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Letter to the Editor

Screening of hospital-manhole sewage using MacConkey agar with cefotaxime reveals extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*

Sir,

The dissemination of multidrug-resistant (MDR) *Enterobacteriaceae*, such as New Delhi metallo-β-lactamase (NDM)- or extended-spectrum β-lactamase (ESBL)-producing isolates, is unquestionably a major concern for infection control both in community and hospital settings [1]. Unfortunately, due to lack of consistent measures, controlling the emergence of MDR bacteria leading to hospital-acquired infections has remained as a significant challenge [1]. Meanwhile, it is believed that hospital-manhole sewage closely reflects activities in the hospital and can indicate the emergence of MDR bacteria in the hospital wards. Therefore, hospital-manhole sewage has a potential scientific value to monitor the emergence of MDR bacteria [2], although its full potential has not been precisely evaluated. Thus, to validate the usefulness of hospital-manhole sewage for monitoring of MDR bacteria, in this study the prevalence of MDR bacteria was compared between hospital-manhole sewage and other manhole sewage from a university environment.

Hospital-manhole sewage ($n=5$ different manholes) as well as other manhole sewage [$n=8$; library ($n=1$), economics department ($n=2$), science department ($n=2$) and dormitory ($n=3$)] from a university located in Hokkaido–Tohoku area, Japan, was collected. As controls, rainwater (manhole structurally unconnected inside building) was also collected from a similar location [hospital-manhole rainwater ($n=2$) and other manhole rainwater ($n=5$)]. The depth of all water flows was <3 cm and the samples were easily collected using sterile plastic tubes. All samples (approximately 50 mL) were collected in December 2017. Samples were kept in ice coolers (approximately 4 °C) and were processed within 4 h after sampling. To remove excessive impurities, the samples were mixed in an equal volume of phosphate-buffered saline (PBS) and, following sedimentation, 1 mL of the resultant supernatant was used for bacterial isolation. The processed sample was immediately inoculated on nutrient agar for estimating total bacteria and on MacConkey agar without or with 2 mg/L cefotaxime (CTX) for CFU enumeration and isolation of CTX-resistant bacteria. Isolated CTX-resistant bacteria were identified to species level using a MALDI Biotyper system (Bruker Daltonics, Billerica, MA, USA) according to the manufacturer's instructions, as follows: score value 2.300–3.000, highly probable species identification; score value 2.000–2.299, secure genus identification with probable species identification; score value 1.700–1.999, probable genus identification; and score value 0.000–1.699, unreliable identification. The antimicrobial susceptibility of the isolates to CTX, ceftazidime, imipenem, tetracycline, ciprofloxacin, tigecycline and colistin was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines [3]. PCR was performed to detect ten different antimicrobial resistance genes (bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, *tetA, tetB, tetC, tetD, tetE* and *tetG* [4]. In addition, PCR-based phylogenetic typing for *Escherichia coli* was performed and the isolates were classified into seven phylogenetic groups/subgroups (A_0, A_1, B_1, $B2_2$, $B2_3$, D_1 and D_2) [4].

Fig. 1A shows prevalence of *E. coli* and the CFU number of bacteria from different sources in different culture conditions. When estimated using nutrient agar and MacConkey agar (plain), the total number of bacteria was consistent [mean ± standard deviation (S.D.) $3.1\times10^4 \pm 4.3\times10^4$ per mL ($1.5\times10^6 \pm 2.1\times10^6$ per sample)] between the hospital-manhole sewage and the other manhole sewage samples, indicating no bias in the amount of loading bacteria introduced on the culture plates. Interestingly, on MacConkey agar with 2 mg/L CTX, the CFU number in the hospital-manhole sewage was significantly higher than in the other manhole sewage ($P=0.0278$), indicating the high number of CTX-resistant bacteria, which may be related to the emergence of MDR bacteria in the hospital wards. Meanwhile, there was no significant difference in the number of CFUs with rose-red colonies estimated on MacConkey agar with 2 mg/L CTX (presumptively assigned as *E. coli* into Enterobacterales, according to the BD manual: https://www.bdj.co.jp/tw/pi/111-251270-N-00.pdf). A total of 44 isolates from MacConkey agar with or without CTX were assigned as the following scores (mean ± S.D.) by MALDI Biotyper: *E. coli* ($n=25$), 2.40 ± 0.06; *Citrobacter gillenii* ($n=1$), 2.15; *Citrobacter braakii* ($n=1$), 2.43; *Citrobacter freundii* ($n=1$), 2.27; *Serratia fonticola* ($n=4$), 2.16 ± 0.16; *Aeromonas hydrophila* ($n=1$), 2.31; *Klebsiella oxytoca* ($n=2$), 2.34; *Enterobacter asburiae* ($n=1$), 2.42; *Pseudomonas koreensis* ($n=2$), 2.13; *Pseudomonas monteilii* ($n=2$), 2.20; *Pseudomonas nitroreducens* ($n=2$), 2.34; *Pseudomonas putida* ($n=1$), 2.10; and *Acinetobacter baumannii* ($n=1$), 2.26. Nine CTX-resistant isolates, including eight *E. coli* [minimum inhibitory concentration (MIC) >4 mg/L] and one *P. monteilii* (MIC > 64 mg/L), were identified. All of the *E. coli* isolates possessed bla_{CTX-M} genes. Interestingly, *E. coli* was likely to be preferentially isolated from hospital-manhole sewage but not from manhole rainwater (see the upper pie charts in Fig. 1A). *Escherichia coli* isolates were characterised according to the following indicators: strain ID (location); β-lactamase gene type; phylogenetic group; and MIC (in mg/L)] (Fig. 1B). Fortunately, no strains were isolated exhibiting carbapenem resistance, a serious public-health threat associated with significant morbidity and mortality [5].

In this study, screening of hospital-manhole sewage using MacConkey with 2 mg/L CTX revealed ESBL-producing *E. coli*, indicating their persistence in the hospital environment. We believe that hospital-manhole sewage has potential in monitoring the emergence of MDR bacteria. However, the significance of this work for public health requires further study with a higher number of

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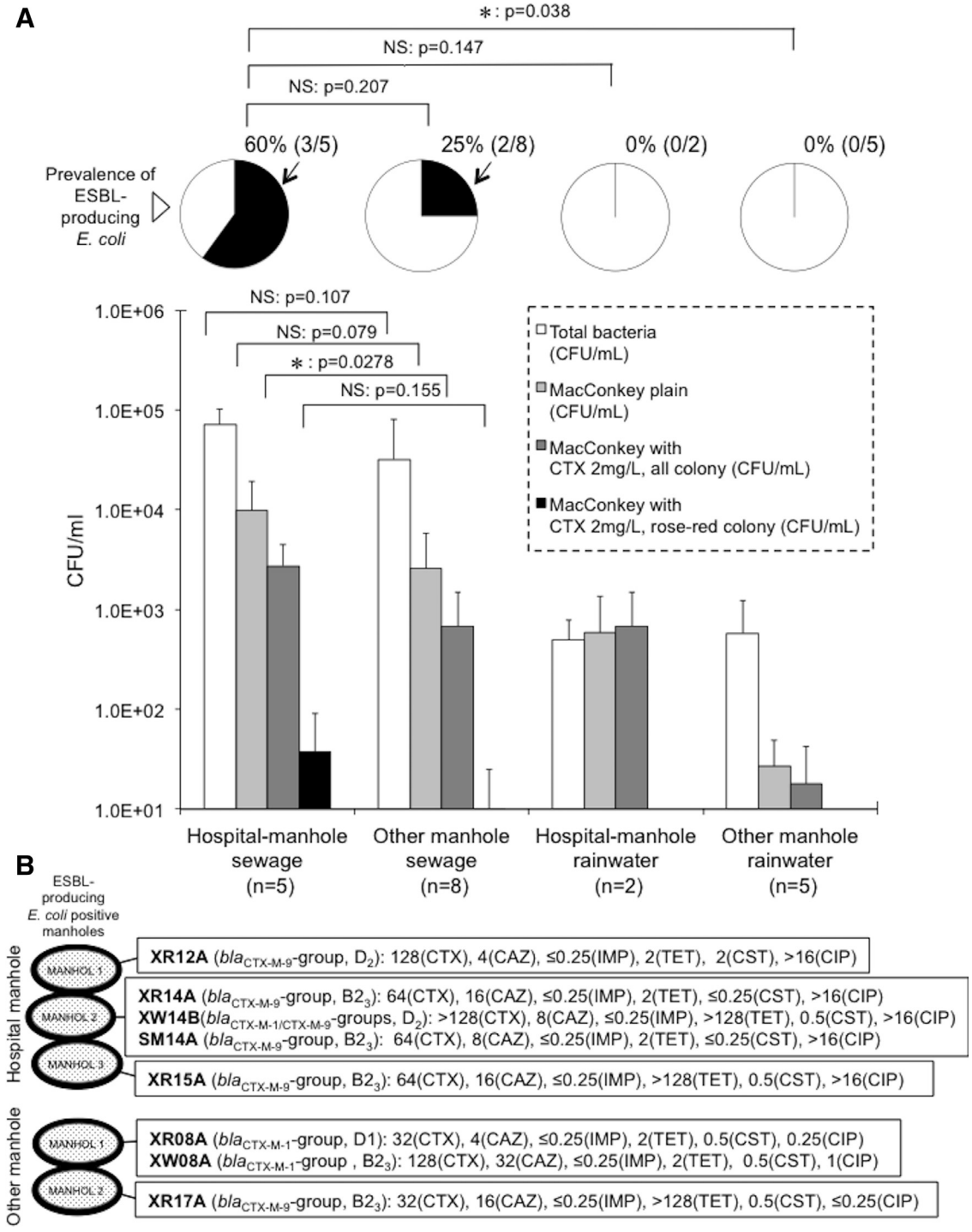

Fig. 1. Hospital-manhole sewage with a significant impact on screening extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*. (A) The upper pie charts show the prevalence of ESBL-producing *E. coli* (black) in sewage and rainwater. The prevalence is indicated as a percentage (%). The lower graph shows a comparison of CFU numbers (mean ± standard deviation) from each sewage on nutrient agar for total bacteria, MacConkey agar (plain), MacConkey agar with 2 mg/L cefotaxime (CTX) (total) and MacConkey agar with CTX (rose-red colonies). Comparison of the prevalence of CTX-resistant *E. coli* between hospital-manhole sewage and other manhole sewage was performed by χ^2 test of independence. Comparison of the total CFU counts between hospital-manhole sewage and other manhole sewage was also performed by Mann–Whitney *U*-test. NS, not significant. * Statistically significance at $P < 0.05$. (B) Characterisation of ESBL-producing *E. coli* isolated from three hospital-manhole sewage samples and two other manhole sewage samples, with strain ID (location), β-lactamase gene type, phylogenetic group, and MICs (mg/L) to CTX, ceftazidime (CAZ), imipenem (IMP), tetracycline (TET), colistin (CST) and ciprofloxacin (CIP).

manhole sewage samples as well as inclusion of samples from other hospitals and sources.

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Torahiko Okubo, Takaki Hasegawa
Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido University Graduate School of Health Sciences, Nishi-5 Kita-12 Jo Kitaku, Sapporo 060-0812, Japan

Akira Fukuda
Laboratory of Food Microbiology and Food Safety, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
Osaka Institute of Public Health Microbiology Section, 8-34 Tojotyo, Tennoji, Osaka 543-0026, Japan

Jeewan Thapa
Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido University Graduate School of Health Sciences, Nishi-5 Kita-12 Jo Kitaku, Sapporo 060-0812, Japan

Masaru Usui, Yutaka Tamura
Laboratory of Food Microbiology and Food Safety, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

Hiroyuki Yamaguchi*
Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido University Graduate School of Health Sciences, Nishi-5 Kita-12 Jo Kitaku, Sapporo 060-0812, Japan

*Corresponding author. Tel.: +81 11 706 3326; fax: +81 11 706 3326.
E-mail address: hiroyuki@med.hokudai.ac.jp (H. Yamaguchi)

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