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 Soga¹, Miki Akiyama¹, Yusuke Ohsaki¹**, Wataru Hayashi¹, Takehisa Matsumoto², Kozue Oana³,⁴, Noriyuki Nagano²,⁴, and Yoshiyuki Kawakami¹,³,⁴*

¹Department of Laboratory Medicine, Nakatsugawa Municipal General Hospital, Nakatsugawa 508-8502; ²Department of Laboratory Sciences, Gunma University Graduate School of Health Sciences, Maebashi 371-8543; and ³Department of Health and Medical Sciences and ⁴Department 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 CO₂-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 CO₂ 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 CO₂ 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.

Received May 31, 2018. Accepted November 16, 2018.
J-STAGE Advance Publication November 30, 2018.
DOI: 10.7883/yoken.JJID.2018.201

**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 \textit{P. mirabilis} NA2609 harbored the gene encoding \textit{ISEcp1-}\textit{bla}_{\textit{CTX-M-2}}\textit{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 \textit{P. mirabilis} NA2609. Sheep blood agar media were seeded with \textit{P. mirabilis} NA2609 and the 2 reference strains \textit{P. mirabilis} ATCC29906 and ATCC43071 (purchased and stocked in Shinshu University) and then incubated overnight. The plates were incubated overnight in 5% CO\textsubscript{2} at 30, 35, and 42 °C. As shown in Table 1, \textit{P. mirabilis} NA2609 grew at 30 °C and 35 °C, although both \textit{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 \textit{P. mirabilis} NA2609 and the reference strains \textit{P. mirabilis} ATCC29906 and ATCC43071 by seeding the strains in blood agar. Growth was measured after overnight incubation at 35 °C in the CO\textsubscript{2} incubator with CO\textsubscript{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, \textit{P. mirabilis} NA2609 showed significant growth at 35 °C with 4.0% CO\textsubscript{2} atmosphere, which was also consistent with our previous findings (3), although the 2 reference \textit{ATCC} strains grew well at all CO\textsubscript{2} concentrations.

Given that \textit{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 agar (Eiken Chemical Co.) by incorporating varying concentrations of the dyes using the 2 \textit{ATCC} strains as the growth controls. As shown in Table 3, \textit{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 \textit{P. mirabilis} NA2609 or the 2 \textit{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 \textit{P. mirabilis} NA2609 was assessed to determine the emergence of revertants capable of growing without the addition of CO\textsubscript{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\textsuperscript{-9} and was verified to produce \textit{ISEcp1-}\textit{bla}_{\textit{CTX-M-2}}\textit{ESBL}. Genetic clonality between the revertant and \textit{P. mirabilis} NA2609 was confirmed by Dienes test (4) and pulsed-
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 CO₂ transport.

The Krebs cycle is the primary metabolic pathway responsible for CO₂ production, and the inducible decarboxylases supply CO₂ under conditions of reduced CO₂ levels (7). Cellular levels of CO₂ and HCO₃⁻ are important for cell growth (8). Intracellular levels of CO₂/bicarbonate are generally low, and spontaneous reactions cannot support growth in ambient air. Therefore, the *can* gene is essential for bacterial growth under low CO₂ 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 10⁻⁵. 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 CO₂ 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.

**REFERENCES**

1. Eykyn S, Phillips I. Carbon dioxide-dependent *Escherichia coli*. Br Med J. 1978; 1: 576.
2. Tena D, González-Praetorius A, Sáez-Nieto JA, et al. Urinary tract infection caused by capnophilic *Escherichia coli*. Emerg Infect Dis. 2008; 14: 1163-4.
3. Oana K, Yamaguchi M, Nagata M, et al. First isolation of carbon dioxide-dependent *Proteus mirabilis* from an uncomplicated cystitis patient with Sjögren’s syndrome. Jpn J Infect Dis. 2013; 66: 241-4.
4. Bale MJ, Hollis RJ. Characterization of organisms for epidemiologic purposes: serotyping, pyocin typing, and Dienes test. In: Isenberg HD, editor. Clinical Microbiology Procedures Handbook, vol. 2. 1st ed. Washington, D.C.: American Society for Microbiology; 1992. p. 11.14.1-11.14.2.
5. Merlin C, Masters M, McAteer S, et al. Why is carbonic anhydrase essential to *Escherichia coli*? J Bacteriol. 2003; 185: 21: 6415-24.
6. Hashimoto M, Kato J. Indispensability of the *Escherichia coli* carbonic anhydrases YadF and CinT in cell proliferation at a low CO₂ partial pressure. Biosci Biotechnol Biochem. 2003; 67: 919-22.
7. Takayama M, Ohyama T, Igarashi K, et al. *Escherichia coli* cad operon functions as a supplier of carbon dioxide. Mol Microbiol. 1994; 11: 913-8.
8. Guilloton MB, Lamblin AF, Kozlinski EI, et al. A physiological role for cyanate-induced carbonic anhydrase in *Escherichia coli*. J Bacteriol. 1993; 75: 1443-51.
9. Hashimoto M, Kato J. Indispensability of the *Escherichia coli* carbonic anhydrases YadF and CinT in cell proliferation at a low CO₂ partial pressure. Biosci Biotechnol Biochem. 2003; 67: 919-22.
10. Sahuquillo-Arce JM, Chouman-Arcas R, Molina-Moreno JM, et al. Capnophilic *Enterobacteriaceae*. Diagn Microbiol Infect Dis. 2017; 87: 318-9.
11. Nishino K, Yamaguchi A. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J Bacteriol. 2001; 183: 5803-12.