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Comparison of ViTEK 2, MALDI-TOF and Partial Sequencing of 16S rRNA Gene in Identification of *Brevibacterium* Species with its Antibiotic Susceptibility Pattern

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background - *Brevibacterium* species were formerly viewed as apathogenic, however, recent reported cases of infectious states caused by *Brevibacterium* sp. have renewed interests in the genus as infectious agents. However, several authors have reported difficulties in identification of different species in the genus and resistance to some antibiotics.
Objectives – This study is therefore designed to compare accuracies of 3 methods for identification of *Brevibacterium* spp. and their antibiotic susceptibility pattern.
Methods – Ten *Brevibacterium* spp. were presumptively identified from staphylococci collections due to their characteristic cheesy smell. The selected isolates were identified by MALDI TOF, Vitek 2 identification system and partial sequencing of 16S rRNA gene by standard procedures. The antibiotic susceptibility was determined by disc diffusion method.
Results - Vitek 2 identification system misidentified all the organisms while MALDI TOF identification system correctly identified the *Brevibacterium* strains to the genus level but with scores less than 1.7 for all tested strains. 16S rRNA identification identified the 10 strains as 3 species: 5 *B. epidermidis,* 3 *B. iodinum* and 2 *B. oceani.* All the strains were susceptible to Vancomycin, Linezolid and Rifampicin while they were all resistant to Penicillin, Fusidic acid, and Trimethoprim. *Brevibacterium epidermidis* were generally resistant to Erythromycin and Clindamycin while *B. iodinum* and *B. oceani* were susceptible.
Conclusion - 16S rRNA identification is the only method that could correctly identified *Brevibacterium* sp to the species level in this study. High susceptibility was shown by all tested strains to Vancomycin, Linezolid and Rifampicin
Keywords – *Brevibacterium*, Identification, Antibiotic susceptibility

INTRODUCTION

Many microorganisms were previously viewed as contaminants in clinical specimens but recent advances in bacterial identifications have revealed that these organisms may be responsible for various infectious states. Brevibacteria are naturally found on skin surfaces and some dairy foods e.g. cheese. They are in the order Actinomycetales and the only genus in the family Brevibacteriacrae. The genus consists of more than 20 species including *B. casei, B. epidermidis, B. iodinum, B. linens* among others. They are Gram positive, non acid fast, non spore former, non motile, obligate aerobes, salt tolerant, irregular rods which can segment into cocci in older cultures. (Gruner *et al*, 1993). They have a distinctive odor sometimes described as cheesy, inhabit the skin and have been described to be probably responsible for some foot and body odour. (Holt *et al*., 1986). They also contribute to the aroma of various cheese.
They have been considered as non pathogenic and isolates from previous clinical specimens has been described as skin flora. (Gruner *et al*, 1993, Ulrich *et al*, 2006). However, several authors have reported cases of infections from the genus e.g. neurosurgical site infection Talento *et al.*, (2013), relapsing peritonitiss Poesem *et al.*,

(2012), catheter-related bloodstream infection Bal *et al* (2015), bacteremia and line sepsis Janda *et al*., (2003) and human clinical specimen (Wauters *et al*., 2004). These reports and more have aroused interest in the genus *Brevibacterium*. As with all pathogens and potential pathogens, their general antibiotic susceptibility or resistance is of interest to health practitioners as a means to evaluate the best treatment options for the bacteria in infectious states.

However, the organisms are very difficult to identify by conventional identification method. Poesem *et al.,* (2012) reported difficulties in identifying the bacteria by API Coryne system (bioMerieux, Marcy l`Etoile, France), also Talento *et al.,* (2013) reported inability to identify the organism by API Coryne system (bioMerieux, Marcy l`Etoile, France) which is the best available common biochemical identification kit for coryneform bacteria.

In the last few decades, there have been great advances in bacterial identification. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a very rapid method of bacterial identification in which protein fingerprinting are obtained from whole bacterial cells and compared to a reference database through various algorithms. VITEK 2 is the most-used automated identification technique in current microbiology laboratories. (Guo *et al*., 2014). Sequencing of 16S rRNA gene is a gold standard for the identification of a many microorganisms due to large reference databases.

Therefore, this study is designed to identify *Brevibacterium* species obtained from skin swabs and evaluate the effectiveness of MALDI-TOF, ViTEK 2 and partial sequencing of 16S rRNA gene in identifying the organisms and also evaluate the antibiotic susceptibility pattern of species of *Brevibacterium*.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used for the study were obtained from our research group Gram positive culture collections. They were isolated from humans. Non-staphylococci strains with characteristic cheesy smell were further grown on Blood agar and selected for further studies.

Identification of Strains

Identification of Bacterial Strains with ViTEK 2 System

The bacterial strains were streaked on Blood agar media and incubated at 37^0C for 24 hours. Single colonies were picked on the agar plates and resuspended in saline solutions to make 0.5-0.65 Macfarland standard. The resulting solutions were used in VITEK® 2 system (bioMerieux, Paris, France) with GP ID cards according to the manufacturer\s instruction. Acceptable identification is 85% accuracy according to the manufacturer\s standard.

Identification of Bacterial Strains with Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry

Bacterial strains were grown on Blood agar for 18 h at 37^0C. Each grown colonies were used to make a thin smear on MALDI plate. The bacterial smears were overlaid with 1 µl of matrix solution (saturated solution of cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). The bacterial smears with matrix sample were air drying at room temperature. Measurements were performed with Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany) using FlexControl software (version 3.0). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1voltage, 20 kV; ion source 2 voltage, 18.4 kV; lens voltage, 9.1 kV; mass range, 2,000 to 20,000 Da).

The highest score of a match against a spectrum in the database was used for identification according to the proposed benchmark by the manufacturer. Scores below 1.7 were considered not to have generated a reliable identification; a score of 1.7 was considered identification to genus, and a score of 2.0 was used for species identification.

Identification of Bacterial Stains by Partial Sequencing of 16s rRNA gene

Bacterial DNA was extracted by standard boiling method. 5 µl DNA template was then used as a template in polymerase chain reaction (PCR) amplification containing 0.32 mM dNTP, 10 pmol of each PCR primer, 2.5 units AmpliTaq Gold DNA Polymerase, LD (Applied Biosystems 4338856) 5 µl GeneAmp 10X PCR Gold Buffer (Applied Biosystems 4338856) 5 mM MgCl2 (Applied Biosystems 4338856). BSF8N 5'-AGAGTTTGATCMTGGCTCAG-3' and BSR534 5'-ATTACCGCGGCTGCTGGC-3' were the primers used in a 50 µl reactions other PCR components. The PCR conditions were as follows: 10 min of initial denaturation at 95°C, followed by 45 cycles of annealing of 15s at 95°C, 30s at 55°C, 30s at 72°C followed by a single 7-minute extension at 72°C and finally set on hold at 4°C. Gel electrophoresis was used to detect the PCR amplicon which was purified and sequenced using standard procedures. The basic local alignment search tool program was used to compare the identity of the sequences obtained with those held in GenBank database. The sequences were deposited in Genbank with ascension numbers KX580046- KX580052

Antibiotic susceptibility testing

Antibiotic susceptibility of all *Brevibacterium* spp. to the following antibiotics (Oxoid, UK) were done by Kirby Bauer technique according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015) guidelines: Fusidic Acid, Linezolid, Norfloxacin, Rifampicin, Penicillin, Clindamycin, Erythromycin, Cefoxitin, Tobramycin, Trimethoprim and Vancomycin. The plates were incubated at 37°C for 24 hrs and examined for clear zones of inhibition around the discs. The diameter of inhibition was measured and compared with standard zones to determine resistance according to EUCAST breakpoint for *Staphylococcus* species. (EUCAST, 2015).

RESULTS

All the 10 selected strains with the cheesy odour were identified by Vitek 2, MALDI TOF and partial sequencing of 16SrRNA gene. Identification by sequencing of 16SrRNA gene identified all the strains as *Brevibacterium* sp. namely 5 *B. epidermidis,*3 *B. iodinum* and 2 *B. oceani.* Vitek 2 was unable to identify any of the isolates while MALDI-TOF identified 6 of the isolates as *Brevibacterium* to the genus level but with wrong species identification. However, the scores for the isolates were less than 1.7. (Table 1)

The antibiotic resistance of all isolates was evaluated. All tested strains were susceptible to Vancomycin, Linezolid and Rifampicin with Cefoxitin (90%) while all strains were resistant to Penicillin, Fusidic Acid, and Trimethoprim with Norfloxacin (90%). 70% of the strains were susceptible to Tobramycin. *Brevibacterium epidermidis* were generally resistant to Erythromycin and Clindamycin while *B. iodinum* and *B. oceani* were susceptible to the 2 antibiotics (Table 2).

Table 1: Comparison of *Brevibacterium* Identification by Vitek 2, MALDI-TOF and Partial Sequencing of 16S rRNA gene

S/N	Identification Methods			
	Strains	16 S rRNA score %	V.TEK	MALDI-TOF	% MAL
1	*B. epidermidis*	100	Slashline VG	*B. linens*	1.626
2	*B. epidermidis*	100	LD	*Arthrobacter pyridinolis*	1.335
3	*B. epidermidis*	98	LD	*B. linens*	1.641
4	*B. epidermidis*	99	Unidentified	*Pseudomonas mucidolens*	1.264
5	*B. epidermidis*	98	Slashline VG	*B. iodinum*	1.520
6	*B. iodinum*	99	Slashline VG	*S. lutrae*	1.237
7	*B. iodinum*	99	Slashline VG	*B. linens*	1.523
8	*B. iodinum*	98	Slashline VG	*B. linens*	1.410
9	*B. oceani*	97	LD	*Actinomyces odontolyticus*	1.280
10	*B. oceani*	98	Slashline VG	*Rhizobium tropici*	1.240

Table 2: Antibiotic Susceptibility/ Resistance of *Brevibacterium* Isolates

		ZONE OF INHIBITION (MM)										
		ANTIBIOTICS										
No.	Strain	P	D	E	FOX	VA	FD	LZD	TOB	NOR	RD	TMP
76	*B. epidermidis*	R	R	R	S	S	R	S	S	R	S	R
79	*B. epidermidis*	R	R	R	S	S	R	S	S	R	S	
80	*B. epidermidis*	R	I	R	S	S	R	S	R	R	S	
141	*B. epidermidis*	R	R	R	R	S	R	S	R	R	S	R
176	*B. epidermidis*	R	R	R	S	S	R	S	S	R	S	R
126	*B. iodinum*	R	S	S	S	S	R	S	R	S	S	
139	*B. iodinum*	R	S	S	S	S	R	S	S	R	S	R
145	*B. iodinum*	R	S	S	S	S	R	S	S	R	S	R
89	*B. oceani*	R	S	S	S	S	R	S	S	R	S	R
169	*B. oceani*	R	S	S	S	S	R	S	S	R	S	R

DISCUSSION

Brevibacterium spp. normally inhabits the human skin where they are opportunistic pathogens. Attentions have not been given to this genus as infectious agents rather if seen in clinical samples were dismissed as skin contaminants (Poesem *et al.,* 2012) However, as more authors report their involvement in infectious state, more attentions are being given to this group of organism

In the present study, 3 species were identified as *B. epidermidis, B. iodinum* and *B. oceani.* Vitek 2 and was unable to identify the entire organism. This is a rare feat for Vitek 2 apparatus as it is one of the commonest machines in diagnostic laboratories for identification of infectious organisms. However, Navas *et al*, (2014) reported correct identification of 3 *Brevibacterium casei* and species misidentification of *Brevibacterium otitidis* by Vitek. Several authors have reported inability to identify *Brevibacterium* sp with API Coryne system (bioMerieux, Marcy l`Etoile, France) (Poesem *et al.,* 2012, Talento *et al.,* 2013) but this is the first study reporting inability of Vitek 2 apparatus to identify *B. epidermidis, B. iodinum* and *B. oceani.*

MALDI TOF was able to identify the strains to genus level as *Brevibacterium* in 60% of the isolates but not to species level. The major drawback in the MALDI identification is that the confidence level is less than acceptable 1.7. A clinician working with unknown organisms might have discarded the identification because of the low identification score even though some of the identification was correct to the genus level. MALDI TOF identification is not very effective for *Brevibacterium* identification. Senga *et al*. (2013) grouped *Brevibacterium massiliense, Brevibacterium otitidis, Brevibacterium paucivorans, Brevibacterium ravenspurgense, Brevibacterium sanguinis, Brevibacterium stationis* into rare bacteria in clinical setting that they could not be identified by MALDI but were identified by molecular identification

Partial sequencing of 16S rRNA gene gives correct identification in all strains. This has been reported as the choice method for identification of Brevibacteria when other methods fail (Talento *et al.* 2013); Poesem *et al.* 2012). Sequencing is the standard identification method, however, it is not ideal in clinical situation that needs rapid identification of infectious bacteria in order to commence rapid therapy. Therefore, other accurate and faster methods are constantly exploited (Guo *et al*., 2014). Some molecular methods such as multilocus typing, amplified ribosomal DNA restriction enzyme analysis, pulsed-field gel electrophoresis, ribotyping have previously been used in identification of Brevibacteria, (Forquin *et al*, 2009.Hoppe-Seyler *et al*, 2007, Lima *et al* 2000, Oberreuter *et al*., 2002) but they may not have practical application in clinical setting. Bal *et al* (2015) recorded success in identifying *B. casei* with MALDI-TOF but we are unable to identify the different species use in this study with MALDI.

All Brevibacteria in this study were susceptible to Vancomycin, Linezolid ; Rifampicin and Cefoxitin. Poesem *et al.,* (2012) also noted full susceptibility of *Brevibacterium* to quinolones, aminoglycosides, tetracyclines, glycopeptides, and linezolidS. However, we discovered a little variation from Poesem *et al* (2012) result because our organisms have 30% resistance to Tobramycin.

Gruner *et al* (1993) also reported that all tested *Brevibacterium* strains were susceptible to erythromycin. However, in this study, all *B. epidermidis* were resistant to Erythromycin while *B. iodinum* and *B. oceani* were susceptible to the antibiotics. Perhaps *Brevibacterium* susceptibility to Erythromycin is species related. We noted very high resistance *to* Penicillin, Fusidic acid, Trimethoprim and Norfloxacin in this study. This agrees with the work of Poesem *et al.,* (2012) who reported reduced susceptibility to beta-lactam antibiotics in tested *Brevibacterium* strains. Troxler *et al*., (2001) also reported natural resistant of *Brevibacterium casei* to cotrimoxazole and natural susceptibility to glycopeptides and Rifampicin as reported also in this study.

CONCLUSION

Our study reports inability of ViTEK 2 apparatus to identify all the tested *Brevibacterium* strains while MALDI TOF was able to identify 60% of tested species only to genus level with low confidence level. Partial sequencing of 16s rRNA gene correctly identifies all strains. We also report resistance of *Brevibacterium* species to Penicillin, Fusidic acid, Trimethoprim and Norfloxacin and susceptibility to Vancomycin, Linezolid, Rifampicin and Cefoxitin. Resistance to Erythromycin were only observed in *B. epidermidis* while *B. iodinum* and *B. oceani* were susceptible.

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