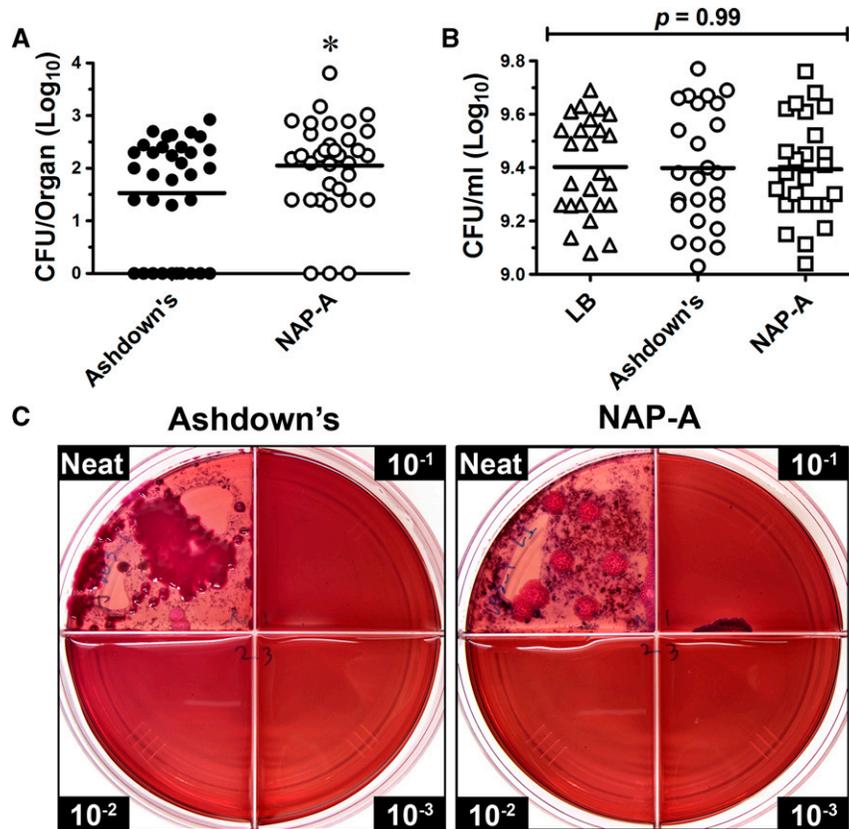


FIGURE 3. Increased sensitivity of NAP-A compared with ASH medium. (A) NAP-A medium improves isolation of *B. pseudomallei* from tissues with low-level *B. pseudomallei* colonization (titers $\leq 1,000$ cfu/organ). Data from GI organs plated on ASH and NAP-A media are graphed as individual log₁₀ cfu per organ values, with bars representing mean values ($N = 34$). Data were pooled from seven independent experiments. Statistical differences were determined by a two-tailed Student *t* test ($*P < 0.05$). (B) Overnight cultures of 25 *B. pseudomallei* isolates (12 clinical, 10 environmental, and 3 laboratory strains) were grown in LB broth. Serial dilutions were plated on LB, ASH, or NAP-A medium agar plates. Data are presented as individual log₁₀ cfu per 1 mL titers, with bars representing the mean titer. Bacterial titers were compared by a one-way ANOVA. Data are representative of two independent experiments. (C) BALB/c mice were infected orally with 9.1×10^4 cfu *B. pseudomallei* strain 2685a, and mice were euthanized 3 days after infection. Colon tissue was processed for determination of bacterial burden as described in *Materials and Methods*, and growth after culture of the same tissue sample on ASH or NAP-A media is presented. Agar plates were incubated at 37°C for 5 days. Enteric bacteria on ASH medium can be seen as a lawn of mucoid purple colonies. *B. pseudomallei* can be seen on both ASH and NAP-A media as red ruffled colonies.

10 environmental, and 3 laboratory strains) (Table 1) were assessed for their ability to grow on NAP-A medium. Overnight cultures of each strain were prepared in LB broth, and stationary phase cultures were diluted in sterile PBS and plated on each agar medium. We found nearly equivalent growth of all 25 strains on all three media (LB, ASH, or NAP-A; $P = 0.99$) (Figure 3). The growth of additional *Burkholderia* spp. was also investigated by streaking each strain onto agar media. Growth was equivalent on both media, with *B. thailandensis*, *B. oklahomensis*, *B. dolosa*, and *B. ubonensis* capable of growth on both ASH and NAP-A, whereas neither *B. gladioli* pathovar *cocovenenans* nor *B. vietnamiensis* was able to grow on either medium (data not shown). In summary, the addition of four antibiotics to the NAP-A medium did not reduce recovery of multiple different *B. pseudomallei* strains from infected tissues. In addition, NAP-A medium improved isolation of *B. pseudomallei* from sparsely colonized tissues without suppressing growth of a panel of clinical isolates of *B. pseudomallei*.

Comparison of culture of specimens from suspected human melioidosis patients on ASH and NAP-A medium. Lastly, we compared the effectiveness of NAP-A with conventional

ASH medium for recovery of *B. pseudomallei* from clinical specimens from humans with suspected *B. pseudomallei* infection (melioidosis). A total of 359 consecutive clinical specimens (119 pus/pus swab specimens, 172 throat swabs, 2 mouth swabs, 2 sputum specimens, and 64 fluid specimens [31 ascitic, 19 pleural, 5 joint, 2 pericardial, 6 Pouch of Douglas, and 1 gastric aspirate]) obtained from patients in Laos was plated in parallel on ASH medium and NAP-A medium; 16 positive samples were obtained (7 pus/pus swab, 7 throat swabs, 1 ascitic fluid, and 1 pleural fluid), and all 16 samples grew on both ASH and NAP-A medium either on direct plating or through

TABLE 3
Isolation from clinical samples

	Total samples	ASH	NAP-A	<i>P</i> value*
<i>B. pseudomallei</i>	359†	16	16‡	1.0
Commensal flora	150§	6	0	0.014

*Growth rates on ASH and NAP-A were compared by a two-tailed Fisher exact test.

†Samples were composed of 119 pus/pus swab, 172 throat swab, 64 fluids, 2 mouth swabs, and 2 sputum samples.

‡One sample was not tested by direct plating but positive when plated after broth culture.

§Samples were composed of 69 throat swab, 48 pus/pus swab, 29 fluids, 2 mouth swabs, and 2 sputum samples.

enrichment (Table 3). One of the samples that was positive by direct plating on ASH was not tested by direct plating onto NAP-A agar in error, but it was positive when broth enrichment cultures were plated on NAP-A medium. In contrast, there was one sample that was positive by direct plating on NAP-A but negative on direct plating on ASH, although *B. pseudomallei* was isolated after SBCT enrichment on both media. In general, the NAP-A and ASH plates were read as positive on the same day of incubation, with the exception of one each that was read as positive on ASH and NAP-A 24 hours earlier than the other medium. During the second

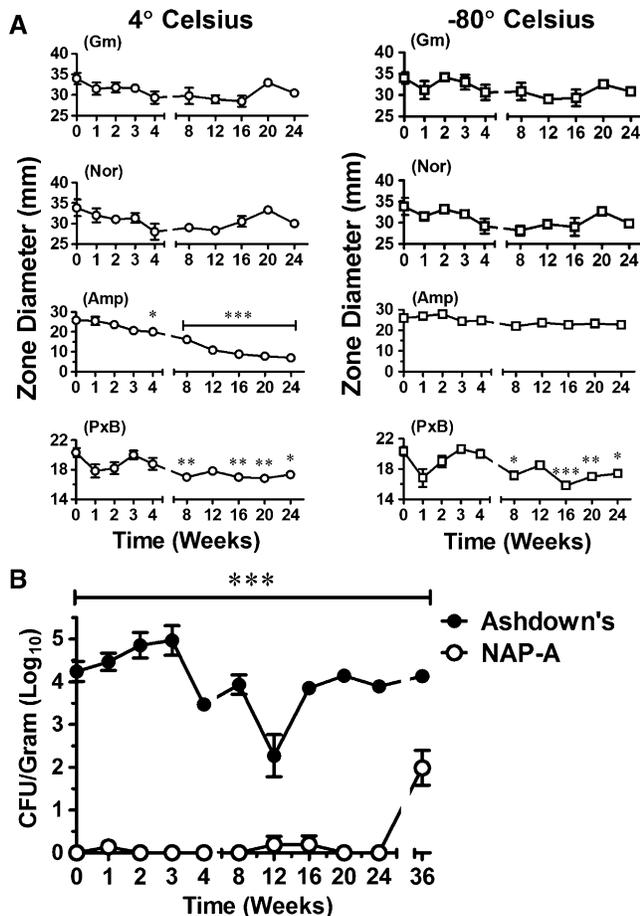


FIGURE 4. Stability of antibiotic stock solutions and NAP-A medium agar plates. (A) Antibiotic stock solutions were prepared as described in *Materials and Methods*. Stock solutions were aliquoted and stored at either 4°C or -80°C. At various time points after preparation, aliquots were thawed and assayed by the disk diffusion assay as described in *Materials and Methods*. Data at each time point represent data pooled from two independent experiments with three technical replicates per experiment ($N = 6$). Data are graphed as the mean \pm SEM zone of inhibition diameter in millimeters. Statistical differences compared with time zero samples were determined by a one-way ANOVA followed by a Tukey multiple means test. (B) Stability of NAP-A medium agar plates stored at 4°C. Agar plates were prepared as described in *Materials and Methods* and stored at 4°C. To test the stability of NAP-A medium, fecal bacteria from BALB/c mice was plated on ASH and NAP-A media at various time points after storage at 4°C. Data are graphed as mean \pm SEM \log_{10} cfu per 1 g titers. Statistical differences in fecal titers between ASH and NAP-A media at each time point were determined by a two-way ANOVA followed by a Bonferroni post-test. Data were pooled from two independent experiments, with six samples per experiment ($N = 12$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

phase, there were no differences seen between the semiquantitative growth of *B. pseudomallei* colonies from each sample on the two media (data not shown). These results showed, therefore, that *B. pseudomallei* was recovered from patient specimens equivalently when grown on either ASH or NAP-A media ($P = 1.0$).

In the second phase, the ability of NAP-A medium to suppress growth of commensal bacteria in human clinical specimens was assessed by semiquantitatively comparing growth of contaminating bacteria on NAP-A and ASH plates using a previously described technique.³⁹ Growth of contaminating flora was investigated in a total of 150 samples (48 pus/pus swab specimens, 69 throat swabs, 29 fluid specimens, 2 mouth swabs, and 2 sputum specimens). Contaminating flora growth was not observed on NAP-A medium, whereas six samples (four throat swabs and two pus samples) grew contaminating flora on ASH medium (two samples on direct culture and all six samples on SBCT subculture). One specimen had 1–10 colonies, one specimen had > 50 colonies on the direct culture, two specimens had 10–50 colonies, and four specimens had > 50 colonies on SBCT subculture. Statistical comparison of the overall frequency of contaminating growth on the two media (Fisher exact test) revealed a significant reduction in the isolation of contaminating flora on NAP-A medium compared with ASH (Table 3) ($P = 0.014$).

Stability of NAP-A medium. The stability of NAP-A medium constituent antibiotic solutions was investigated using the disk diffusion method. These studies showed that gentamicin, norfloxacin, and ampicillin stock solutions were stable for 6 months when stored at -80°C, whereas polymyxin B was stable for 3 months (Figure 4). At 4°C, gentamicin and norfloxacin remained stable for at least 6 months, whereas polymyxin B was stable for 3 months. A small but significant reduction in polymyxin B stability was observed after 3 months of storage at 4°C or -80°C, although a high degree of stability was still observed at 6 months. In contrast, ampicillin was only stable for 2 weeks at 4°C (Figure 4).

The stability of NAP-A plates was investigated by using fecal bacteria to screen the stability of NAP-A medium compared with ASH medium after storage at 4°C. After 6 months of storage at 4°C, enteric bacteria from BALB/c mice was unable to grow on NAP-A medium (limit of detection = 10 cfu/g), whereas an average titer of 2.5 \log_{10} cfu/g was isolated when the same samples were plated on 6-month ASH medium (Figure 4). However, the stability of NAP-A medium declined by 9 months of storage at 4°C (Figure 4). However, the 9-month NAP-A enteric bacterial titer was still significantly less than the enteric bacterial titer on ASH medium ($P < 0.001$). In summary, NAP-A medium remained stable for at least 6 months when stored at 4°C, and stock solutions of antibiotics used to prepare NAP-A medium could also be stored for at least 6 months at -80°C.

DISCUSSION

In this report, we describe the development and clinical evaluation of a modified ASH medium (NAP-A) designed to suppress growth of commensal and contaminating bacteria in clinical specimens, while still allowing the growth of *B. pseudomallei*, even when present in very low numbers. We found that addition of norfloxacin, ampicillin, and polymyxin B to conventional ASH medium resulted in increased specificity

and sensitivity for isolation of *B. pseudomallei* from GI tissues of mice. Moreover, NAP-A was comparable with ASH in recovery of *B. pseudomallei* from human clinical specimens, while still effectively suppressing growth of contaminating bacterial flora. In addition, NAP-A medium did not suppress the growth of 25 different clinical isolates of *B. pseudomallei*. These results suggest, therefore, that NAP-A medium may be a suitable alternative to ASH medium for isolation of *B. pseudomallei* from clinical specimens with high levels of contamination with endogenous flora, such as gastrointestinal specimens.

Although the oral route of infection with *B. pseudomallei* in humans has received relatively little attention, there is growing epidemiological and clinical evidence that oral infection may be a primary route of *B. pseudomallei* infection. For example, culture-positive drinking water and breast milk have been associated with melioidosis cases, including two separate outbreaks in Australia and a case of infant melioidosis.^{44–47} In addition, ulcers have been reported in the stomach, small intestine, and colon of melioidosis patients, and *B. pseudomallei* has been isolated from gastric fluids, intestinal contents, and rectal swabs of melioidosis patients.^{28,48–53} Moreover, two mouse models of melioidosis have recently shown that oral infection results in persistent colonization of the stomach together with fecal shedding and dissemination to extraintestinal organs.^{35,54} Our studies in the murine model of chronic GI infection also indicated that murine enteric bacteria often outcompeted *B. pseudomallei* when grown on ASH. Thus, we reasoned that overgrowth of enteric bacteria on ASH would also likely complicate investigation of enteric *B. pseudomallei* infection in humans. Until truly effective selective media for culture of *B. pseudomallei* from GI and fecal samples are developed, the true prevalence of GI infection with *B. pseudomallei* in humans cannot be determined. Thus, the new NAP-A medium may be particularly useful as a means to begin to assess GI chronic infection with *B. pseudomallei*.

Although the current study used ASH as the basal medium, other selective media have been developed for specific isolation of *B. pseudomallei*. For example, *B. pseudomallei* selective agar (BPSA) has been reported to improve isolation of mucoid colonies of *B. pseudomallei*, whereas growth of these mucoid colonies has been reported to be inhibited on ASH.⁵⁵ In one clinical study, however, BPSA medium was found to be equivalent to ASH in its ability to grow *B. pseudomallei* but also found to be less selective.¹⁰ Additional studies may be warranted to determine whether the addition of norfloxacin, ampicillin, and polymyxin B would improve the selectivity of BPSA medium.

NAP-A medium may also improve isolation of *B. pseudomallei* from environmental specimens, such as soil or water, by suppressing growth of the environmental microbiota. Although ASH and a basal salt solution supplemented with threonine have been used successfully for the isolation of *B. pseudomallei* from soil and water, contaminating bacteria often complicate environmental studies.^{56,57} Larger volumes of soil could potentially be processed, allowing for more readily performed quantitative analyses and a reduced need for initial broth culture. Indeed, studies in Laos are currently underway to determine whether NAP-A medium could improve isolation of *B. pseudomallei* from soil samples.

In summary, culturing heavily contaminated specimens on NAP-A medium resulted in more effective recovery of isola-

tion of *B. pseudomallei* from fecal specimens of mice, whereas recovery of *B. pseudomallei* from human clinical specimens was equivalent between ASH and NAP-A medium. We also determined that NAP-A medium and antibiotic stock solutions could be stored for extended periods without loss of activity. Although only a small-scale study comparing clinical *B. pseudomallei* isolation with ASH or NAP-A was performed, the success of these studies warrant additional investigation of NAP-A medium for recovery of *B. pseudomallei* from wound and fecal specimens of melioidosis patients as well as environmental specimens.

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Authors' addresses: Andrew Goodyear, Linda Strange, Herbert P. Schweizer, and Steven Dow, Department of Microbiology, Immunology, and Pathology, Rocky Mountain Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research, Colorado State University, Fort Collins, CO, E-mails: goody13@colostate.edu, lindamariestranger@gmail.com, Herbert.Schweizer@ColoState.edu, and sdow@colostate.edu. Drew A. Rholl, Department of Biology, North Park University, Chicago, IL, E-mail: drewrholl@gmail.com. Joy Silisouk and David A. B. Dance, Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao People's Democratic Republic, E-mails: joy@tropmedres.ac and david.d@tropmedres.ac.

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