EFFICACY EVALUATION OF DETECTING AND IDENTIFYING BACTERIAL ENDOCARDITIS AGENTS FROM BLOOD CULTURES BY MATRIX-ASSISTED LASER DESORPTION IONIZATION-TIME OF FLIGHT MASS SPECTROMETRY

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Abstract. Early identification of etiologic agents of infective endocarditis is important for reducing morbidity and mortality. Therefore, we aimed to evaluate the efficacy of the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for the early identification of the etiologic bacterial agents of infective endocarditis in Thailand from blood cultures compared to the VITEK 2 (bioMérieux) method. Nine causative agents of endocarditis were retrieved from Siriraj hospital bacterial culture collection and evaluated: *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus oralis*, *Haemophilus parainfluenzae*, *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis* and *Eikenella corroden* with the last 4 species being fastidious. *E. coli* cannot be differentiated from *Shigella* spp with the MALDI-TOF MS method therefore it was not tested in our study. Artificially created positive blood cultures were used for this study. Each bacterial suspension mixed with human blood was injected into a blood culture bottle (BACTEC™ FX blood culture system). An initial bacterial concentration of 10-100 CFU/ml in the blood culture was used to simulate the bacterial concentration typically found in the blood of a bacterial endocarditis patient. Time to bacterial identification of these cultures using the VITEK 2 method and the MALDI-TOF MS method were compared. Since the manufacturer (Bruker Daltonics) MALDI-TOF MS database is limited, we created our own in-house (Siriraj Hospital) MALDI-TOF MS database and combined it with the manufacturer's database. Only non-fastidious bacteria were consistently identified to the species level with the MALDI-TOF MS method but both fastidious and non-fastidious bacteria were detected with the VITEK 2 method. The MALDI-TOF MS method identified the studied non-fastidious bacteria to the species level from the blood cultures faster than the VITEK 2 method (7.2-12.7 hours versus 57.2 – 63.8 hours; $p < 0.001$). The optimal time to identification of *H. parainfluenzae* could not to be determined for the MALDI-TOF MS method since the

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times varied too much (SD = 8.9 hours). The MALDI-TOF MS method identified *E. corrodens* only to the genus level and did not identify *A. actinomycetemcomitans* or *C. hominis* at all. The VITEK 2 method identified all studied organisms, both fastidious and non-fastidious, to the species level, but took time longer than the MALDI-TOF MS method. The VITEX 2 method gave sensitivity results, which the MALDI-TOF MS method cannot. These findings indicated the MALDI-TOF MS method should never be used by itself for identification of bacteria from blood cultures, but only in combination with other methods. The MALDI-TOF MS also requires an additional step and expense of removal of contaminated protein from the blood culture prior to being conducted. Further studies are needed to determine if this early identification of species without sensitivity testing can make a significant difference in patient management and outcomes.

Keywords: bacterial infections, endocarditis, diagnosis

INTRODUCTION

Infective endocarditis is an endovascular infection causing valvular and perivalvular destruction (Thanavaro and Nixon, 2014). The pathology of infective endocarditis includes detachment of septic vegetations with emboli, metastatic infections and septicemia (Thiene and Basso, 2006). The mortality rate of in-hospital patients with infective endocarditis in one study was found to be 19.7% (Bannay *et al*, 2011).

The etiology of infective endocarditis includes bacteria, fungi and some viruses, with bacteria being the most common cause (Thanavaro and Nixon, 2014). The bacteria causing infective endocarditis include *Staphylococcus aureus*, the viridans streptococci, coagulase-negative staphylococci, other *Streptococcus* spp, enterococci, the HACEK group (*Haemophilus* species, *Aggregatibacter* species, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella* species) group and *Coxiella burnetii* (Murdoch *et al*, 2009; Watt *et al*, 2014).

Modified Duke criteria have been used as the gold standard to define infective endocarditis, with a positive blood culture as a major criterion (Thanavaro and Nixon, 2014). The next step is species identification, which is crucial, since only typical organisms known to cause infective endocarditis are included in the modified Duke criteria: the viridans streptococci, *Streptococcus bovis*, the HACEK species, *S. aureus* and enterococci without other primary sites (Thanavaro and Nixon, 2014). Subcultivation of a positive hemoculture from the bottle onto an agar plate is needed to obtain a pure culture and then the species is identified after which sensitivity testing is generally performed based on specific criteria. Various methods have been used to identify bacterial species from pure cultures, such as conventional biochemical tests, automated systems and DNA sequencing methods (Cullimore, 2010). To shorten identification time, a new method able to identify bacteria directly from the blood culture bottle without subcultivation should be investigated. This new method could give earlier species identification and earlier diagnosis of bacterial endocarditis (Brouqui and Raoult, 2001).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a reliable, rapid, modestly priced method that allows iden-

tification of a wide variety of bacterial species (Jamal *et al*, 2013). The MALDI-TOF MS analyzes protein molecules of each organism based on mass spectrometry and gives distinct protein patterns for each organism (Jamal *et al*, 2013). This distinct biological spectral pattern is then compared with a MALDI-TOF spectral database either supplied by the manufacturer or in combination with an in-house spectral database (Jamal *et al*, 2013). Species identification is made by matching organism spectral patterns with database spectral patterns. This method can be used to identify bacterial species directly from blood culture bottles. However, information about the efficacy of the MALDI-TOF MS method is limited.

In Thailand, the etiologic agents of infective endocarditis during 1982–2001 at Siriraj Hospital, Bangkok, Thailand were identified in one study: non-fermentative gram-negative bacilli, coagulase-negative staphylococci, the viridans streptococci, *S. aureus* and *Escherichia coli* were responsible for infective endocarditis in 20.94%, 12.47%, 10.23%, 9.29% and 8.59% of the cases, respectively (Srifuengfung *et al*, 2004). In northeastern Thailand during 2010-2012, *Enterococcus faecalis, C. burnetii, S. aureus* and the viridans streptococci were found to be the etiologic agents of endocarditis in 10%, 8.3%, 8.3% and 8.3%, respectively (Watt *et al*, 2014).

A review of worldwide literature (Brouqui and Raoult, 2001) found the HACEK group to be the etiologic agents of endocarditis in 1.4-3%. Fastidious and slow-growing bacteria often take longer to be identified (Baron *et al*, 2005) using modified Duke criteria.

We aimed to evaluate the efficacy of the MALDI-TOF MS method to identify bacteria causing infective endocarditis from blood cultures and the time needed for identification compared to the VITEK 2 method already used to identify blood culture organisms at our study institution.

MATERIALS AND METHODS

Bacterial strains

One isolate each of the following strains were used for this study: *Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus* and *Streptococcus oralis, Haemophilus parainfluenzae, Aggregatibacter actinomycetemcomitans, Cardiobacterium hominis* and *Eikenella corrodens.* The latter four species belong to HACEK group. All isolates were obtained from the clinical culture collection of the Microbiology Laboratory, Department of Microbiology, Faculty of Medicine, Siriraj Hospital. *E. coli*, a common etiology of infective endocarditis, was excluded from our study because the MALDI-TOF MS method cannot distinguish between *E. coli* and *Shigella* spp (Khot and Fisher, 2013). All isolates were kept at -70°C in 10% skim milk until used. Prior to use, each organism was subcultured twice onto an appropriate agar plate (sheep blood agar for *K. pneumoniae, P. aeruginosa, E. faecalis, S. aureus, S. oralis, A. actinomycetemcomitans*, and *E. corrodens* and chocolate agar for *H. parainfluenzae* and *C. hominis*).

Creating artificially positive blood cultures

The BACTEC™ FX blood culture system (Becton, Dickinson, San Jose, CA) was used in this study. We simulated a positive blood culture as follows.

The HACEK group bacteria were grown on agar plates then suspended in phosphate-buffered saline. The other bacteria were grown in broth media (Luria-Bertani broth for *K. pneumoniae*,

P. aeruginosa, *S. aureus*, and brain-heart infusion broth for *E. faecalis* and *S. oralis*). The bacterial suspensions were adjusted to a concentration of 100-1,000 CFU/ml using absorbance values on a spectrophotometer at an optical density of 600 nm. The bacterial suspensions were checked for purity by subsequent growth on appropriate agar plates.

Whole blood and the prepared bacterial suspensions were then added to the blood culture bottles (BACTEC™ Plus Aerobic/F Medium; Becton, Dickinson). The blood was obtained from the Department of Transfusion Medicine, Faculty of Medicine, Siriraj Hospital. Four milliliters of packed red blood cells, 5 ml plasma and 1 ml bacterial suspension (Elfath *et al*, 2000) were added to each blood culture bottle to give an estimated final bacterial concentration of 10-100 CFUs/ml blood; the concentration typically found in the blood of bacterial endocarditis patients (Werner *et al*, 1967. Ten milliliters of sterile phosphate-buffered saline was added to a blood culture bottle as a negative control.

All the cultures were then incubated following standard procedures (BACTEC™, Becton, Dickinson). Negative control bottles were incubated for up to five days to check for sterility. The concentrations of bacteria (CFU/ml) in the positive bottles were determined using the plate count method, on suitable media for each bacterial species.

Species identification with the VITEK 2 method

The positive BACTEC blood cultures were examined with the VITEK 2 (bioMérieux, St Louis, MO) method according to the manufacturer's instructions. gram-positive (GP), gram-negative (GN), anaerobe and *Corynebacterium* (ANC) and *Neisseria-Haemophilus* (NH) identification cards were used in this study.

Bacterial protein preparation and species identification with the MALDI-TOF MS method

Protein preparation for MALDI-TOF MS analysis of blood cultures requires additional steps to remove contaminated protein from the blood culture broth; therefore, the commercial Sepsityper™ kit (Bruker Daltonics, Bremen, Germany), was used for sample preparation. This lysis-based method was selected because it has been reported to give a better result than the traditional centrifugation-based method for blood culture protein extraction (Juiz *et al*, 2012). The kit was used according to the manufacturer's instructions (Bruker Daltonics). Briefly, 200 µl of lysis buffer was added to 1 ml blood culture broth taken from the blood culture bottle. This solution was then mixed with a vortex mixer for 10 seconds and then centrifuged at 17,000*g* for 2 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml washing buffer and then, centrifuged at 17,000*g* for 2 minutes. The supernatant was discarded and the pellet was used for protein extraction using the ethanol–formic acid extraction method according to the manufacturer's instructions (Bruker Daltonics) as follows: the remaining pellet was dried at room temperature and resuspended in 50 µl 70% formic acid (Sigma-Aldrich, St Louis, MO). An equal volume of acetonitrile (Sigma-Aldrich) was added and then the solution was centrifuged at 13,500 rpm for 3 minutes. One microliter of the supernatant was placed as a spot on the MALDI target plate (Bruker Daltonics) and let dry at room temperature. Three spots were done for each specimen. One microliter of α-cyano-4-hydroxycinnamic acid (HCCA) matrix (saturated HCCA

in 50% acetonitrile-2.5% trifluoroacetic acid) (Bruker Daltonics) was then added to each of the spots and let dry at room temperature.

The prepared MALDI target plate was then inserted in the MALDI-TOF MS instrument. The laser of the instrument was directed at each spot and the resulting protein spectra of each spot was obtained.

MALDI Biotyper RTC (realtime classification) software (Bruker Daltonics) was then used for spectra analysis. This software identifies the bacterial species by comparing (matching) the protein spectra from the studied bacteria with the spectral database. Protein spectra were analyzed in a mass-to-charge ratio (m/z) range of 3,000 to 15,000. The bacterial spectra database used in this study was a combination of the manufacturer's database with our in-house database previously created (Ekcharoenkul *et al*, 2014). The results of the spectra-matching process were expressed as a logarithmic score (log score) which quantified the similarity to the database entries. The log scores ranged from 0 to 3 and used for identification of bacterial species. The log scores of > 1.7 and > 2.0 were indicative of a genus level and a species level, respectively (Richter *et al*, 2012).

Determination of the total time to identification

Hemoculture broth for each bacterial species was examined with the MALDI-TOF MS before the BACTEC blood culture machine gave a positive result at specific time points. The time points were chosen based on the time to obtain a positive culture with the BACTEC blood culture machine (time to positivity with the BACTEC blood culture).

The time to positivity of BACTEC blood culture for each bacterial species was determined in triplicate except for *H. parainfluenzae* which was done with 11 repetitions, since the time to identify organism varied widely with each repetition. Depending on the mean time to positivity of the BACTEC blood culture for each bacterial species, the blood culture broth for each bacterial species was collected for MALDI-TOF MS analysis at those time points (Table 1). The shortest collection time point that gave the highest log score was selected for the subsequent MALDI-TOF MS identification for each bacterial species. The concentrations of bacteria (CFU/ml) for each positive blood culture at each time point were also determined with the standard plate count method on media suitable for each bacterial species. The "total time to identification" for each bacterial species was determined based on the spent time from initial inoculation of each bacterial species into the blood culture bottle to species identification with both the MALDI-TOF MS or VITEK 2 methods.

Statistical analysis

Significant differences in time to identification using the VITEK 2 method versus the MALDI-TOF MS method were determined with the Student's *t*-test, calculated using Predictive Analytics SoftWare (PASW), version 18 (IBM Thailand, Bangkok, Thailand). A p-value < 0.05 was considered statistically significant.

Ethical considerations

Ethical approval for this study was obtained from the Siriraj Institutional Ethical Review Board (approval no. Si147/2013).

The manufacturer's spectra database (Bruker Daltonics) is an open database, the right to use this database comes with the purchase of the MALDI-TOF MS in-

Table 1
Collection time points from the blood culture broth for each bacterial species analyzed using the MALDI-TOF MS method.

Species	Collection time points in hours (mean)
K. pneumoniae	6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 (9.2)
P. aeruginosa	11, 12, 13, 14, 15, 16 (15.8)
E. faecalis	9.0, 9.5, 10.0, 10.5, 11 (10.7)
S. aureus	8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0 (11.6)
S. oralis	10, 11, 12, 13, 14, 15 (14)
E. corrodens	18, 19, 20, 21, 22 (21)

Table 2
Comparison of the studied methods for identifying a positive blood culture.

Species	The VITEK 2 method	The MALDI-TOF MS method	
	Identification level	Mean (±SD) log scores	Identification level[a]
K. pneumoniae	Species	2.15 (±0.06)	Species
P. aeruginosa	Species	2.04 (±0.04)	Species
E. faecalis	Species	2.31 (±0.04)	Species
S. aureus	Species	2.08 (±0.05)	Species
S. oralis	Species	2.24 (±0.06)	Species
E. corrodens	Species	1.74 (±0.17)	Genus
H. parainfluenzae	Species	2.34 (±0.06)	Species
A. actinomycetemcomitans	Species	1.34 (±0.03)	Not identified
C. hominis	Species	1.56 (±0.20)	Not identified

[a]A log score ≥2.000 indicates identification to the species level and a log score of 1.700-1.999 indicates identification to the genus level. SD, standard deviation; MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry.

strument. The database may be updated by the user or the manufacturer and can be used for diagnosis and study purposes.

RESULTS

Of the 9 studied bacteria, MALDI-TOF MS method successfully identified 6 to the species level (*K. pneumoniae*, *P. aeruginosa*, *E. faecalis*, *S. aureus*, *S. oralis* and *H. parainfluenzae*), 1 to the genus level (*E. corrodens*) and was not able to identify 2 species (*A. actinomycetemcomitans* and *C. hominis*) (Table 2). The VITEK 2 method identified all 9 studied bacteria to the species level (Table 2) and gave antimicrobial sensitivity result (data not shown).

Six of the 7 bacteria identified with the MALDI-TOF method were identified in a shorter time than with the VITEK 2 method (Table 3). The times required to identify *K. pneumoniae*, *P. aeruginosa*, *E. faecalis*, *S. aureus*, *S. oralis*, and *E. corrodens* were significantly ($p < 0.001$) shorter than the times to identify them with the VITEK 2 method by 50 hours, 51.1 hours, 49.2

hours, 50.4 hours, 50.3 hours, and 49.3 hours, respectively (Table 3). *E. corrodens* was identified to only the genus level. Because of the variability in the lengths of the time required to identify *H. parainfluenzae* using the MALDI-TOF MS method during the various repetitions of the culture, it was determined this method could not adequately be quantified or compared (Table 3).

DISCUSSION

The MALDI-TOF MS method gave a faster identification time for 6 out of 9 studied bacteria compared to the VITEK 2 method but cannot identify antimicrobial sensitivities like the VITEK 2 method can. *E. coli* could not be examined because it cannot be differentiated from *Shigella* species by the MALDI-TOF MS method. It could not identify *A. actinomycetemcomitans* or *C. hominis*. The MALDI-TOF MS method gave widely varying results in identifying *H. parainfluenzae*. *E. corrodens* could only be identified to the genus level by MALDI-TOF MS method in our study. It also requires an additional step and expense to remove contaminated protein before being conducted. A previous study also found MALDI-TOF MS method could only identify some selected bacteria, *K. pneumoniae*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *S. oralis,* from blood culture more rapidly than the BACTEC FX method, a method similar to the VITEK 2 method, by up to 2 hours earlier (Wang *et al*, 2015). In our study, the MALDI-TOF MS method only identified some selected non-fastidious bacteria up to 50 hours earlier than the VITEK 2 method. Therefore, the MALDI-TOF MS method is inappropriate as a single method for diagnosing the etiology of bacterial endocarditis directly from the blood culture.

Previous studies found the MALDI-TOF MS method correctly identified 66-100% of HACEK bacteria species from colonies on chocolate agar plates (Couturier *et al*, 2011; Powell *et al*, 2013). In our study of blood cultures, the MALDI-TOF MS method failed to detect 3 out of 4 HACEK species and inconsistently detected a forth species. Typically, the unmatched peaks are caused by high background noise, as reported earlier when using MALDI-TOF MS method to identify bacteria directly from blood culture (Buchan *et al*, 2012). However, this phenomenon only occurred with the HACEK bacteria in our study suggesting the HACEK bacteria might produce different types of protein from other bacteria which could not be removed with the Sepsityper™ kit. Therefore, a more efficient sample preparation kit for blood cultures is needed for the HACEK bacteria. However, since the type of bacteria is unknown prior to the test, a better sample preparation is needed for the test to be useful.

In conclusion, the MALDI-TOF MS method should not be used by itself to identify the organisms causing endocarditis. It must be used in combination with other methods. There are other problems with the method: first, it is not suitable for detecting HACEK bacteria in hemoculture broth directly. This is one of the main reasons for using this method but our results do not support this use. Second, antimicrobial susceptibility cannot be determined with this method. Further studies are needed to determine if the added expense of this method is warranted and if its use in addition to the VITEK 2 method can result in better treatment outcomes.

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Table 3
Comparison of times required to identify the studied bacteria using the studied methods.

Species	VITEK 2 method			MALDI-TOF MS method			*p*-value[c] (95% confidence interval)
	Mean (±SD) incubation time in hours	Mean (±SD) bacterial concentration in CFU/ml[b]	Mean (±SD) total time to identification in hours	Incubation time in hours	Mean (±SD) bacterial concentration in CFU/ml	Total time to identification in hours	
K. pneumoniae	9.2 (±0.09)	5.5 (±1.9) x 10^8	57.2 (±0.1)	6.5	1.8 (±0.4) x 10^6	7.2	$p < 0.001$ (49.8-50.2)
P. aeruginosa	15.8 (±0.16)	1.3 (±0.2) x 10^8	63.8 (±0.2)	12	7.0 (±0.4) x 10^5	12.7	$p < 0.001$ (50.8-51.4)
E. faecalis	10.7 (±0.42)	4.3 (±1.0) x 10^8	58.9 (±0.4)	9.5	3.2 (±0.3) x 10^7	9.7	$p < 0.001$ (48.6-49.8)
S. aureus	11.6 (±0.13)	7.5 (±1.0) x 10^8	59.6 (±0.1)	8.5	2.1 (±1.6) x 10^6	9.2	$p < 0.001$ (50.2-50.6)
S. oralis	14.0 (±0.28)	9.5 (±1.6) x 10^8	62.0 (±0.3)	11	1.7 (±0.4) x 10^7	11.7	$p < 0.001$ (49.8-50.8)
E. corrodens	21.0 (±0.30)	2.8 (±0.4) x 10^8	69.0 (±0.3)	19	4.4 (±2.2) x 10^7	19.7	$p < 0.001$ (48.8-49.8)
H. parainfluenzae[a]	55.0 (±8.9)	9.8 (±0.4) x 10^7	N/A	N/A	N/A	N/A	N/A

[a]time to identify the organism varied too widely with repetition to give an accurate value; [b]The mean bacterial concentration at the time if yielded a positive blood culture result; [c]Comparison of the difference in time to identify the studied organism with the VITEK 2 method versus the MALDI-TOF MS method; SD, standard deviation, CFU, colony forming unit, MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry.

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