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Short Communication

Isolation of a Capnophilic and Extended-Spectrum β-Lactamase-Producing *Proteus mirabilis* Strain from the Urine of an Octogenarian Male Patient with Acute Pyelonephritis

Eiji Soga[1], Miki Akiyama[1], Yusuke Ohsaki[3]**, Wataru Hayashi[3], Takehisa Matsumoto[2], Kozue Oana[3,4], Noriyuki Nagano[3,4], and Yoshiyuki Kawakami[3,4]*

[1]Department of Laboratory Medicine, Nakatsugawa Municipal General Hospital, Nakatsugawa 508-8502; [2]Department of Laboratory Sciences, Gunma University Graduate School of Health Sciences, Maebashi 371-8543; and [3]Department of Health and Medical Sciences and [4]Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University Graduate School of Medicine, Matsumoto 390-8621, Japan

SUMMARY: A capnophilic Gram-negative rod-shaped bacterium was recovered from the urine of an octogenarian male patient with acute pyelonephritis. The isolate was found to produce CTX-M-2-type extended-spectrum β-lactamase. Interestingly, the isolate failed to grow on modified Drigalski (BTB) and MacConkey agar media, even under CO_2-enriched atmosphere. Our analysis revealed that the pH-indicator dyes, bromothymol blue, and/or crystal violet that were incorporated into the agar media inhibited the growth of the isolate. Although routine identification methods using Vitek® 2 Compact systems were unsuccessful, the isolate was identified as *Proteus mirabilis* by 16S rRNA sequencing and MALDI-TOF MS analysis. The carbonic anhydrase (CA) region spanning approximately 2,000 bp upstream to 2,000 bp downstream, which is responsible for the CO_2 requirement, was not amplified, which could be attributed to the large-scale deletion or mutation of the DNA sequences containing the CA gene region. In fact, revertants with the ability to grow without CO_2 were not detected. However, a revertant that was capable of growing in both BTB and MacConkey agar was detected at frequencies less than 10^{-9}. Therefore, the genes responsible for the highly sensitive reactions of the isolate to pH indicator dyes is not likely to be linked to the CA genes.

Previous studies have reported the isolation of *Escherichia coli* and *Proteus mirabilis* from urine samples (1–3). In the present study, we successfully isolated capnophilic *P. mirabilis* capable of producing extended-spectrum β-lactamase (ESBL), a causative agent of acute pyelonephritis. In addition, the isolate was not capable of growing in modified Drigalski (BTB) (Kyokuto Pharmaceutical Ind., Tokyo, Japan) and/or MacConkey agar media (Eiken Chemical Co., Ltd., Tokyo, Japan).

The patient was an octogenarian Japanese male suffering from urinary tract infection with a body temperature ranging from 38 ˚C to 40 ˚C. The patient was admitted in June 2016 to Nakatsugawa Municipal General Hospital, Nakatsugawa, Japan because of fever (38.5 ˚C). The ethical committee waived the need for written consent regarding the research of bacterial isolates; the personal data related to the clinical information were anonymized. Direct microscopy of the Gram-stained urine preparation revealed an abundance of leucocytes with Gram-negative rod-shaped cells, thereby confirming previous results showing high white blood cell counts (1.05×10^9 cells/L) and higher C-reactive protein levels (9.62 mg/L). After incubation in CO_2 incubator (Sanyo Electric Co., Tokyo, Japan) at 35 ˚C for 48 h, non-hemolytic and swarming Gram-negative short-sized rods were visible at cell counts of 10^7 cfu/mL on sheep blood agar (Eiken Chemical Co.). Interestingly, the isolate did not grow on BTB or MacConkey agars after overnight incubation in CO_2-enriched atmosphere.

Biochemical characterization and evaluation of the antimicrobial susceptibility of the isolate using the Vitek® 2 Compact (bioMérieux Japan Ltd., Tokyo, Japan) system were unsuccessful because the isolate did not proliferate. Comparative sequence analysis of the isolate revealed 99.9% similarity of the 16S rRNA sequence to that of *P. mirabilis* AOUC-001. The isolate was further analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Microflex mass spectrometer; Bruker Daltonics Japan, Yokohama, Japan). Results showed that the isolate had almost the same spectrum as that of *P. mirabilis*, with an excellent score of 2.289. Therefore, the isolate was identified as *P. mirabilis* and was designated as *P. mirabilis* NA2609. Antimicrobial susceptibility was determined by incubating the isolate at 35 ˚C for 18 h in 5% CO_2 using a Dry-Plate Eiken DP-31 (Eiken Chemical Co.) system. *P. mirabilis* NA2609 showed resistance to various β-lactams,

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*Corresponding author: Mailing address: Department of Health and Medical Sciences, Shinshu University Graduate School of Medicine, Matsumoto 390-8621, Japan. Tel: 81-263-37-3510 (ex.3510), E-mail: yk23724@shinshu-u.ac.jp

**Present address: Department of Laboratory Medicine, Toyohashi Municipal Hospital, Toyohashi 441-8570, Japan.

including piperacillin, cefazoline, cefotiam, cefotaxime, ceftazidime, cefepime, sulbactam/ampicillin, and aztreonam, but was found to be susceptible to flomoxef and meropenem. Successive disc-diffusion screening tests demonstrated positive results for ESBL production. ESBL gene analysis showed that *P. mirabilis* NA2609 harbored the gene encoding IS*Ecp1*-*bla*$_{CTX-M-2}$ ESBL. The empirical prescription was changed from ampicillin/sulbactam on admission to meropenem. Afterwards, the patient's fever and symptoms subsided after 5 days of administration.

Then, we examined the effect of temperature on the growth of *P. mirabilis* NA2609. Sheep blood agar media were seeded with *P. mirabilis* NA2609 and the 2 reference strains *P. mirabilis* ATCC29906 and ATCC43071 (purchased and stocked in Shinshu University) and then incubated overnight. The plates were incubated overnight in 5% CO_2 at 30, 35, and 42 ˚C. As shown in Table 1, *P. mirabilis* NA2609 grew at 30 ˚C and 35 ˚C, although both *P. mirabilis* ATCC29906 and ATCC43071 showed significant growth at all tested temperatures, consistent with our previously reported findings (3).

We investigated the effect of carbon dioxide on the growth of *P. mirabilis* NA2609 and the reference strains *P. mirabilis* ATCC29906 and ATCC43071 by seeding the strains in blood agar. Growth was measured after overnight incubation at 35 ˚C in the CO_2 incubator with CO_2 concentrations of 0.5%, 1.0%, 1.5%, 2.0%, 3.0%, 4.0%, 5.0%, 10%, and 20%. As shown in Table 2, *P. mirabilis* NA2609 showed significant growth at 35 ˚C with 4.0% CO_2 atmosphere, which was also consistent with our previous findings (3), although the 2 reference ATCC strains grew well at all CO_2 concentrations.

Given that *P. mirabilis* NA2609 failed to grow in BTB agar as well as MacConkey agar, we investigated the growth inhibitory effects of the pH indicator dyes bromothymol blue, crystal violet, and neutral red in nutrient agars (Eiken Chemical Co.) by incorporating varying concentrations of the dyes using the 2 ATCC strains as the growth controls. As shown in Table 3, *P. mirabilis* NA2609 did not grow on agar media with bromothymol blue concentrations greater than 0.004% or those containing more than 0.0002% crystal violet, which were the dye concentrations used in BTB and MacConkey agar media, respectively. By contrast, the addition of neutral red to MacConkey agar at concentrations higher than 0.003% did not exert a growth inhibitory effect on *P. mirabilis* NA2609 or the 2 ATCC reference strains. However, growth was suppressed at slightly higher concentrations of neutral red dye. The above findings clearly demonstrated that failure of NA2609 to grow on BTB and/or MacConkey media was caused by the presence of bromothymol blue and crystal violet dyes.

In the subsequent study, the stability of *P. mirabilis* NA2609 was assessed to determine the emergence of revertants capable of growing without the addition of CO_2 and/or those capable of growing in BTB or MacConkey agar. No revertants exhibiting capnophilic property were detected. By contrast, a single revertant that grew on both BTB and MacConkey agar media was detected at cell frequencies less than 10^{-9} and was verified to produce ISEcp1-*bla*$_{CTX-M-2}$-type ESBL. Genetic clonality between the revertant and *P. mirabilis* NA2609 was confirmed by Dienes test (4) and pulsed-

Table 1. Effects of temperatures under 5% CO_2-added circumstances on the proliferation after overnight-incubation on sheep blood agars

Incubation temperature	*Proteus mirabilis* NA2609 (this study)	*Proteus mirabilis* ATCC29906[1)]	*Proteus mirabilis* ATCC43071[1)]
30˚C	+	++	++
35˚C	+	+++	+++
42˚C	±	++	++

±, subtle growth; ±, weak growth; ++, good growth; +++, ample growth.
1): Reference *Proteus mirabilis* ATCC strains for growth control.

Table 2. Effect of CO_2 concentration on the proliferation of the capnophilic isolate and the reference ATCC strains after overnight-incubation at 35˚C on sheep blood agars

	Ambient air	Growth at following CO_2 (%) concentrations								
		0.5	1.0	1.5	2.0	3.0	4.0	5.0	10	20
Proteus mirabilis NA2609 (this study)	–	–	–	p.p.[2)]	p.p.[2)]	p.p.[2)]	+	+	+	+
Proteus mirabilis ATCC29906[1)]	+	+	+	+	+	+	+	+	+	+
Proteus mirabilis ATCC43071[1)]	+	+	+	+	+	+	+	+	+	+

1): See footnote of Table 1.
2): pin-point colonies.

Table 3. Growth inhibitory behavior in the presence of pH-indicator dyes, bromothymol blue, crystal violet, and neutral red, at concentrations incorporated in isolation agar media against the isolate and the reference ATCC strains

	pH-indicator dyes	Bromothymol blue	Crystal violet		Neutral red		
Concentration (%)	0	0.004[1)]	0.0001[2)]	0.0002	0.003[2)]	0.024	0.096
Proteus mirabilis NA2609 (this study)	+++	No-growth	±	No-growth	+++	±	No-growth
Proteus mirabilis ATCC 29906[3)]	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis ATCC 43071[3)]	+++	+++	+++	+++	+++	+++	+++

1): concentration (%) incorporated into modified Drigalski-agars.
2): concentration (%) incorporated into MacConkey agars.
3): See footnote of Table 1.

field gel electrophoresis (3) (data not shown).

To investigate the capnophilic property of *P. mirabilis* NA2609, we analyzed its carbonic anhydrase (CA)-encoding *can* gene and the neighboring regions.

Results revealed that the *can* gene was present in ATCC 29906 and the 2 clinical *P. mirabilis* strains (data not shown), although it was undetected in *P. mirabilis* NA2609. Moreover, the segment spanning from the 2,000 bp upstream to 2,000 bp downstream regions of the *can* gene was successfully amplified from the DNA isolated from ATCC 29906 and the 2 clinical strains. By contrast, the corresponding region was not amplified from *P. mirabilis* NA2609 (data not shown).

A recent study showed that proliferation of *Escherichia coli* was dependent on the presence of bicarbonate (5). Indeed, CA metalloenzymes have been assumed to catalyze the hydration-dehydration of carbon dioxide-bicarbonate (5,6). These enzymes are well known to support various physiological functions, including respiration and CO_2 transport.

The Krebs cycle is the primary metabolic pathway responsible for CO_2 production, and the inducible decarboxylases supply CO_2 under conditions of reduced CO_2 levels (7). Cellular levels of CO_2 and HCO_3^- are important for cell growth (8). Intracellular levels of CO_2/bicarbonate are generally low, and spontaneous reactions cannot support growth in ambient air. Therefore, the *can* gene is essential for bacterial growth under low CO_2 concentrations (9).

Sahuquillo-Arce et al. (10) recently showed that the *can* gene of the 2 capnophilic *E. coli* strains cannot be detected by PCR. Likewise, the *can* gene of *P. mirabilis* NA2609 was undetectable by PCR, although the *can* genes of ATCC 29906 and the 2 clinical strains were amplifiable. Even after repeated trials, we could not detect revertants that were capable of growing in ambient air, which suggested that the capnophilic property of *P. mirabilis* NA2609 was caused by a large-scale deletion or mutation of sequences containing the CA gene region.

Meanwhile, 5 drug transporters in *E. coli* strains, namely, ABC-, RND-, MF-, SMR-, and MATE-types, have been genetically investigated for sensitivity to dyes (11). Of these, the RND-type-transporter is known to be involved in the release of compounds, including dyes. Presumably, *P. mirabilis*, like *E. coli*, encodes a drug transporter; however, it is likely that the gene encoding the drug transporter of *P. mirabilis* NA2609 isolate was disrupted, although mechanisms other than the RND-type transporter might be involved.

Notably, only one revertant capable of growing on BTB and MacConkey media was detected at cell frequencies less than 10^{-9}. The above findings implied that the mutated region was smaller than the *can* gene.

The isolation of capnophilic *Enterobacteriaceae* from urine samples has been rarely documented. The low isolation frequency could be attributed to the fact that routine incubation of bacterial cultures from urine samples is not performed using CO_2 incubators. Moreover, the inhibitory effect of the dyes on growth cannot be ruled out, since crystal violet or bromothymol blue are incorporated into the isolation media. Considering that the failure of the isolates to grow on the tested media is an unusual finding, such strains could be overlooked. Although the strain NA2609 was successfully isolated because the blood agar without dye was used, strains from fecal samples of suspected pathogens, such as diarrheagenic *E. coli*, cannot be isolated because such blood agars are not routinely used as isolation media. Our findings indicated that clinical microbiologists should pay appropriate attention to the isolation of fastidious bacteria. Our case report has provided important findings in the field of clinical microbiology.

Conflict of interest None to declare.

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