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Evaluation of the Disk Diffusion Test for *Bacteroides fragilis* Group Clinical Isolates

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Background: *Bacteroides fragilis* group (BFG) isolates are the most frequently isolated gram-negative anaerobic bacteria and exhibit higher levels of antimicrobial resistance than other anaerobic bacteria. Reliable susceptibility testing is needed because of reports of resistance to the most active antibiotics. Recently, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) introduced disk zone diameter breakpoints. We evaluated the disk diffusion test (DDT) for susceptibility testing of BFG isolates compared with the agar dilution method.

Methods: In total, 150 BFG isolates were collected from three institutes in Korea. The agar dilution method was conducted according to the CLSI guidelines. DDT was performed following the EUCAST guideline. Fastidious anaerobe agar supplemented with 5% defibrinated horse blood was used as the culture medium. Nine antimicrobials were evaluated: penicillin, cefoxitin, cefotetan, imipenem, meropenem, piperacillin-tazobactam, clindamycin, moxifloxacin, and metronidazole.

Results: The categorical agreement (CA) between the two methods was >90.0% for imipenem, meropenem, clindamycin, and metronidazole. However, the CA for piperacillin-tazobactam was low, at 83.2%. Major errors were found: 5.4% for imipenem, 7.4% for meropenem, and 12.8% for piperacillin-tazobactam. All minor errors were <10%. We propose using the area of technical uncertainty (ATU) zone-overlapping area for susceptible and resistant strains to reduce errors in the DDT. Outside the ATU, the CAs of cefoxitin, cefotetan, and piperacillin-tazobactam were >90.0%, whereas that of moxifloxacin was increased to 88.5%.

Conclusions: The DDT can be a useful alternative antimicrobial susceptibility test for BFG isolates when using the ATU zone to reduce errors.

Key Words: Area of technical uncertainty, *Bacteroides fragilis*, Disk diffusion antimicrobial tests, Microbial sensitivity tests, Uncertainty

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INTRODUCTION

Bacteroides fragilis group (BFG) isolates are the most frequently isolated gram-negative anaerobic bacteria and exhibit higher levels of antimicrobial resistance than other anaerobic bacteria [1-3]. The resistance patterns of many anaerobes have changed significantly over recent decades, both within and among countries [4-6]. Antimicrobial susceptibility testing (AST) should be considered for specific infections such as bacteremia, brain abscesses, endocarditis, osteomyelitis, and prosthetic device infec-

tions [1, 3]. Despite the clinical significance, routine AST for anaerobes is not commonly performed because it is difficult, expensive, and inflexible. Furthermore, reports of resistance to the most active antibiotics, such as carbapenem, piperacillin-tazobactam, clindamycin, and metronidazole, highlight the urgent need for reliable susceptibility testing.

Although the agar dilution method (ADM) is the gold standard for AST of anaerobes, the labor and skill requirements limit its widespread use. Gradient strips are available for critical cases, but their cost-effectiveness in the routine laboratory setting is limited. Consequently, researchers have explored the disk diffusion test (DDT) for anaerobes, culminating in the publication of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [7-9]. The Committee of the Antibiogram of the French Society of Microbiology (CA-SFM) proposed DDT breakpoints for anaerobes in 2011 [10]. The introduction of the area of technical uncertainty (ATU) in the CA-SFM documents made it possible to meet the United States Food and Drug Administration (FDA) criteria using this method, as few errors were observed outside the ATU zone, whereas within the ATU zone, the determination of the minimum inhibitory concentration (MIC) resolves most problems [11]. In 2023, the EUCAST revised DDT breakpoints [12]. However, EUCAST and CA-SFM DDT breakpoints are correlated to EUCAST MIC breakpoints. In Korea, the CLSI MIC breakpoints are usually used in routine laboratories, and no study has compared the DDT and CLSI MIC breakpoints. Therefore, we evaluated the DDT for susceptibility testing of BFG isolates by comparing DDT and ADM CLSI breakpoints.

MATERIALS AND METHODS

Bacterial isolates

In total, 150 BFG isolates were collected at Hanyang University Hospital, Hanyang University Guri Hospital, and Seoul Clinical Laboratory in Korea between January 2022 and December 2023. Non-duplicate clinical isolates were obtained from blood, abscesses, and body fluids from 150 patients. This study received ethical approval from the Institutional Review Board of Hanyang University Seoul Hospital, Seoul, Korea (approval No. 202207037).

Agar dilution method

The ADM was conducted according to the CLSI guideline M11-A8 [13]. Brucella agar supplemented with hemin and vitamin K1 (Sigma-Aldrich, Seoul, Korea) and 5% laked sheep blood was used as the culture medium. We used the following antimicrobial powders: penicillin, piperacillin (Sigma-Aldrich, Seoul, Korea), tazobactam (Yuhan, Seoul, Korea), cefoxitin (Merck Sharp & Dohme, West Point, PA, USA), cefotetan (Daiichi Pharmaceutical, Tokyo, Japan), clindamycin, imipenem, meropenem, moxifloxacin, and metronidazole (Sigma-Aldrich). A constant concentration of 4 μg/mL tazobactam was used for the piperacillin-tazobactam combination. An inoculum of 10^5 colony-forming units was applied using a Steers replicator (CMI-Promex Inc., Pedricktown, NJ, USA), and plates were incubated in an anaerobic chamber (Bactron, Cornelius, OR, USA) at 35°C for 48 hrs. MICs were determined as the concentration at which a marked reduction in growth occurred, such as from confluent colonies to a haze, < 10 tiny colonies, or several normal-sized colonies [13]. *B. fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC29741, and *Clostridioides difficile* ATCC 700057 were used as QC strains.

Disk diffusion test

The DDT was performed following the EUCAST guidelines [12]. Fastidious anaerobe agar (MB Cell, Seoul, Korea) supplemented with 5% defibrinated horse blood (FAA-HB) was used as the culture medium. FAA-HB plates were dried at 25°C overnight and were not pre-reduced. Bacterial suspensions were prepared in normal saline at a McFarland density of 1. Antimicrobial disks were used as follows: penicillin 1 unit, cefoxitin 30 μg, cefotetan 30 μg, imipenem 10 μg, meropenem 10 μg, piperacillin-tazobactam 30/6 μg, clindamycin 2 μg, moxifloxacin 5 μg, and metronidazole 5 μg; all disks were purchased from Oxoid (Hampshire, UK). Plates were incubated in an anaerobic chamber (Bactron) at 35°C for 16–20 hrs. Clindamycin plats were incubated for up to 40–44 hrs. Zone diameters were measured following the EUCAST guidelines [14].

Method comparison

Clinical categorization was based on the CLSI MIC and EUCAST inhibition zone breakpoints. Very major error (VME) was defined when isolates were susceptible in the DDT and resistant in the ADM; major error (ME) was defined when isolates were resistant in the DDT and susceptible in the ADM; and minor error (mE) was defined when isolates were intermediate in the ADM and resistant or susceptible in the DDT. The ATU is defined by a range of inhibition zone diameters, which is an area where difficulties in interpretation exist.

RESULTS

Bacterial collection

In total, 150 clinical isolates of BFG were tested, including 100 *B. fragilis*, 24 *B. thetaiotaomicron*, nine *Bacteriodes ovatus*, five *Bacteriodes vulgatus*, three *Bacteriodes pyogenes*, two *Bacteriodes uniformis*, two *Bacteriodes intestinalis*, two *Bacteriodes salyersiae*, two *Bacteriodes cellulosilyticus* isolates, and one *Bacteriodes faecis* isolate. One *B. fragilis* isolate failed to grow, even after three attempts.

Penicillin, imipenem, and meropenem

The distribution of MICs and inhibition zone diameters for the 149 BFG isolates for the nine antibiotics is presented in Fig. 1. Table 1 lists the CLSI MIC breakpoints and EUCAST zone diameter breakpoints. We provided the zone diameter breakpoints with ATU zones for BFG isolates (Table 1). All isolates were resistant to penicillin according to the CLSI MIC breakpoints (Fig. 1A). Four and five isolates were resistant to imipenem and meropenem, respectively (Fig. 1B and 1C). The CA between CLSI ADM and DDT was 93.3% for imipenem and 90.6% for meropenem (Table 2). No isolates were present in the VME region for imipenem and meropenem compared to the CLSI MIC breakpoints. The mEs of imipenem and meropenem were 1.3% and 2.0%, respectively. However, compared with the EUCAST MIC breakpoints, seven (4.7%) and four (2.7%) isolates exhibited VME for imipenem and meropenem, respectively.

Piperacillin–tazobactam, clindamycin, and metronidazole

Six isolates were resistant to piperacillin-tazobactam according to the CLSI MIC breakpoints (Fig. 1D). The CA between the CLSI ADM and DDT was 83.2% for piperacillin-tazobactam. Compared with the CLSI MIC breakpoints, no isolates were present in the VME region for piperacillin-tazobactam. However, 19 isolates (12.8%) had ME, and 4.0% had mE. The wide inhibition zone diameters for the clindamycin-susceptible strains ranged from 13 to 40 mm (Fig. 1E). The CA of clindamycin was 93.3%. Both VME and ME were 0%; mE was 6.7%. No metronidazole-resistant isolates were detected (Fig. 1F). For metronidazole, one isolate (0.7%) had ME, with a low MIC of 2 μg/mL and an inhibition zone diameter of 24 mm. The CA of metronidazole was 99.3%.

Cefoxitin, cefotetan, and moxifloxacin

The EUCAST guidelines do not include breakpoints for inhibition zone diameters for cefoxitin, cefotetan, or moxifloxacin. The inhibition zone diameter breakpoints with ATU zones used in this study are listed in Table 1. We defined ATU ranges of 19–21 and 19–22 mm for cefoxitin and cefotetan, respectively, which overlapped with resistant, intermediate, and susceptible isolates (Fig. 1G and 1H). The breakpoints proposed in this study resulted in CAs of 93.8% and 90.8% for cefoxitin and cefotetan, respectively. The VMEs were 0% and 3.1%, whereas the ME was 1.6% and 1.5% for cefoxitin and cefotetan, respectively. For moxifloxacin, the 19–21-mm range was defined as the ATU overlapping with resistant, intermediate, and susceptible isolates (Fig. 1I). The CA was 88.5%. VME and mE were 1.5% and 10.1%, respectively.

DISCUSSION

The EUCAST guidelines for DDT suggest that a correct inoculum should produce a confluent lawn of growth evenly distributed over the agar surface. If growth is not confluent, it is difficult to read the inhibition zone, resulting in interpretation error, and the test must be repeated. In this study, one *B. fragilis* isolate failed to grow even when the DDT was reattempted. The growth failure rate was 0.6%, which was acceptable according to FDA recommendations (< 10%) [15].

According to CLSI guideline M23, the VME should be < 1.5% and ME < 3% for a large collection of unselected clinical isolates [16]. According to the FDA, CA must be ≥90%, VME < 1.5%, and ME < 3% [15]. In this study, overall, the CA between methods was high, although several discrepancies were observed, especially for piperacillin-tazobactam. Compared to the CLSI MIC breakpoints, the CA between the MIC and DDT methods was > 90.0% for imipenem, meropenem, clindamycin, and metronidazole (Table 2). The CA of piperacillin-tazobactam was low, at 83.2%. The MEs were 5.4% for imipenem, 7.4% for meropenem, and 12.8% for piperacillin-tazobactam. All mEs were < 10%.

Although the VME and mE rates were acceptable according to the CLSI and FDA recommendations, the ME rate was unacceptable, at > 3%, for imipenem, meropenem, and piperacillin-tazobactam. To minimize errors in categorization, we proposed the overlapping zone of susceptible and resistant strains in the 3–4-mm zone in Table 1. This area was defined as the ATU, where difficulties in interpretation and reading by staff can occur. Nagy, *et al*. reported that the the difference in the zone diameter between repeated measurements within 0–3 mm was 88.5%, and the standard deviation of diameters for the *B. fragilis* QC strain based on parallel measurements were 0.5 and 2.2 mm [8]. The ATU zones for imipenem, meropenem, and piperacillin-tazobactam were proposed as 3-mm intervals relative to the EUCAST

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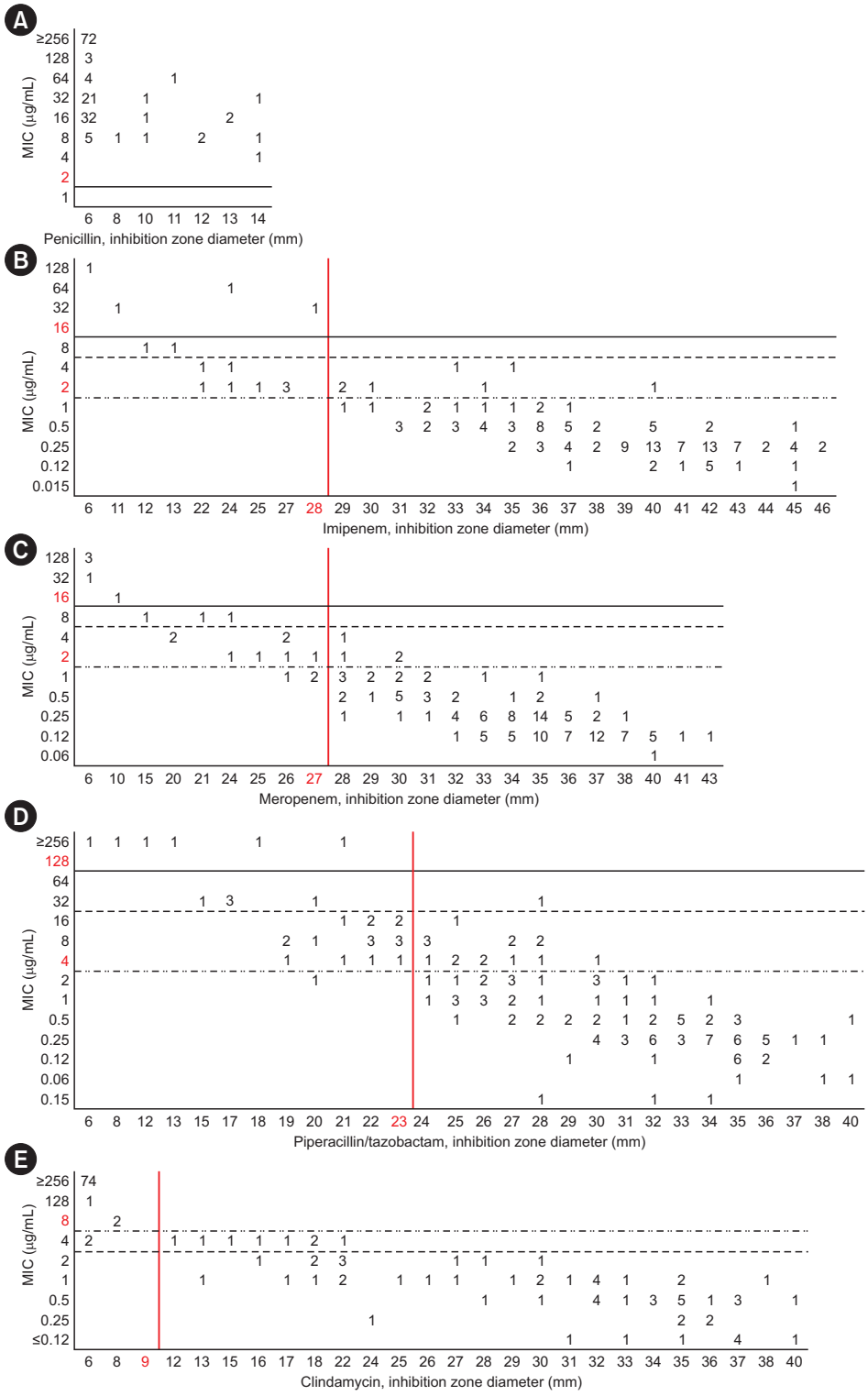

Fig. 1. Distribution of inhibition zone diameters and MICs of 149 BFG isolates for nine antibiotics. The numbers in the figures represent isolate counts. Red letters on the axis represent MIC or zone diameter breakpoints for resistance. Solid black and dashed lines represent CLSI MIC values for resistance and intermediate resistance, respectively. Dash-dot lines indicate EUCAST MIC values for resistance (A–I). Red vertical lines represent zone diameter breakpoints for resistance (A–F). The gray zone represents the ATU in this study (G–I).
Abbreviations: BFG, *B. fragilis* group; MIC, minimum inhibitory concentration; EUCAST, European Committee on Antimicrobial Susceptibility Testing; ATU, area of technical uncertainty.

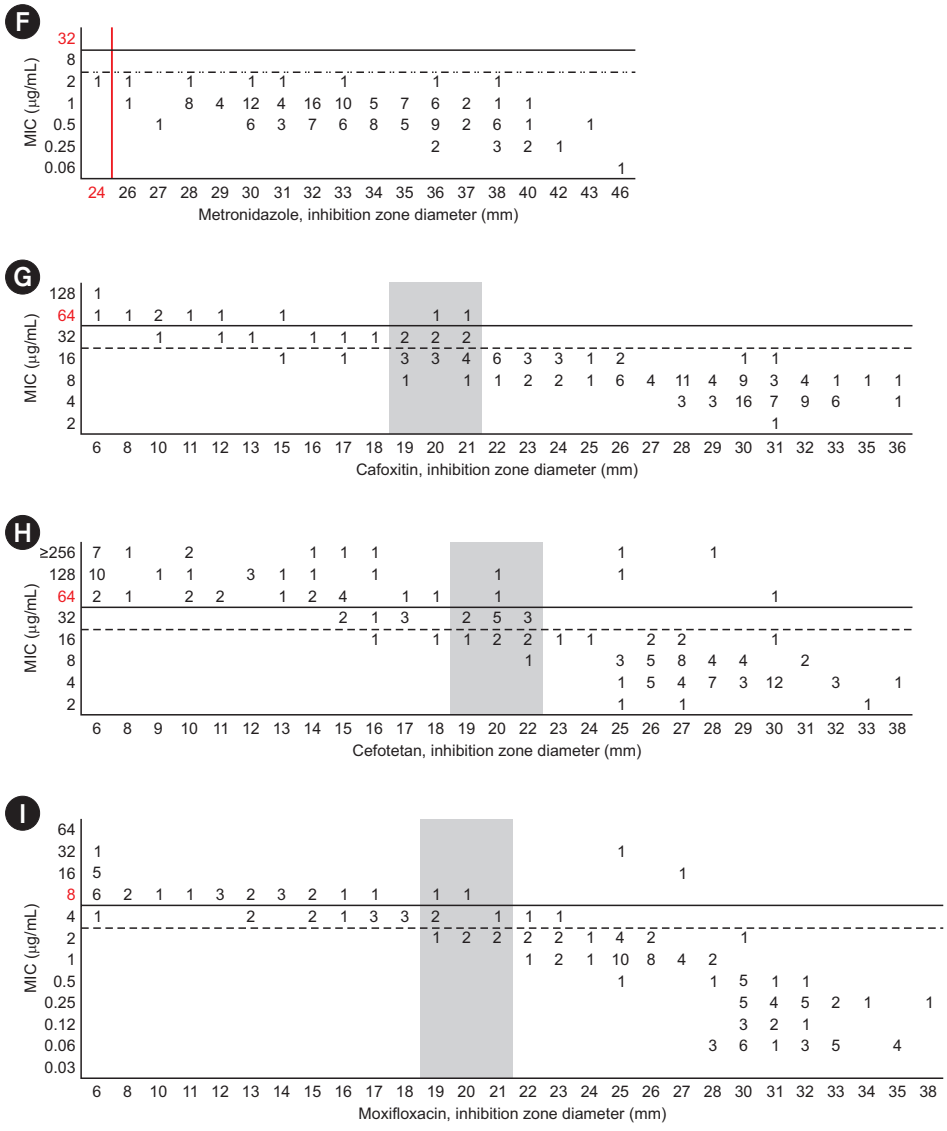

Fig. 1. Continued.

breakpoints. We suggest that the ATU zones for cefoxitin, cefotetan, and moxifloxacin would improve the CA and reduce VME and ME. Therefore, the ATU zone could be a buffer zone to reduce errors in the DDT. Except for the ATU isolates, the CAs of cefoxitin, cefotetan, and piperacillin-tazobactam were >90.0%, and that of moxifloxacin was increased to 88.5%. When the 27–29-mm ATU zone was used for imipenem, the ME decreased from 5.4% to 3.5%. When ATU zones of 26–28 mm for meropenem and 22–24 mm for piperacillin-tazobactam were applied, the ME decreased from 7.4% to 3.0% and from 12.8% to 5.4%, respectively. The introduction of the ATU zone makes the DDT more convenient in routine testing and generates fewer VMEs and MEs. However, the isolates in the ATU zone require several MIC determinations via the E-test. Dubreuil, *et al*. [11] suggested that MIC measurements within the ATU zone can be limited. They recommended reporting "resistant" in cases of intrinsic resistance and leaving this field blank when the strain is susceptible to other antibiotics.

Resistant isolates were separated from susceptible ones for clindamycin and metronidazole. The EUCAST guidelines suggest that it is crucial to carefully examine zones for colony growth for clindamycin. Clindamycin-susceptible isolates in a 24-hr incubation showed resistant results after incubation for up to 40 hrs [9]. In the present study, three isolates were identified as susceptible to clindamycin via the DDT and showed resistance after retesting or after a 40-hr incubation. The wide inhibition zone di-

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Table 1. Breakpoints proposed in CLSI, EUCAST, and this study

Antimicrobial agent	MIC breakpoints (μg/mL)					Zone diameter breakpoints (mm)				
	CLSI			EUCAST		EUCAST		This study		
	S≤	I	R≥	S≤	R>	S≥	R<	S≥	ATU	R≤
Penicillin	0.5	1	2	-	-	-	-	15	-	14
Cefoxitin	16	32	64	-	-	-	-	22	19–21	18
Cefotetan	16	32	64	-	-	-	-	23	19–22	18
Imipenem	4	8	16	1	1	29	29	30	27–29	26
Meropenem	4	8	16	1	1	28	28	29	26–28	25
Pip/tazobactam	16	32–64	128	2	2	24	24	25	22–24	21
Clindamycin	2	4	8	4	4	10	10	10	-	9
Moxifloxacin	2	4	8	-	-	-	-	22	19–21	18
Metronidazole	8	16	32	4	4	25	25	25	-	24

Abbreviations: MIC, minimum inhibitory concentration; EUCAST, European Committee on Antimicrobial Susceptibility Testing; Pip, piperacillin; S, susceptible; R, resistant; ATU, area of technical uncertainty; -, not applicable.

Table 2. Categorical agreement and error rates between agar dilution method and disk diffusion test

Antimicrobial agent	N (%) of isolates with (CLSI)				N (%) of isolates with (EUCAST)			N (%) of isolates outside the ATU zone in this study			
	CA	VME	ME	mE	CA	VME	ME	CA	VME	ME	mE
Penicillin	-	-	-	-	-	-	-	149 (100)	0 (0)	0 (0)	0 (0)
Cefoxitin	-	-	-	-	-	-	-	121 (93.8)	0 (0)	2 (1.6)	6 (4.7)
Cefotetan	-	-	-	-	-	-	-	119 (90.8)	4 (3.1)	2 (1.5)	6 (4.6)
Imipenem	139 (93.3)	0 (0)	8 (5.4)	2 (1.3)	142 (95.3)	7 (4.7)	0 (0)	135 (95.1)	0 (0)	5 (3.5)	2 (1.4)
Meropenem	135 (90.6)	0 (0)	11 (7.4)	3 (2.0)	142 (95.3)	4 (2.7)	3 (2.0)	127 (94.8)	0 (0)	4 (3.0)	3 (2.2)
Pip/tazobactam	124 (83.2)	0 (0)	19 (12.8)	6 (4.0)	131 (87.9)	17 (11.4)	1 (0.7)	117 (90.0)	0 (0)	7 (5.4)	6 (4.6)
Moxifloxacin	-	-	-	-	-	-	-	123 (88.5)	2 (1.4)	0 (0)	14 (10.1)
Clindamycin	139 (93.3)	0 (0)	0 (0)	10 (6.7)	147 (98.7)	0 (0)	2 (1.3)	139 (93.3)	0 (0)	0 (0)	10 (6.7)
Metronidazole	148 (99.3)	0 (0)	1 (0.7)	0 (0)	148 (99.3)	0 (0)	1 (0.7)	148 (99.3)	0 (0)	1 (0.7)	0 (0)

Abbreviations: EUCAST, European Committee on Antimicrobial Susceptibility Testing; Pip, piperacillin; ATU, area of technical uncertainty; CA, categorical agreement; VME, very major error; ME, major error; mE, minor error; -, not applicable.

ameter range for clindamycin-susceptible strains was in accordance with that in a previous study [8]. A limitation of this study is its reliance on small sample size and restricted representativeness, as data were only collected from three domestic institutions.

In conclusion, the DDT can be a useful alternative AST method for BFG isolates when using the ATU zone to reduce errors. The number of MIC measurements can be limited to isolates within the ATU zone in routine laboratories. The accuracy and reliability of antibiotic susceptibility testing for BFG could lead to better clinical outcomes.

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AUTHOR CONTRIBUTIONS

Conceptualization: Lee Y and Lee K; Methodology: Lee Y, Lee K, Kim M, Bae MH, Lee H; Investigation: Lee Y and Lee K; Funding acquisition: Lee Y and Lee K; Supervision: Lee Y and Lee K; Writing – original draft: ee Y, Lee K, Kim M, Bae MH, Lee H, Writing – review and editing: ee Y, Lee K, Kim M, Bae MH, Lee H.

CONFLICTS OF INTEREST

None declared.

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