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Evaluation of 3 (4, 5 dimethyl-2-thiazolyl)-2, 5 diphenyl tetrazolium bromide method for rapid detection of vancomycin-resistant enterococci

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ABSTRACT: Vancomycin-resistant enterococci (VRE) have become a global problem of hospital-associated infections. A rapid VRE detection and early isolation of the positive cases are essential for prevention and control of their transmission in hospitals. The aim of this study was to develop and evaluate a simple and rapid method for detection of VRE by using 3 (4, 5 dimethyl-2-thiazolyl)-2, 5 diphenyl tetrazolium bromide (MTT) colorimetric method compared with standard broth microdilution (BMD) and Epsilometer test (Etest). A total of 85 *Enterococcus* isolates (75 vancomycin-non-susceptible, 2 vancomycin-intermediate and 8 vancomycin-susceptible) were studied. The minimum inhibitory concentrations (MICs) of vancomycin for all isolates were tested by BMD, MTT and Etest methods. The MTT method gave results within 9 h with good category agreement, essential agreement, very major, major, and minor errors (100, 92.9, 0, 0 and 7.1%, respectively), which were comparable to those of the Etest (97.6, 95.3, 2.4, 0 and 4.7%, respectively). The Cohen's kappa coefficient (κ) of the MTT method compared with the BMD showed perfect agreement (1.000) ($p < 0.001$). The MTT method is simple and rapid for early VRE detection to support prevention and control the spread of the enterococci in hospitals.

KEYWORDS: vancomycin resistance, enterococci, Etest, broth microdilution, MTT

INTRODUCTION

Enterococci are common bacteria found in gastrointestinal tract of humans and animals, soil, foods, and water. They cause various infections in humans including endocarditis, intra-abdominal infections, pelvic infections, urinary tract infections, skin infections, and central nervous system infections [1]. Most enterococcal infections in humans were caused by *Enterococcus faecalis* with the rate accounted around 80%, while *Enterococcus faecium* caused about 20% of the infections [2]. Enterococci are intrinsically resistant to several common antimicrobials and have high potential to acquire antimicrobial resistance factors, which cause serious problems in patient therapy [1, 3]. In the recent decades, the emergence of vancomycin-resistant enterococci (VRE) has become a global public health concern [4, 5].

There are several acquired determinants, which are responsible for glycopeptide resistance in enterococci. These include *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*. The *vanA* and *vanB* are two vancomycin resistance gene clusters of clinical relevance, which are located on the transposable elements, Tn*1546* and Tn*1549* [6, 7], being transferred between strains by plasmids and conjugative transposons. The *vanA* was responsible for most of VRE in human cases around the world [8]. It was predominantly carried by *E. faecium*, followed by *E. faecalis*, whereas for the non-transferable, *vanC* was a unique intrinsic resistance in *E. gallinarum* and *E. casseliflavus* [4]. The ability of transferable *vanA* and *vanB* led to the emerging of VRE [9]. In addition, bacteria carrying *vanA* usually have high minimum inhibitory concentrations (MICs) of vancomycin (> 64 μg/ml), leading to a high-level resistance in most clinical VRE. In contrast, the intrinsic vancomycin resistance *E. casseliflavus* and *E. gallinarum*, carrying *vanC*, have low MICs of

vancomycin ($\leqslant 32$ μg/ml) [4].

The vancomycin susceptibility test for *Enterococcus* spp. by a disk diffusion method was performed by incubation for 24 h. Subsequently, the inhibition zones should be observed carefully with transmitted light for small colonies or haze inside the zone. Any growth found in the inhibition zone may indicate the resistance [10]. The vancomycin-intermediate enterococci and VRE should be then confirmed by using vancomycin agar screen plate method or broth microdilution method (BMD) for their MIC determination. Intermediate resistance to vancomycin is usually found among *E. gallinarum* and *E. casseliflavus* [11]. When VRE was detected, it should be reported immediately for prompt infection control in the hospital [12].

Several studies have reported on VRE detection. The *vanA*-carrying VRE were detected by both conventional disk diffusion or agar dilution, and automatic system such as Vitek GPS-101 and MicroScan system, whereas some *vanB*-carrying VRE were not detected by the automatic system [13]. The agar screen test was the most reliable and an easy method for routine screening; but it took up to 24 h. The modern methods, such as Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and real time PCR, gave results more rapidly; but they needed specific experiences and expensive equipments [14–16].

3 (4, 5 dimethyl-2-thiazolyl)-2, 5 diphenyl tetrazolium bromide (MTT) is a yellow chemical substance, which becomes blue formazan when dehydrogenated by mitochondria of living cells [17]. The MTT assay had been applied for assessing cell metabolic activity, cytotoxicity or loss of viable cells [17–19]. Vancomycin is a bactericidal agent which kills the susceptible bacterial cells. Therefore, we applied the MTT in BMD method for VRE testing to shorten the incubation time.

This study aimed to evaluate an MTT method for detection of VRE compared with standard broth microdilution (BMD) and Etest. The information would be useful for VRE detection and control in hospitals with low resources.

MATERIALS AND METHODS

A total of 85 *Enterococcus* isolates (77 vancomycin non-susceptible *Enterococcus* and 8 vancomycin-susceptible *Enterococcus*) collected between January and December 2017 from patients of hospitals in the upper area of northeastern Thailand were studied. They were identified by biochemical tests and kept at −20 °C in skimmed milk plus 20% glycerol. Reference strains of vancomycin-resistant *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus*, carrying *vanA*, *vanB*, *vanC1*, and *vanC2* genes, respectively, and vancomycin-susceptible *E. faecalis* ATCC 29212 were used as control strains.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Khon Kaen University (project no. HE591546).

MIC determination

The MICs of vancomycin (Sigma-Aldrich, St. Louis, USA) for all isolates were determined by BMD method using vancomycin concentrations ranging from 0.125 to 1024 μg/ml according to the standard CLSI [10]. In addition, the Etest MICs of vancomycin for all isolates were performed according to the manufacturer description (AB Biodisk, Solna, Sweden). The results were interpreted according to the CLSI [10].

MTT method

The MICs of vancomycin were determined by rapid MTT method in a 96 well microplate. The vancomycin concentrations ranging from 0.125 to 1024 μg/ml were mixed with 10^5 CFU/ml of fresh culture of each isolate and incubated for 8 h at 37 °C. After the incubation, 20 μl of MTT solution (5 mg/ml in phosphate buffer) (Sigma-Aldrich, St. Louis, USA) were added to each well and re-incubated for 1 h. The formation of blue color in the solution indicated the presence of viable bacterial cells [18]. The MICs were determined by visual minimum drug concentration that still had yellow color. The MTT test was carried out in triplicate for each isolate.

The time-kill value of *Enterococcus* for vancomycin from a previous study was around 8 h [20], thus in this study, the bacteria were exposed to vancomycin for 8 h before detection with the MTT solution.

Detection of species specific and *van* genes

The total bacterial DNA was prepared by a rapid alkaline lysis method as described previously [21]. The *Enterococcus* species specific for *E. faecium*, *E. faecalis*, and the *vanA*, *vanB* and *vanC1* genes (for *E. gallinarum*) were amplified by multiplex polymerase chain reaction (PCR) using oligodeoxynucleotide primers according to a previous report [22].

Data analysis

The results were evaluated in terms of the following parameters: categorical agreement (CA, the total number of isolates tested that yielded the same categorical interpretation as the reference method) and essential agreement (EA, the obtaining MIC value was within 1 $\log_2$ dilution of the reference value). The CA is subdivided into 3 types of error: very major errors, major errors, and minor errors. A very major errors was defined as an isolate that was resistant by the reference BMD method, but susceptible with the test method (false susceptibility). A major error was defined as an isolate that was susceptible by the reference BMD method, but resistant with the test method (false resistance). A minor error was defined as a discrepancy between the results of the reference BMD and the test method corresponding to one interpretation category. The acceptable rate for CA and EA is $\geqslant$ 90%. Acceptable minor error rates are $\leqslant$ 10%, while acceptable major error and very major errors rates are $<$ 3% of the susceptible and resistant isolates tested, respectively [23].

Statistical analysis was performed using SPSS version 13 (Statistical Package for the Social Sciences, Chicago, IL, USA). Cohen's kappa coefficient test was used to compare the MIC results between the standard BMD method and the MTT method. Kappa value was calculated to analyse the level of agreement between the standard BMD method and MTT method. A probability of < 0.05 was considered significant.

RESULTS

Species identification

A total of 85 catalase-negative, gram-positive cocci were subjected for biochemical tests and confirmed by PCR technique. The 85 *Enterococcus* isolates were identified as 80 *E. faecium* (94.1%), 3 *E. faecalis* (3.5%), 1 *E. gallinarum* (1.2%) and 1 non-specified *Enterococcus* species (1.2%).

Determination of vancomycin MIC and detection of vancomycin resistance genes

The MICs of vancomycin for the 85 *Enterococcus* isolates determined by standard BMD method showed 75 vancomycin-resistant enterococci (VRE) (88.2%), 2 vancomycin-intermediate (2.4%), and 8 vancomycin-susceptible enterococci (9.4%). All of the 75 VRE isolates were *E. faecium* carrying *vanA*, which had high vancomycin MICs of 128 to $>$ 1024 μg/ml. Eight *Enterococcus* isolates had low vancomycin MICs (1-4 μg/ml) and no *vanA* gene. One isolate of *E. gallinarum* had low vancomycin MIC of 16 μg/ml. Most of *E. faecalis* were vancomycin-susceptible.

Comparison of MICs from MTT method and Etest with BMD method

The results obtained from the MTT method and Etest versus those of the standard BMD were shown in Table 1. The CA, EA, VME, ME, and mE of the MTT method were within the acceptance criteria (100, 92.9, 0, 0, and 7.1%, respectively), and were comparable to those of the Etest (97.6, 95.3, 2.4, 0 and 4.7%, respectively). The error rates of both methods were shown in Table 2. The Cohen's kappa coefficient (κ) of MTT method compared with BMD showed perfect agreement ($\kappa = 1.000$; $p < 0.001$). The correlations between the Etest and the BMD method was almost perfect with Cohen's kappa coefficient (κ) = 0.88 ($p < 0.001$).

DISCUSSION

VRE have been reported increasingly and become a major problem in nosocomial infections [24]. It caused a significant increasing rate of bloodstream infection from 5.9 to 16.7% during a 10 year – surveillance of a hospital in Germany [25]. The prevalence of VRE had also been increased in other countries. In China, a 2015 report from Beijing showed up to 14.3% increasing incidence of VRE–*E. faecium*, and 1.3% of VRE–*E. faecalis* [26]. In Thailand, most VRE were *E. faecium* carrying *vanA*. The annual report in Thailand between 2014–2016 showed increasing VRE infection rates of 1.7, 2.2 and 3.6%, respectively. The rates of VRE–*E. faecium* were 4.3, 4.5 and 9.9%, while those of VRE–*E. faecalis* were 0.3, 1.3 and 0.4%, respectively [27]. In addition, most of the *Enterococcus* isolates in Thailand with high MICs of vancomycin carried *vanA*, similar to those in China [26]. In contrast, most VRE isolates in Australia were *E. faecium*, which related to *vanB* [21].

The prevention and control of VRE transmission in hospitals require an early detection of infected or colonized patients. It is crucial to have a rapid VRE detection and early isolation of the positive cases. Therefore, the rapid and accurate detection of vancomycin resistance is essential. In this study we evaluated a simple rapid method for detection of VRE compared with the standard BMD method and Etest. The MTT assay has become a gold standard for determination of cell viability and proliferation since 1983 [28]. The assay measures cell viability from reductive activity of enzymes that converses

Table 1 Comparison of vancomycin MICs of *Enterococcus* spp. by BMD, MTT and Etest methods.

Method	Number of *Enterococcus* spp. with MIC of vancomycin (μg/ml)										
	1	2	4	8	16	32	64	128	256	512	1024
BMD	1*	6*	1*	1	1	0	0	2	6	45	22
MTT	2*	6*	0*	1	1	0	0	2	10	42	21
Etest	8*	0*	2*	0	0	0	0	0	0	75	0

* indicates the vancomycin-susceptible isolates.

Table 2 Error rates and agreements of MTT and Etest methods for detection of vancomycin-resistant *Enterococci* compared to BMD method.

Method	Error rate (%)[a]			Category	Essential
	Very major	major	minor	agreement (%)	agreement (%)
MTT	0 (0)	0 (0)	6 (7.1)	100	92.9
Etest	2 (2.4)	0 (0)	4 (4.7)	97.6	95.3
Acceptance criteria	$< 3\%$	$< 3\%$	$\leqslant 10\%$	$\geqslant 90\%$	$\geqslant 90\%$

[a] Relative to the CLSI reference BMD method; very major error, false susceptibility; major errors, false resistance; minor errors, a discrepancy between the results of the reference method and the test method corresponding to one interpretation category.

the tetrazolium compound to water insoluble formazan crystals. The MTT has been used as an indicator to evaluate cell proliferation and microbial growth [29, 30]. For example, an MTT assay for rapid detection of rifampicin-resistant *Mycobacterium tuberculosis* gave high sensitivity, specificity, positive and negative predictive values of 100%, 86%, 100%, 99%, respectively [31]. In the present study, the MICs of vancomycin for *Enterococcus* using MTT method gave congruent results to those of the reference BMD method. The CA and EA were over 90% and the VME, ME and mE were within the acceptance criteria. Similarly, Etest also gave good results with the CA, EA, VME, ME, and mE values within the acceptance criteria. Etest is an easy method for MIC determination without performing the serial dilution. However, it needed to incubate for 24 h to detect the vancomycin resistance, while the MTT method in this study needed a shorter period of incubation.

The time-kill value of *Enterococcus* for vancomycin from a previous study was around 8 h [20], thus in this study the bacteria were exposed to vancomycin for 8 h before detection with the MTT solution. The MICs of vancomycin for both VRE and non-VRE isolates were clearly observed by naked eye within one day. This method is simple and fast. It does not require any sophisticated equipments or additional tests to confirm the antibiotic resistance. The cost per test is 1.21 USD (for triplicate test).

The present study was a primary test; thus the MTT concentration was not optimized but followed a previous report [18]. Therefore, further study with more samples and optimization of MTT concentrations should be performed. This MTT method should be used carefully when testing with slow-growth rate strains. The MTT reagent is easy to prepare and has a long shelf life. Stored properly at 4 °C and protected from lights, the kit components would remain stable for 12 months. For in-house MTT solution, once prepared, the solution when stored for four weeks at 4 °C and protected from lights can be used. The DMSO-formazan solution stored in the dark would provide stable absorption readings for up to 1 h. In this study, the optical density of bacterial growth at 520 nm of each well was > 0.24; whereas those with no bacterial growth were < 0.24, which was clearly consistent to those inspected by naked-eye (data not showed). Therefore, it is appropriate for VRE prevention and control in low-resource settings.

CONCLUSION

The vancomycin susceptibility for *Enterococcus* was performed by using MTT colorimetric method. The result was obtained within 9 h and in concordance with the standard BMD method (Cohen's kappa coefficient (κ) = 1.000). This method may be a rapid and economical test for routine service. Most VRE isolates in this area were *vanA*-carrying *E. faecium*.

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