Underdiagnosis of urinary tract infection caused by Methylobacterium species with current standard processing of urine culture and its clinical implications

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INTRODUCTION

Methylobacterium species are environmental opportunistic bacteria, and urinary tract infection (UTI) caused by these pathogens has not yet been documented. Four cases of UTI with Methylobacterium bacteraemia in immunocompetent female patients are reported. Their urine cultures, processed according to standard procedures (i.e. incubation at 35 °C in ambient air for 24 h before incubation at room temperature for a further 24 h), were either negative or positive for Escherichia coli. Specially designed experiments indicated that colonies of Methylobacterium species were visualized on blood agar only after incubation at 35 °C for at least 40 h, and growth was completely suppressed when concurrently incubated with much smaller inocula of E. coli. The isolates were variably susceptible to cephalosporins, but 100% susceptible to aminoglycosides. This study suggests an underdiagnosis of UTI caused by Methylobacterium species when the standard procedure of processing urine cultures is used, and implies that administration of aminoglycosides is important when treatment of UTIs with cephalosporin fails.

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INTRODUCTION

Methylobacterium species are pink-pigmented, Gram-negative rods and are normally distributed in environmental sources such as leaf surfaces, soil and sewage (Smith et al., 1985). Infections caused by Methylobacterium species have been increasingly reported and clinical sources of these bacteria include blood (Kaye et al., 1992), dialysate (Rutherford et al., 1988), lymph node, bone marrow (Fernandez et al., 1997), skin (Strazzi et al., 1992) and synovium (Liu et al., 1997). All patients reported so far in the literature suffering from invasive infections with Methylobacterium isolated from normally sterile sites were immunocompromised (Fernandez et al., 1997; Kaye et al., 1992; Korvick et al., 1989; Liu et al., 1997; Rutherford et al., 1988; Sanders et al., 2000; Smith et al., 1985; Zabransky et al., 1997). It is noteworthy that these environmental bacteria have not yet been reported to cause urinary tract infection (UTI). Recent observations from our institute were to the contrary. Between August 1999 and December 2000, a total of four patients were diagnosed, based on their clinical manifestations and laboratory data, with UTI at Chang Gung Memorial Hospital-Kaohsiung (CGMH-KS), Taiwan. All these patients had blood cultures positive for Methylobacterium species, and their urine cultures obtained before initiation of antimicrobial therapy were either negative or positive for Escherichia coli; surprisingly, these four patients had no overt evidence of immunoincompetence.

CGMH-KS is a 2500-bed medical centre in southern Taiwan serving as a primary care and tertiary referral centre, whose clinical microbiology laboratory processes urine specimens in accordance with standard procedures (Pezzio, 1988). Briefly, the collected urine specimens are inoculated on blood agar plates (BAP) and eosin-methylene blue (EMB) agar. These plates are then incubated in ambient air for 24 h and then in ambient air at room temperature for a further 24 h. If no visible colony growth is found, or the colony count is found to be < 1000 c.f.u. ml⁻¹ after 48 h, the urine culture report will be ‘negative’, or ‘colony count < 1000 c.f.u. ml⁻¹’, with no further identification of the bacterium.

The clustered cases of UTI with Methylobacterium bacteraemia found in our institute raised questions concerning the pathogenesis of Methylobacterium species in UTI. Are they opportunistic pathogens, or are they commonly colonizing bacteria, if not normal flora, having the ability, like other environmental bacteria, to cause UTI in immunocompetent hosts? Furthermore, does the processing of urine specimens in a clinical microbiology laboratory lead to a negative urine culture and, in turn, to an underestimation of the incidence...
and clinical importance of UTI caused by Methylobacterium species? We report these cases and, to clarify these puzzles, performed experiments in an attempt to determine whether the standard procedure in clinical microbiology laboratories leads to an underdiagnosis of UTI caused by Methylobacterium species.

**METHODS**

**Case reports**

**Case 1.** A 3-year-old girl who had played with other children on the soil of a front yard 1 week before, presented at our Emergency Department (ED) in September 1999 with chief complaints of a 2-day fever, dysuria and abdominal pain. Physical examination disclosed suprapubic tenderness. Urinalysis revealed pyuria with a white cell count of 40–45 cells per high-power field (HPF) and a negative nitrite reaction. A diagnosis of UTI was thus made and renal-bladder sonography disclosed ureter-vesicle reflux. The patient received antimicrobial therapy with parenteral cephalothin and gentamicin for 3 days and oral cotrimoxazole for the following 10 days. Urine culture subsequently revealed *E. coli* with a bacterial load of 8000 c.f.u. ml⁻¹ and a Gram-negative bacillus with a bacterial load of 1000 c.f.u. ml⁻¹, which was not identified. However, blood culture grew *Methylobacterium* species. This little girl had an uneventful clinical course under antimicrobial therapy.

**Case 2.** A 55-year-old woman was hospitalized in April 2000 having suffered from fever for 2 days, with dysuria and progressive left flank pain. Her medical history was unremarkable. Urinalysis disclosed pyuria with a white cell count of 60–65 cells per HPF and a negative nitrite reaction. Because of the possibility of acute pyelonephritis, she received treatment with parenteral cefazolin for 1 week and oral cotrimoxazole for the following 10 days. Urine culture subsequently showed *Methylobacterium* species. This patient recovered uneventfully under antimicrobial therapy.

**Case 3.** A 2-year-old girl was presented to our ED in May 2000, because of a 1-day high fever up to 39°C. Urinalysis showed pyuria with a white cell count of 50–55 per HPF with a positive nitrite reaction. The patient received an empirical antibiotic treatment with ampicillin and gentamicin. Urine culture subsequently showed *E. coli* with a bacterial load of 75 000 c.f.u. ml⁻¹ and another Gram-negative bacillus with a bacterial load of 9000 c.f.u. ml⁻¹, which was not identified. Blood culture subsequently grew *Methylobacterium* species. The empirically used antibiotics were switched to cefotaxime according to the susceptibility of the pathogen from blood culture. The girl recovered after antibiotic treatment.

**Case 4.** A 43-year-old female farmer presented to our ED in July 2000 due to a 1-week fever, dysuria and right flank pain. She had underlying bilateral renal calyceal stones. Urinalysis showed pyuria with a white cell count of 11–13 cells per HPF, microhaematuria with a red cell count of 21–23 cells per HPF and a negative nitrite reaction. Because of the possibility of acute pyelonephritis, she received treatment with parenteral cefuroxime and gentamicin for 3 days and subsequent oral cotrimoxazole therapy for 10 days. Her urine culture was negative; however, her blood culture grew *Methylobacterium* species. She recovered uneventfully under antimicrobial therapy.

**Bacterial identification.** The four isolates of *Methylobacterium* species from blood of the four separate patients reported above were numbered corresponding to case number. In a 48 h culture, each isolate grew Gram-negative vacuolated rods producing pink pigment on nutrient agar (NA; Difco) at 25°C but not at 42°C, and did not grow on MacConkey agar (MAC; BBL Microbiology). Biochemical tests showed that all strains were positive for indophenol oxidase, catalase and urease, except for *Methylobacterium* species no. 3 which was negative for urease. They reduced nitrate and were motile. For each isolate, acid production in oxidative fermentation base with 1% glucose, xylose, fructose, mannitol and lactose was negative; it was positive for 1% methanol. The morphology and biochemical testing of these four isolates fulfilled the criteria for identification of *Methylobacterium* species (Schrenkemberger & von Graevenitz, 1999; Rutherford et al., 1988; Wallace et al., 1990; Smith et al., 1985). In a further phenotypic identification (Zaharatos et al., 2001), imipenem (10 μg) and meropenem (10 μg) disks (BBL SensiDisc, Becton Dickinson) were placed on Sabouraud dextrose agar (SDA; BBL Microbiology) and incubated at 25°C for 72 h. All strains were susceptible to imipenem, but resistant to meropenem (NCCLS, 2002).

**Observations of growth of *Methylobacterium* species and *E. coli* in tryptic soy broth (TSB) and in urine at 35°C.** Bacterial suspensions of the respective isolates of *Methylobacterium* species and *E. coli* ATCC 25922 were independently adjusted with 0.9% saline to obtain a turbidity visually comparable to that of 0.5 McFarland nephelometer standards. *E. coli* strain ATCC 25922 was used for all experiments. One millilitre of each of the above suspensions was independently mixed with 3 ml TSB (BBL Microbiology) and 3 ml sterile urine. Sterile urine was from a volunteer clinician, who provided urine for all experiments. The urine was proven to be sterile as it showed negative bacterial growth on concurrently spread BAP agar plates and BAP (both from BBL Microbiology). The TSB- and urine-diluted bacterial suspensions were inoculated at 35°C for a total of 72 h. Bacterial counts of each isolate of *Methylobacterium* species and *E. coli* were measured at 0, 4, 8, 12, 24, 36, 48, 60 and 72 h by enumerating the number of colonies from 10-fold serially diluted specimens of 100 μl aliquots of the suspensions plated on BAP and MB agar. The plates were then incubated in ambient air at 35°C for 72 h. Although colonies were found at each time-point on both agar media, the colonies of *Methylobacterium* species were bigger and visibly clearer on BAP than on EMB, suggesting that BAP was more favourable for the growth of *Methylobacterium* species. For facilitating counting of bacterial colonies, BAP was therefore used for the growth of *Methylobacterium* species in all of the following experiments.

**Observations of growth behaviour of *Methylobacterium* species in urine under conditions simulating the routine processing in a clinical microbiology laboratory and at 35°C for a total of 72 h.** (i) One millilitre of bacterial suspensions of the isolates of *Methylobacterium* species at 0.5 McFarland standard were independently mixed with 3 ml sterile urine and then spread on separate BAPs. The inoculated BAPs were incubated in ambient air at 35°C for an initial 24 h and at room temperature for a subsequent 48 h. (ii) Each isolate of *Methylobacterium* species was prepared and spread onto BAPs as described in (i); however, the inoculated BAPs were incubated in ambient air at 35°C for a total of 72 h. Bacterial growth of *Methylobacterium* species in (i) and (ii) were measured at 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68 and 72 h by counting the visible colonies.

**Observations of growth behaviour of *Methylobacterium* sp. 3 and *E. coli* in respective urines concurrently inoculated with a fixed inoculum of *Methylobacterium* sp. 3 and different inocula of *E. coli*.** Because similar growth behaviour of these four isolates of *Methylobacterium* species was observed, *Methylobacterium* sp. 3 was arbitrarily chosen and was adjusted with 0.9% saline to 0.5 McFarland standard. *E. coli* suspension at 0.5 McFarland standard was likewise obtained. One millilitre of *Methylobacterium* sp. 3 suspension was mixed with 1 ml of *E. coli* suspensions with a bacterial load of about 10⁵, 10⁴ and 10³ c.f.u. ml⁻¹. The suspensions of *E. coli* with different bacterial loads were obtained by serial 10-fold dilution of the original suspension at 0.5 McFarland standard. Urines mixed with a fixed inoculum of *Methylobacterium* sp. 3 and different inocula of *E. coli* were
incubated in ambient air at 35 °C for a total of 72 h. Bacterial counts of *Methylobacterium* sp. 3 and *E. coli* were measured at 0, 4, 8, 12, 24, 36, 48, 60 and 72 h by counting the number of colonies from 10-fold serially diluted specimens of 100 μl aliquots of the mixed suspensions plated on BAPs. All experiments were performed at least twice for confirmation of the result.

**Antimicrobial susceptibility.** Susceptibility testing was performed using the disk diffusion method in accordance with NCCLS guidelines for *Enterobacteriaceae* (NCCLS, 2002). Mueller–Hinton agar (Difco) was used and the following 14 antimicrobial disks were included: cefazolin (30 μg), cefuroxime (30 μg), cefazidime (30 μg), ceftriaxone (30 μg), ampicillin (10 μg), amoxycillin/clavulanate (20/10 μg), ciprofloxacin (5 μg), piperacillin (100 μg), imipenem (10 μg), gentamicin (10 μg), amikacin (30 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). All disks were purchased from BBL Becton Dickinson. *E. coli* ATCC 25922 was used as a control.

**RESULTS**

**Growth behaviour of *Methylobacterium* species and *E. coli* in TSB and in urine at 35 °C**

In contrast to the rapid growth of *E. coli*, the trends of growth of the four isolates of *Methylobacterium* species in either TSB (Fig. 1a) or urine (Fig. 1b) at 35 °C were similarly slow, reflective of the micro-organisms’ fastidious characteristics.

**Fig. 1.** Growth curves of four isolates of *Methylobacterium* species and *E. coli* on TSB agar (a) and in urine (b) in ambient air at 35 °C. *Methylobacterium* species isolate 1, ◆; 2, ▶; 3, △; 4, □; mean of the four *Methylobacterium* species isolates, ■; *E. coli*, ○.

**Growth behaviour of *Methylobacterium* sp. 3 and *E. coli* in urine concurrently inoculated with a fixed inoculum of *Methylobacterium* sp. 3 (about 10⁷ c.f.u. ml⁻¹) and different inocula of *E. coli* (about 10³, 10⁴ and 10⁵ c.f.u. ml⁻¹) at 35 °C**

No colonies of *Methylobacterium* sp. 3 could be detected at any time-point throughout the 72 h incubation in ambient air, whereas colonies of *E. coli* developed rapidly at a speed in proportion to the inoculum size (Fig. 3).

**Susceptibilities**

Antibiotic susceptibilities of the isolated *Methylobacterium* species are shown in Table 1. Briefly, they were variably resistant to β-lactam antibiotics including cephalosporins, nalidixic acid, nitrofurantoin and trimethoprim/sulfamethoxazole and were 100 % susceptible to ciprofloxacin, imipenem and aminoglycosides.
environmental bacteria, *Methylobacterium* species are theoretically more likely to cause UTI in people with environmental exposure, especially women. However, as mentioned before, no UTI caused by *Methylobacterium* species has previously been documented. Zabransky et al. (1997) reported a case involving a patient with underlying multiple sclerosis who suffered from UTI and *Methylobacterium* bacteraemia; although this patient’s urine culture grew *Pseudomonas aeruginosa* (>10^5 c.f.u. ml^-1) and a non-fermenting Gram-negative bacillus (<10^3 c.f.u. ml^{-1}) that was not identified, the authors speculated that the non-fermenting Gram-negative bacillus isolated from patient’s urine was a *Methylobacterium* species, which caused the secondary bacteraemia. Our experiments indicated that, according to the current standard of processing urine cultures, within 24 h of incubation in ambient air at 35 °C colonies of *Methylobacterium* species are unlikely to be seen, and the small number of colonies found after an additional 24 h of incubation in ambient air at room temperature will only suggest that a *Methylobacterium* species is a contaminant. In particular, when *Methylobacterium* species are introduced into the urinary reservoir where *E. coli*, the most commonly found uropathogen, is present, the overwhelming suppression of the growth of *Methylobacterium* species by *E. coli* would make detection of this fastidious micro-organism in urine impossible. The present study supports a conclusion of the paradoxical fact that absence of reported UTI caused by the environmental *Methylobacterium* species results, at least in part, from the shortcoming of the current standard processing of urine culture adopted by the vast majority of clinical microbiology laboratories. The other possible explanation for the absence of reported UTI due to *Methylobacterium* species is the relatively low clinical virulence of this pathogen (Kaye et al., 1992; Liu et al., 1997; Sanders et al., 2000), which makes UTI caused by this pathogen self-limiting or perhaps cured with prescribed antibiotics aimed at the presumed uropathogens belonging to *Enterobacteriaceae*. For this reason, the need for a test for *Methylobacterium* species in urine has not been recognized.

Although patients with *Methylobacterium* infections reported previously were immunocompromised, the patients with UTI and secondary *Methylobacterium* bacteraemia in this report were apparently immunocompetent. Our report suggests that UTI caused by *Methylobacterium* species has long been undiagnosed. Murray et al. (1992) reported that urine specimens containing members of *Enterobacteriaceae* can be reliably incubated for a full 24 h, and then all specimens with uropathogen growth <10^4 c.f.u. ml^{-1} should be incubated for an additional day to ensure accurate quantification. Specimens from patients with suspected slow-growing organisms such as fungi should be incubated for a minimum of 2 days at 35 °C. Our experiments in growth characteristics of the fastidious *Methylobacterium* species further support the conclusion made by Murray and colleagues. Patients considered to be at risk for development of UTI due to *Methylobacterium* species should include those involved in gardening, farming, as well as outdoor activities.

**DISCUSSION**

The fact that UTI is much more common in women than in men supports the notion that the ascending route is the most important pathogenesis in this infection. The female urethra is comparatively short and its proximity to the moist vulva and perineum renders it easily contaminated. As a rule, pathogens frequently colonize vaginal introitus and perineum before ascending to cause UTI (Hooton, 2000). All patients reported in the present study are female with or without a traceable history of environmental exposure. Being

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**Table 1. Susceptibilities of four *Methylobacterium* species**

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<th>Antibiotic</th>
<th><em>Methylobacterium</em> species</th>
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<td>Cefazolin</td>
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<td>Trimethoprim/sulfamethoxazole</td>
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<td>Nalidixic acid</td>
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<td>Nitrofurantoin</td>
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**Fig. 3.** Growth curves of *Methylobacterium* sp. 3 in urine in ambient air at 35 °C (●) (results identical for all three *E. coli* inocula), when mixed with *E. coli* at different inoculum concentrations. Starting inocula of *E. coli* were about 10^5 c.f.u. ml^-1 (○), about 10^4 c.f.u. ml^-1 (△) and about 10^3 c.f.u. ml^-1 (×). The lower limit of the viable counts on agar plate was set at 30 c.f.u. ml^-1.
with soil or plant contact and, perhaps, those from the rural community. Previous underdiagnosis of UTI due to *Methylobacterium* species has limited clinicians’ awareness of UTI caused by this pathogen. The clinical characteristics of UTI due to *Methylobacterium* species and the ability of *Methylobacterium* species from the urinary tract to invade the bloodstream deserve further study.

In agreement with a previous report, aminoglycosides were extremely active against *Methylobacterium* species in vitro, whereas β-lactam drugs showed variable inhibitory effects (Brown et al., 1992). Half of our isolates were susceptible to trimethoprim/sulfamethoxazole. All isolates were resistant to nalidixic acid and all but one were resistant to nitrofurantoin. The present study implies that, in patients with UTI who show a poor clinical response to treatment with commonly used antibiotics such as cephalosporins, trimethoprim/sulfamethoxazole, nalidixic acid and nitrofurantoin, *Methylobacterium* species should be considered as a potential pathogen and managed accordingly by empirically adding the universally effective aminoglycosides until the pathogen is proven to be otherwise. This is especially true for patients with environmental exposure. The development of a cost-effective procedure for processing urine culture for commonly seen uropathogens in general, and which is sensitive for the detection of *Methylobacterium* species in particular, is required.

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**REFERENCES**


