

Original Article

임상 뇨 검체에서 변법 EUCAST 신속 직접 항균제
감수성 시험법의 평가저스틴 무지라네자¹, 권미정², 김대원², 이상국², 이혁민², 용동은²연세대학교 ¹보건대학원 글로벌 보건안보학과, ²의과대학 진단검사의학교실 및 세균내성연구소Performance of Modified-EUCAST Rapid
Direct Antimicrobial Susceptibility Testing on
Clinical Urine SamplesJustin Mugiraneza¹, Mijung Kwon², Daewon Kim², Sang-Guk Lee², Hyukmin Lee², Dongeun Yong²¹Department of Global Health Security, Yonsei University Graduate school of Public Health, Seoul,²Department of Laboratory Medicine and Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul, Korea

ABSTRACT

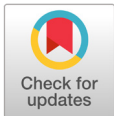
Background: The rapid antimicrobial susceptibility testing (AST) performed on urine samples would guide the adequate choice of antibiotics for obtaining better treatment outcomes in patients. Our study aimed to evaluate the performance of the modified-EUCAST (European Committee on Antimicrobial Susceptibility Testing) rapid direct AST on urine samples.

Methods: From >2,000 urine samples, a total of 128 urine samples containing bacterial counts of $\geq 2 \times 10^4$ CFU/mL with a uniform bacterial shape were initially included based on flow cytometry (Sysmex UF-1000i, Japan) and Gram staining, respectively. A total of 103 samples showing the presence of *Enterobacteriaceae* were finally selected in this study. The urine samples were directly inoculated on Mueller-Hinton agar, which was used in the current EUCAST rapid direct AST on blood samples. The size of the growth inhibition zones around antimicrobial disks was measured using a digital scanner (BIOMIC vision analyzer, Giles scientific, USA) and further confirmed by visualization with naked eyes after incubation for 4, 6, and 8 hours. The AST interpretations were compared to those of the conventional VITEK 2 AST system (bioMérieux, France) and the discrepancies between both tests were confirmed with the E-test.

Results: The antibiotics, namely ampicillin, cefazolin, aztreonam, ceftazidime, cefotaxime, ceftiofur, cefepime, gentamicin, ciprofloxacin, and cotrimoxazole showed excellent correlations with modified-EUCAST rapid direct test and conventional ASTs with >0.75 weighted kappa values. The categorical agreement of the rapid direct AST was 1,442 (93.3%), with 76 (4.9%) minor error, 9 (0.6%) major error and 18 (1.2%) very major error, implicating the reliability of this method for clinical application.

Conclusion: Performing the modified-EUCAST rapid direct AST on urine samples can predict reliable AST results within 8 hours. The rapid direct AST can help the physicians to initiate adequate antimicrobial treatment for urinary tract infections.

Keywords: Anti-bacterial agents, Antibiotics, Rapid methods



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INTRODUCTION

Urinary tract infections (UTIs) are a medical threat with the high morbidity of 150 million people worldwide a year [1]. Among the bacteria and fungi causing UTIs, *Enterobacteriaceae* is the most common [2]. Especially, *Escherichia coli* cause 80% of the uncomplicated UTI [3]. UTIs like other infections are treated with broad-spectrum antibiotics which consequently lead to an increase of resistance. Hence, the rapid antimicrobial susceptibility testing (AST) would increase the adequate choice of antibiotics for the better outcome of the patients [4]. Disk diffusion methods and commercial broth micro dilution kits are widely used for antimicrobial susceptibility testing in many clinical settings [5]. To ensure accurate and timely antimicrobial therapy we need rapid AST methods as it takes 2 to 3 days from the sample collection to the AST results by using the conventional methods [6].

To speed up the AST results, many methods have been developed such as Vitek Classic (bioMérieux, Marcy L'Etoile, France), the more automated VITEK 2 (bioMérieux) and Microscan Walkaway (Dade-Behring Microscan, Sacramento, CA, USA), and Phoenix system (BD Diagnostic Systems, Sparks, MD, USA) [7]. Despite the introduction of these methods, many laboratories in low-income settings cannot use them due to their high cost [8]. Currently, European Committee on Antimicrobial Susceptibility Testing (EUCAST) published a guideline for rapid AST using disk diffusion methods in blood culture. But, neither EUCAST nor CLSI (Clinical and Laboratory Standards Institute) has approved the guideline for rapid AST in urine, yet. Hence, we aimed to expand the EUCAST rapid AST from blood to urine culture.

Urine culture method is totally different with blood culture. For urine culture, semi-quantitation for bacterial pathogen using calibrated loop is recommended. The most commonly used criterion to define significant bacteriuria is ' $\geq 10^5$ colony forming unit/mL of urine'. Compared to this, blood culture needs more sensitive methodology using enrichment broth. In each broth bottles, 5-10 mL of blood was inoculated to recover the bacterial pathogen.

Many papers on reducing the turnaround time of antimicrobial susceptibility testing have been published [9], however, limited studies have been done on short incubation time using disk diffusion directly on urine samples. Our study aimed to expand the sample scope and to compare the performance of EUCAST rapid direct disk diffusion AST on urine samples within 8 hr incubation with the conventional AST method.

MATERIALS AND METHODS

1. Specimen collection

The study was carried out in the clinical microbiology laboratory of Severance Hospital from August to November 2019. Urine samples analysis was done by flow cytometry (Sysmex UF-1000i, TOA Medical Electronics, Kobe, Japan) followed by Gram stain. Samples were included in the study only if it contained $\geq 20,000$ bacteria/ml plus the presence of monobacterial Gram-negatives on microscopy. This criteria of 2×10^4 CFU/mL was from the internal validation to have a visible bacterial growth after 8 hours incubation. In detail, using the *E. coli* ATCC25922 strain, we prepared different dilutions (10^6 , 10^5 , 10^4 CFU/mL) using

culture negative urine. Spiked samples were cultured to check how well the growth will be shown. By using the samples showing sufficient growth, the cut off value from Sysmex UF-1000i was set for positive sample to be included in our study.

2. Modified-EUCAST rapid direct AST and species identification

128 urine samples fulfilling the criteria were preselected. Modified-EUCAST rapid direct ASTs were performed immediately or kept at 4°C for no longer than 24 hours before testing depending on the reception time. The identification of all isolates included in our study was performed by using MALDI-TOF MS system, ASTA Micro IDSys (ASTA Inc., Suwon, Korea) according to the manufacturer's recommendation.

For the quality control, *E. coli* ATCC25922 strain was mixed in sterile urine samples. They were diluted 100-, 1000- fold to achieve the starting concentrations of 10^6 , 10^5 CFU/mL, and were inoculated on the Mueller-Hinton agar (MHA) plate using a spreader. Antimicrobial disks, ampicillin, cefazolin, ertapenem, cefepime, cefotaxime, ceftazidime, cefoxitin, aztreonam, trimethoprim-sulfamethoxazole, amikacin, gentamicin, piperacillin-tazobactam, tigecycline, ciprofloxacin, imipenem (Becton, Dickinson and Company, Sparks, MD, USA), were applied and pressed firmly onto the agar surface with sterile forceps. The 15 antibiotics were selected since they were in VITEK 2 AST cards for *Enterobacteriaceae* in the clinical microbiology laboratory.

The sample volume, suggested by EUCAST for direct rapid AST in blood [10], was depending on plates size. Hence, 130 µL and 350 µL of the sample were taken and spread by using cotton swab on the 90 mm- and 150 mm-diameter MHA, respectively. The plates were incubated at $35 \pm 1^\circ\text{C}$ and read on the same day after 8 hours of incubation. The images of inhibition zones were taken and recorded using a digital scanner (BIOMIC vision analyzer, Giles scientific, DC, USA) and confirmed by naked eyes. The interpretations of AST results were performed following the CLSI breakpoints. However, in tigecycline, we used EUCAST breakpoint since there is no CLSI breakpoint for *Enterobacteriaceae*.

3. Conventional urine culture and AST

The urine sample was inoculated on both blood agar and MacConkey agar plates. Plates were incubated at 35°C overnight. The colonies on agar plate were counted and identified using ASTA Micro IDSys. A standardized 0.5 McFarland suspension was prepared and N224 VITEK® 2 cards (bioMérieux, NC, USA) for AST were inoculated following the manufacturer recommendation. Interpretation of AST was performed following the criteria of the CLSI [11].

4. Comparison of modified-EUCAST rapid direct methods and conventional AST

Results from both methods were compared by measuring the agreement kappa with 95% confidence interval analyzed by R studio version 3.3.3. Weighted kappa ranging from 0.75 is classified as excellent, 0.40 to 0.75 as fair to good, and below 0.40 as poor according to Fleiss [12]. The MIC discrepancies showing

major and very major errors were further confirmed by E-test (bioMérieux SA, Marcy-L'Etoile, France) with samples kept in freezer -70°C . Susceptibility results obtained by E-test were used as the golden standard.

RESULTS

We have screened >2,000 urine samples by using flow cytometry and gram stain during the study period. 128 urine samples were preselected, and 103 samples grown with *Enterobacteriaceae* were selected (Fig. 1). They were distributed as 68 *E. coli* (66%), 17 *Klebsiella pneumoniae* (16.5%), 5 *Citrobacter freundii* (4.9%), 2 *Citrobacter braakii* (1.9%), 2 *Enterobacter cloacae* (1.9%), 2 *Klebsiella oxytoca* (1.9%), 2 *Proteus vulgaris* (1.9%), 2 *Klebsiella aerogenes* (1.9%), 1 *Raoultella ornithinolytica* (1%), 1 *Proteus mirabilis* (1%), 1 *Morganella morganii* (1%). Concerning 25 samples excluded, 17 had more than one bacterium grown on the plate and 8 exhibited poor growth after 8 hours to be unreadable.

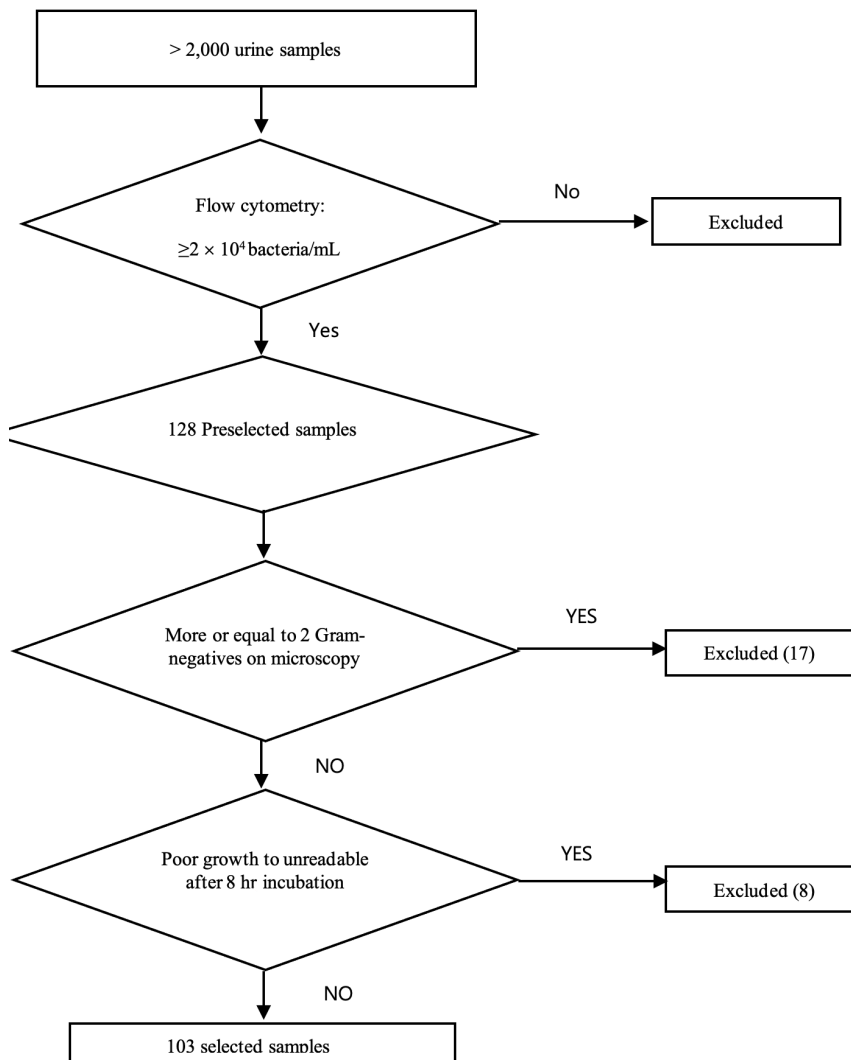


Fig 1. Flowchart of urine samples selection.

This criteria of 2×10^4 CFU/mL in Sysmex UF-1000i was from the internal validation. Direct antimicrobial susceptibility testing on urine from bacteriuria samples ($\geq 2 \times 10^4$ bacteria/mL) showed that the most effective agents were amikacin (98.1%), ertapenem (97.1%), imipenem (96.1%), tigecycline (93.2%) and piperacillin-tazobactam (88.3%) (Table 1). The unweighted kappa scores of 1,545 antibiotics-isolates combinations of modified-EUCAST rapid direct and conventional AST methods ranged from 0.40 to 0.93. The weighted kappa values were from 0.55 to 0.95. Ampicillin, cefazolin, aztreonam, ceftazidime, cefotaxime, ceftazidime, cefepime, gentamicin, ciprofloxacin, cotrimoxazole had excellent correlations between modified-EUCAST rapid direct and conventional ASTs with >0.75 weighted kappa value. Others including piperacillin-tazobactam, ertapenem, imipenem, and tigecycline, showed a fair to good agreement, i.e., 0.68, 0.60, 0.48, and 0.55, respectively.

Table 1. AST performance of modified-EUCAST rapid direct and conventional AST methods

Antimicrobial agents	Modified-EUCAST rapid direct AST			Conventional AST			Unweighted kappa (95% CI)	Weighted kappa (95% CI)
	S	I	R	S	I	R		
Ampicillin	12	3	88	12	0	91	0.83 (0.77 - 1)	0.89 (0.77 - 1)
Piperacillin-tazobactam	91	8	4	86	8	9	0.66 (0.47 - 0.83)	0.68 (0.50 - 0.86)
Cefazolin	44	0	59	41	0	62	0.90 (0.81 - 0.99)	0.90 (0.81 - 0.99)
Aztreonam	65	5	33	67	0	36	0.84 (0.74 - 0.94)	0.89 (0.80 - 0.97)
Ceftazidime	61	14	28	69	1	33	0.74 (0.62 - 0.85)	0.84 (0.75 - 0.92)
Cefotaxime	50	6	47	54	3	46	0.89 (0.81 - 0.97)	0.93 (0.876 - 0.99)
Cefoxitin	77	3	23	70	8	25	0.76 (0.63 - 0.88)	0.86 (0.78 - 0.94)
Cefepime	79	0	24	80	6	17	0.73 (0.59 - 0.88)	0.80 (0.67 - 0.92)
Ertapenem	100	2	1	98	1	4	0.49 (0.18 - 0.79)	0.60 (0.29 - 0.92)
Imipenem	99	2	2	93	6	4	0.40 (0.08 - 0.73)	0.48 (0.12 - 0.83)
Gentamicin	75	1	27	75	1	27	0.93 (0.85 - 1)	0.95 (0.89 - 1)
Amikacin	101	1	1	103	0	0	-	-
Ciprofloxacin	34	21	48	45	10	48	0.76 (0.66 - 0.87)	0.85 (0.78 - 0.92)
Cotrimoxazole	56	1	46	57	0	46	0.90 (0.82 - 0.99)	0.91 (0.83 - 0.99)
Tigecycline	96	0	7	92	2	9	0.52 (0.26 - 0.77)	0.55 (0.29 - 0.82)

Abbreviation: EUCAST, European committee on antimicrobial susceptibility testing; AST, antimicrobial susceptibility test; S, susceptible; I, intermediate; R, resistant; CI, confidence interval.

The overall categorical agreement of the modified-EUCAST rapid direct and the standard ASTs by using additional E-tests was 93.2%, with 4.9% minor errors, 0.6% major error and 1.2% very major errors (Table 2). Concerning major errors observed, 3 (2.9%) were in cefepime. Out of 18 very major errors observed, 5 (4.9%) were shown in tigecycline, 4 (3.9%) in cefazolin, 3 (2.9%) in piperacillin-tazobactam, and 2 (1.9%) in cotrimoxazole.

Table 2. Performance of modified-EUCAST rapid direct AST compared with conventional AST

Antimicrobial agents	Total	CA		MnE		ME		VME	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
Ampicillin	103	99	(96.1)	3	(2.9)	0	(0)	1	(1.0)
Piperacillin-tazobactam	103	94	(91.3)	6	(5.8)	0	(0)	3	(2.9)
Cefazolin	103	98	(95.1)	0	(0)	1	(1.0)	4	(3.9)
Aztreonam	103	96	(93.2)	5	(4.8)	1	(1.0)	1	(1.0)
Ceftazidime	103	89	(86.4)	13	(12.6)	1	(1.0)	0	(0)
Cefotaxime	103	97	(94.2)	5	(4.8)	1	(1.0)	0	(0)
Cefoxitin	103	94	(91.2)	9	(8.7)	0	(0)	0	(0)
Cefepime	103	94	(91.3)	6	(5.8)	3	(2.9)	0	(0)
Ertapenem	103	100	(97.1)	2	(1.9)	0	(0)	1	(1.0)
Imipenem	103	96	(93.2)	6	(5.8)	0	(0)	1	(1.0)
Gentamicin	103	101	(98.1)	2	(1.9)	0	(0)	0	(0)
Amikacin	103	101	(98.1)	1	(1.0)	1	(1.0)	0	(0)
Ciprofloxacin	103	88	(85.4)	15	(14.6)	0	(0)	0	(0)
Cotrimoxazole	103	100	(95.1)	1	(1.0)	0	(0)	2	(1.9)
Tigecycline	103	95	(91.3)	2	(1.9)	1	(1.0)	5	(4.9)
TOTAL	1,545	1,442	(93.3)	76	(4.9)	9	(0.6)	18	(1.2)

Abbreviation: EUCAST, European committee on antimicrobial susceptibility testing; AST, antimicrobial susceptibility test; CA, categorical agreement; MnE, minor error; ME, major error; VME, very major error.

Among 1,545 antimicrobial agents-strain results, 15 were discrepant between modified-EUCAST rapid direct AST and conventional AST; 7 *E. coli* for aztreonam, cefepime, amikacin, cotrimoxazole, and gentamicin, 1 *K. pneumoniae* for tigecycline, 1 *M. morganii* for tigecycline, and 3 *Proteus* spp. for tigecycline and imipenem (Table 3). However, after reanalysis the AST with E-test, the discrepancies reached 2 major and 2 minor errors in *E. coli* for aztreonam, cefepime, amikacin and 2 very major errors in *P. vulgaris* and *P. mirabilis* for tigecycline.

DISCUSSION

The purpose of this study was to evaluate the performance of rapid direct AST on urine compared to the standard method using VITEK 2. *E. coli* and *K. pneumoniae* were the most predominant isolates in bacteriuria with a prevalence of 66%, 16.5%, respectively. EUCAST showed that the rapid AST directly from blood culture bottles was validated only for *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, *E. faecalis*, *E. faecium*, and *A. baumannii*. In this study, we did include other *Enterobacteriaceae*, but not Gram-positives and glucose-non fermenters. Hence, the data in the tables represents the overall performance of modified-EUCAST in *Enterobacteriaceae*, which is not confined to *E. coli* and *K. pneumoniae*.

Table 3. Summary of discrepancies in 13 strains showing discrepant results between modified-EUCAST rapid direct and conventional ASTs

Species	Antimicrobial agent	Modified-EUCAST rapid direct AST	Conventional AST	MIC by E-test (µg/mL)	Interpretation
<i>Escherichia coli</i>	Aztreonam	R	S	0.5	ME
<i>Escherichia coli</i>	Cefepime	R	S	4	MnE
<i>Escherichia coli</i>	Cefepime	R	S	8	MnE
<i>Escherichia coli</i>	Amikacin	R	S	4	ME
<i>Escherichia coli</i>	Cotrimoxazole	S	R	1.5	Agreement
	Gentamicin	R	S	32	Agreement
	Aztreonam	R	S	48	Agreement
<i>Escherichia coli</i>	Cotrimoxazole	R	S	≥32	Agreement
<i>Escherichia coli</i>	Cotrimoxazole	R	S	48	Agreement
<i>Klebsiella pneumoniae</i>	Tigecycline**	S	R	2	Agreement
<i>Morganella morganii</i>	Tigecycline	S	R	1.5	Agreement
<i>Proteus vulgaris</i> *	Tigecycline	S	R	16	VME
<i>Proteus vulgaris</i> *	Tigecycline	S	R	2	Agreement
<i>Proteus mirabilis</i> *	Tigecycline	S	R	24	VME
	Imipenem	S	R	1	Agreement

*The most very major errors were noted in tigecycline for *P. vulgaris* and *P. mirabilis*. **The FDA MIC breakpoints for susceptible (≤ 2 µg/mL), intermediate (4 µg/mL), and resistant (≥ 8 µg/mL) were used to categorize tigecycline susceptibility in *Enterobacteriaceae* strains.

Abbreviation: EUCAST, European committee on antimicrobial susceptibility testing; AST, antimicrobial susceptibility test; MIC, minimal inhibitory concentration; R, resistant; S, susceptible; ME, major error; MnE, minor error; VME, very major error.

Resistant rates to ampicillin, cefazolin, cefotaxime, ciprofloxacin, and cotrimoxazole were more than 40 of the tested strains, whereas piperacillin-tazobactam, ertapenem, imipenem, amikacin, and tigecycline were found to be the most active *in vitro* (Table 1). Our study showed excellent results compared to the conventional AST overall. Weighted kappa values of the results from the comparisons between both methods showed an excellent concordance for some antibiotics, however, others like piperacillin-tazobactam, ertapenem, imipenem, and tigecycline, showed a fair to good agreement (0.68, 0.60, 0.48, 0.55), respectively. Piperacillin-tazobactam or carbapenem was usually recommended for acute pyelonephritis patients with severe conditions such as severe sepsis or septic shock. Therefore, rapid direct AST can help the physicians to start adequate antimicrobial treatment. Kappa statistics of amikacin was not calculated since conventional AST categorized all samples into a single level, i.e, susceptible. Specifically, for cefazolin, cefotaxime, gentamicin, and cotrimoxazole, modified-EUCAST rapid direct AST showed over 0.9 weighted kappa values.

Furthermore, the overall agreement and error rates were within the acceptable limits which gives it the credential to be adopted into routine microbiology laboratory workflow since it helps us to save more than 24 hours (Table 2). According to Jorgensen criteria, the rates of VME (very major error) and the sum of ME (major error) and MnE (minor error) should be $< 3\%$, $< 7\%$ respectively [13]. Cefoxitin, gentamicin, and ciprofloxacin did not show neither major error nor very major error. When considering every single

antibiotic, the results of ciprofloxacin, cefoxitin, cefepime, ceftazidime, tigecycline exceeded the acceptable limits. However, overall ME was 1.2% and the sum of ME and MnE were 5.5% in this study. These findings implicated its usefulness to report rapid AST results of many antibiotics in clinical urine samples.

The modified-EUCAST rapid direct AST is more likely to give the real image of population AST because it was performed with the whole bacterial population in the sample rather than with a single colony which was picked on the plate. Therefore, the results are likely to be clinically relevant [9]. However, the errors in the case of polymicrobial microscope findings are quite high thus it is suggested to be done in monobacterial microscopy findings only [14]. Numbers of urine samples (13.2%, 17/128) have been excluded from this study due to the mixed microscopy of ≥ 2 Gram-negative bacterial findings.

In a study done by Perillaud et al, the direct AST compared to the conventional disk diffusion AST showed 97.9% categorical agreement, 1.5% minor errors, 0.3% major error, and 0.3% vary major error were found [15], which were quite comparable with the results of this study. Though the AST of rapid method seems not to be complete, the result generated in a relatively short period compared to the conventional method and thus can help the physician give the appropriate empirical therapy [9]. For some of the discrepancies, inhibition zones were close to clinical breakpoints, where a slight difference in mm zone of diameter would yield to a major or very major error. The agreement for each antimicrobial agent exceeds 85% in all isolates studied.

The limitation of our study was the spectrum of test strains, because we included *Enterobacteriaceae*, but not other Gram-negative bacilli, such as *P. aeruginosa*. Relatively slow growing bacteria do not allow the reading of inhibition zone diameter before 8 hours incubation. Multiple bacterial infections with similar colony appearance would be also a huddle to this rapid AST method although it is rare, because the discrimination of different isolates on single plate would not be possible by naked eyes. Thus it can make an error in inhibition zone diameter measure.

In conclusion, despite some errors and unstandardized inoculum size, we showed the modified-EUCAST rapid direct AST on urine would be reliable in *Enterobacteriaceae*. The rapid AST reports by using direct susceptibility testing in UTIs can shorten the time for the start of adequate antimicrobial treatment.

요약

배경: 소변 검체 배양 후 신속하게 항균제 감수성 결과를 얻는 것은 적절한 항균제로 환자를 치료하기 위하여 중요하다. 본 연구에서는 EUCAST가 제시한 혈액 신속 직접 항균제 감수성 검사법을 뇨 검체에서 적용하여 평가하고자 하였다.

방법: 유세포 분석기(Sysmex UF-1000i, Japan)와 그람염색법으로 선별검사 후, 2×10^4 bacteria/mL 이상의 단일 세균 형태를 갖는 103개의 뇨 검체를 대상으로 하였다. EUCAST 혈액 신속 직접 항균제 감수성 검사법과 동일하게, Mueller-Hinton 우무 배지에 뇨 검체를 직접 접종한 후, 항균제 디스크를 부착하였다. 이를 4, 6, 8 시간 후에 항균제 디스크 주변 균증식 억제대 지름을 육안과 디지털 스캐너(BIOMIC vision analyzer, Giles scientific, USA)로 측정하였다. 항균제 감수성 검사의 해석은 기존 VITEK 2 시스템과 비교하였으며, 결과가 불일치한 경우 E-test법으로 확인하였다.

결과: Ampicillin, cefazolin, aztreonam, ceftazidime, cefotaxime, cefoxitin, cefepime, gentamycin, ciprofloxacin, cotrimoxazole에서는 신속 직접 항균제 감수성 검사법과 기존의 항균제 감수성 검사법 간의 weighted kappa value가 0.75 이상으로 우수한 상관관계를 보였다. 1,545개 균주-항균제 감수성 시험 결과에서, 신속 직접 항균제 감수성 검사법의 categorical agreement는 1,442 (93.3%), minor errors는 76 (4.9%), major error는 9 (0.6%), very major errors는 18 (1.2%)으로 신뢰성이 있었다.

결론: 신속 직접 항균제 감수성 검사법으로 *Enterobacteriaceae*를 포함한 뇨 검체에서 8시간 내에 신뢰할 수 있는 항균제 감수성 검사 결과를 얻을 수 있었다. 신속 직접 항균제 감수성 검사법은 요로감염에서 임상이가 적절한 항균제 치료를 시작하는데 유용할 것으로 판단하였다.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article were reported.

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