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Comparison of identification methods of vancomycin-resistant enterococci in MALDI-TOF MS, Vitek 2 and API 20 STREP

Porównanie wyników identyfikacji wankomycyno-opornych enterokoków metodą MALDI-TOF MS, Vitek 2 i API 20 STREP

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Słowa kluczowe

enterokoki, VRE, identyfikacja MALDI-TOF MS, Vitek 2

Summary

Introduction. From the clinical point of view, differentiation of the following species in the genus Enterococcus is the most important: *E. faecium* and *E. faecalis* (responsible for over 90% of all enterococcal infections) and *E. gallinarum*, *E. casseliflavus*, *E. flavescens* due to their resistance to low concentrations of vancomycin (VanC phenotype). During research of enterococci strains resistant to vancomycin (VRE), we are observed some of those strains isolated from clinical specimens from hospitalized patients were identified by systems based on biochemical identification: Vitek® 2 and Api 20 STREP (bioMereiux, France) as *E. gallinarum* while other methods demonstrated that they were vancomycin resistant *E. faecium* (VRE).

Aim. Aim of study was to compare system Vitek2 and Api 20 STREP vs MALDI-TOF MS for enterococci identification.

Material and methods. 100 strains of enterococci were identified for species in systems based on biochemical reactions (system Vitek® 2 and API 20 STREP) and mass spectrometry (MALDI-TOF). The detection of *ddl* and *vanC* genes was the reference method.

Results. The differences in identification of enterococci ranged from 2% in Vitek® 2 system to 6% for ATB™ Expression system in relation to MALDI – Biotyper.

Conclusions. High cost and time-consuming of molecular methods, exclude them from the application in routine laboratory diagnosis. However consistency of results in species identification in mass spectrometry MALDI-TOF with genetic methods, abet to use its in routine microbiological diagnosis.

Streszczenie

Wstęp. Z klinicznego punktu widzenia, w zakażeniach wywoływanych przez rodzaj *Enterococcus*, najważniejsze jest różnicowanie gatunków: *E. faecium* i *E. faecalis* (odpowiedzialne za ponad 90% wszystkich zakażeń) oraz *E. gallinarum*, *E. casseliflavus* i *E. flavescens*, ze względu na ich naturalną oporność na niskie stężenia wankomycyny (fenotyp VanC). W trakcie badań nad szczepami enterokoków opornych na wankomycynę (VRE) zaobserwowano, że niektóre enterokoki izolowane z materiałów klinicznych od hospitalizowanych chorych były identyfikowane przez systemy oparte na biochemicznej identyfikacji: Vitek 2 i API 20 Strep (bioMereiux, Francja) jako *E. gallinarum*, podczas gdy inne metody identyfikowały te szczepy jako *E. faecium* oporne na wankomycynę.

Cel. Celem pracy było porównanie systemu Vitek® 2, API 20 STREP oraz MALDI-TOF MS w identyfikacji gatunkowej enterokoków.

Materiały i metody. 100 szczepów drobnoustrojów z rodzaju Enterococcus, poddano identyfikacji do poziomu gatunku w systemach opartych o rozkład cech biochemicznych (systemy Vitek® 2 i API 20 STREP) oraz o spektrometrię masową (MALDI-TOF). Metodami referencyjnymi były metody genetyczne wykrywające geny *ddl* i fragmentu genu *vanC*.

Wyniki. Różnice w identyfikacji enterokoków wynosiły od 2% w systemie VITEK®2 do 6% dla ATB™ Expression względem MALDI – Biotyper oraz metod molekularnych.

Wnioski. Wysoki koszt oraz czasochłonność metoda molekularnych, wyklucza je z zastosowania w rutynowej diagnostyce laboratoryjnej. Natomiast zgodność wyników identyfikacji gatunkowej w spektrometrii masowej MALDI-TOF z metodami genetycznymi, skłania do jej wykorzystania w rutynowej diagnostyce mikrobiologicznej.

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INTRODUCTION

Microorganisms of the *Enterococcus* spp. are Gram-positive, non-spore-forming, relatively anaerobic cocci, in-laying chains or pairs. Most strains could grow at temperatures between 10°C and 45°C, pH 9.6 and in the presence of 6.5% sodium chloride solution (1). They are microorganisms being extremely resistant to harsh environments and are able to survive heating at 60°C for 30 min (1). These bacteria are typical opportunistic pathogens which can cause severe nosocomial infections, they are difficult to treat because of their inherent or acquired resistance to multiple classes of drugs. The term "enterocoque" was for the first time used in the French publication dated 1899 but, due to the morphology of cells, a characteristic image of light microscopy, and the negative reaction to the presence of catalase, to the end of the 70s of the twentieth century enterococci were included among the genus *Streptococcus* (2-4). In 1984, Schleifer proposed separation of a separate genus *Enterococcus* spp., basing on the homology analysis of the 16S rRNA sequence between streptococci and enterococci (5). Until now, more than 40 species of the genus *Enterococcus* have been described, but the following are of clinical relevance: *Enterococcus faecium* and *Enterococcus faecalis*, being responsible for over 90% of all infections and *Enterococcus gallinarum*, *Enterococcus casseliflavus* and *Enterococcus flavescens*, due to their natural resistance to low levels of vancomycin (phenotype VanC1, VanC2 and VanC3) (1, 6, 7).

The principle of MALDI-Biotyper operation is based on laser desorption involving matrix (Matrix Assisted Laser Desorption Ionisation – MALDI), consisting of mild ionization using a laser with a specially constructed matrix that absorbs the laser energy to transfer it onto the analyzed protein. Then, a time of flight (Time of Flight – TOF) analyzer – being a mass analyzer – measures the time of flight of ions from the acceleration of the analyzer to the strike at the detector. Ion time of flight is converted to molecular ion ratio of the weight of its electric charge (m/z), and the greater the charge, the longer the ion flight time. The result is obtained as a result of mass spectrum (in the range of 2 to 20 kDa), which gives a unique pattern of protein (molecular fingerprint) compared to a database for identification of organisms (fig. 1). A computer program in the MALDI-Biotyper system, in addition to the mass spectrum also shows the numerical values specifying the degree of identification of the microorganism. Range: 2300-3000 means a very likely, species identification, 2,000-2,299 is a safe identification of the genus and a probable identification of the species, 1,700-1,999 is a likely determination of the genus, and values between 0,000-1,699 represent an incredible identification.

AIM

The aim of the study was to compare three methods for identification of enterococci: biochemical methods (the ATB Expression System and Vitek 2 systems) and mass spectrometry MALDI-TOF.

MATERIAL AND METHODS

One hundred strains of vancomycin-resistant enterococci (VRE) were analyzed, as isolated from patients after kidney or liver problems, staying in one of the teaching hospitals in Warsaw in the years 2010-2011. The strains were grown from rectal swabs (n = 52), urine (n = 17), wound swabs (n = 9), catheters (n = 7), peritoneal fluid (n = 7), bile (n = 4), and blood (n = 3) and a portion of the tissue (n = 1). Pre-selection and identification of clinical primers were performed based on the procedure by Facklam and Collins (8) and included: analysis of the morphology of colonies on Columbia Agar, as supplemented with 5% of defibrinated sheep blood, performing a Gram-stained preparation, test for the presence of catalase and pyrrolidonyl peptidase. Clinical materials were also seeded on D-Coccosel Agar – in order to isolate enterococci decomposing esculin to esculatin in the presence of bile salts. Rectal swabs in the direction of testing the presence of vancomycin – resistant enterococci were also seeded on selective chromID VRE. The bases used were produced by bioMérieux (France). All of the grown enterococci were subjected to identification by means of the Vitek 2 system and the API 20 STREP biochemical test in the ATB Expression system. All procedures were

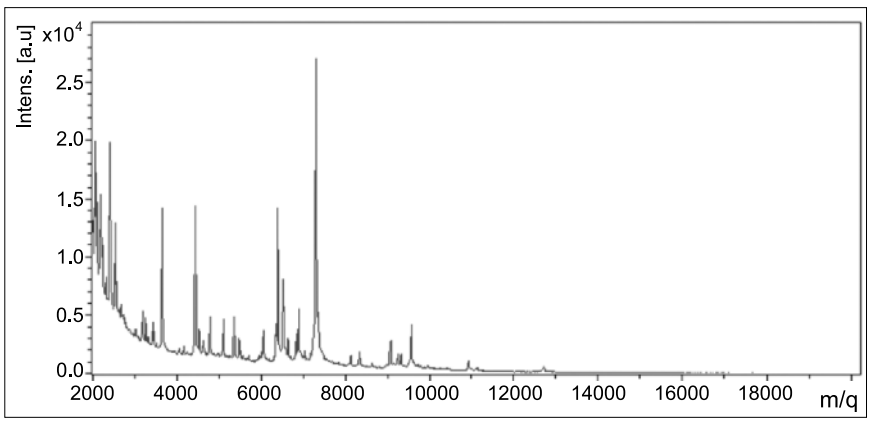

Fig. 1. Sample mass spectrum obtained for *E. faecium* in the MALDI-Biotyper system (Bruker Daltonics, Germany).

performed according to the manufacturer's instructions. For comparison, all the strains were further identified using mass spectrometry (MALDI-TOF) on the MALDI-Biotyper apparatus (Bruker Daltonics, Germany).

Reference methods are methods based on the detection of species-specific *vanC* and *ddl* gene fragments. For all strains, PCR was performed with primers for the gene fragments encoding VanC ligase (VanC1 5'-GAAAGACAACAGGAAGACCGC-3' and VanC2 5'-TCGCATCACAAGCACCAATC-3') (9). Positive control was the strain of *E. gallinarum* (ATCC: BM 4174). The reaction conditions for amplification were as follows: initial denaturation 95°C – 5 min, 30 cycles consisting of denaturation 94°C – 15 s, annealing of primers 55°C – 30 s, elongation 72°C – 15 s, final elongation 72°C – 7 min. Furthermore, identification of *Enterococcus* species was confirmed by the detection of the ddl gene fragment, encoding the D-Ala-D-Ala ligase, basing on the reaction conditions proposed by Domig et al. (10). In the case of *E. faecium* the primers used were: ECIUMF; 5 'GGCAGAGCATGAAGTGTCCA 3' and ECIUMR; 5 'CTTCTGGGTTTTCTGCTTTTGTA 3', positive control was the strain of *E. faecium* (ATCC: BM 4147). In the case of *E. faecalis* the primers used were: ELISF2; 5'-GGCCCTCTTTTATCTGAACGA-3' and ELISR3; 5'-GCGACTTAAGCCACTTCCAT-3', positive control was the strain of *E. faecalis* ATCC 29212.

RESULTS

The results of the comparative analysis performed to identify the species of 100 strains of *Enterococcus* spp. Showed differences ranging from about 2% in the Vitek 2 system to 6% in the ATB Expression system, in regard to the identification based on mass spectrometry MALDI-TOF (fig. 2). The biochemical API 20 STREP test twice misidentified *E. faecium* as *E. gallinarum* and four times as *E. faecalis* (two isolates identified as *E. gallinarum* and *E. faecium*). The automatic Vitek 2 system was characterized by a higher sensitivity than the API 20 STREP identification test, because only two isolates of *E. faecium* were misidentified as *E. gallinarum*. Misidentification of both the Vitek 2 system and the ATB Expression concerned the various isolates of VRE. All of the results of PCR that detected fragments of the gene encoding the VanC1 ligase were negative. Amplification of the ddl gene fragment, encoding the D-Ala-D-Ala ligase *E. faecium* and *E. faecalis* confirmed the correct identification of the MALDI system – Biotyper, the scope for 56 isolates of enterococci ranged between 2300 and 3000, in the case of the remaining 44 isolates, between 2,000 and 2,299.

DISCUSSION

In 1989, Facklam and Collins, on the basis of biochemical assessments, proposed a division of enterococci to 3 groups, according to their capacity of: hydrolysis of arginine, fermentation of alcohols and sugars (mannitol, sorbitol, sorbose, raffinose) and the use of pyruvate by-product in the form of acetoin (8). Test Facklam and Carvalho results from 2002 expanded the enterococci division with two additional groups (11). The scheme of the phenotypic identification of enterococci proposed by Facklam was of commercial use in identification tests that have been successfully used in automated systems in microbiology laboratories. Examples can include: the API 20 STREP system, the Vitek 2 system (bioMérieux, France), Phoenix (Becton-Dickinson, USA) and MicroScan® WalkAway® – 96 Plus System (Siemens, Germany) (12-17).

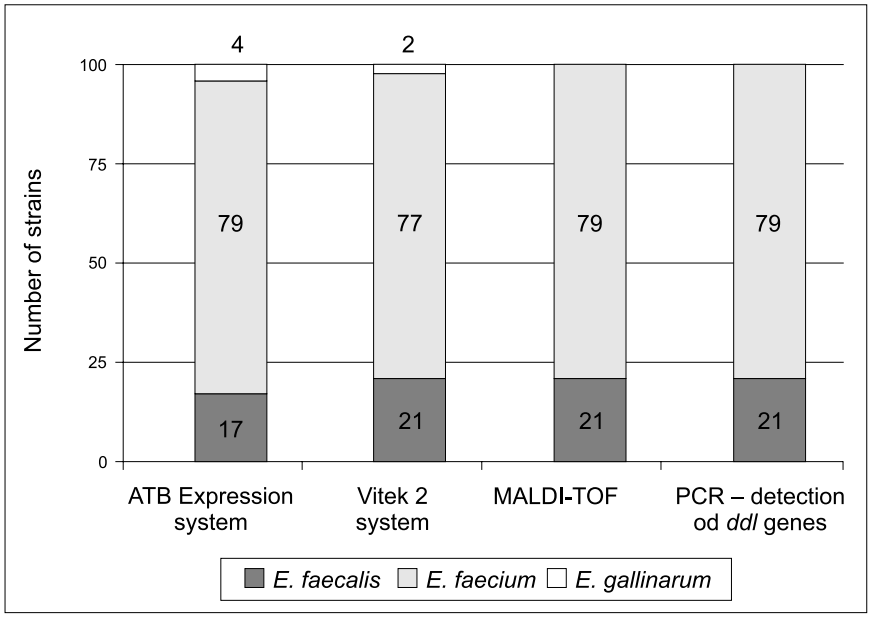

Fig. 2. Comparison of the identification of 100 strains of the genus *Enterococcus* VRE, as determined basing on the MALDI-TOF mass spectrometry and the Vitek 2 system, API 20 STREP test and the detection of *ddl* genes by PCR.

The biochemical API 20 STREP test differentiates between *E. faecium* and *E. gallinarum*, as well as between *E. faecium* and *E. faecalis*, basing, among others, on the hippuric acid hydrolysis capacity (*E. gallinarum* and *E. faecalis* exhibit the capacity) (18). Studying the enzymatic reaction involving sorbitol or raffinose also does not allow to determine the correct identification of species because of the varying capacity of *E. faecium* (18). The Vitek 2 system, in biochemical identification, uses the following reactants: arginine dihydrolase, L-piroydonyl arylamidase, D-sorbitol, the growth-capacity in 6.5% NaCl, D-mannitol, methyl-β-D-glucopyranosidase, D-raffinose, sucrose/sucrose and arginine dihydrolase (thioglicolate sigma). Biochemical analysis of substrates available for identification in GP information shows that the only biochemical constant differentiating between of *E. faecium* from *E. gallinarum* is to detect the methyl-β-D-glucopyranosidase enantiomer (optical isomer). Analysis of the biochemical characteristics of the strain in terms of the distribution of sorbitol or raffinose insufficiently differentiated between *E. gallinarum* and *E. faecium*, as *E. faecium* shows variation or lack of hydrolysis capacity of the above starting materials) (1, 18).

The time of flight test may be useful in differentiation between the species *E. faecium* and *E. gallinarum* (positive for *E. gallinarum*). However, the identification of enterococci should consider the fact that the species *E. casseliflavus* gives a positive result in this assay (18). Thus, it seems that, with the difficulties with the identification of *Enterococcus* species, mass spectrometry (Matrix Assisted Laser Desorption Ionization-Time of Flight – MALDI-TOF) is an alternative for the systems based on biochemical analysis. In this method, identification of species of microorganisms involves the analysis of highly conserved ribosomal proteins. Fang and Griffin have confirmed that the MALDI-TOF mass spectrometry is characterized by a higher sensitivity and specificity than the Vitek 2 system in the rapid identification of enterococci (19, 20).

Due to the natural resistance of enterococci to multiple classes of antibiotics and the ease in acquiring genes conferring resistance to other antibiotics, antimicrobial treatment of enterococcal infections is extremely difficult. Enterococci have natural resistance to cephalosporins, lincosamides, low concentrations of aminoglycosides, semisynthetic penicillins (methicillin), monobactams, streptogramins (for *E. faecalis*) (1, 21). In addition, many authors include the coformulation of sulphametoxazol with trimethoprim to be insufficiently effective in the treatment of infections with enterococcus etiology (1, 21, 22). Conventional therapy for the treatment of invasive infections, consisting of a combination of aminoglycoside and a beta-lactam antibiotic, in most cases, does not apply to hospitals, due to a significant percentage of strains resistant to high levels of aminoglycosides (HLAR, HGLR). This forces the widespread use of glycopeptide antibiotics for enterococci infections. It is, thus, particularly dangerous that enterococci acquired resistance to vancomycin and teicoplanin, until recently considered as drugs of last resort. Formation and selection of strains resistant to glycopeptide antibiotics is promoted by incorrect antibiotic treatment and the ease of transfer of genes conferring resistance to the group of antibiotics, often present in the transposition elements and plasmids, between strains. In addition, a clonal proliferation of "epidemic" strains of VRE can be observed within hospitals and between hospitals.

Due to the reduced susceptibility to vancomycin, *E. gallinarum*, *E. casseliflavus* and *E. flavescens* (genotype: VanC1, VanC2, VanC3), from a clinical point of view, it is very important to guarantee rapid identification of the species of these microorganisms in order to eliminate the empirical therapy of this glycopeptide. What is more, abuse of vancomycin in inpatient further promotes the selection of vancomycin-resistant enterococci. Similarly, species differentiation between *E. faecium* and *E. faecalis* is important not only for epidemiological reasons but mainly due to the species-specific resistance to an important class of drugs. The world records a spread of clonal CC17 *E. faecium* complex resistant to antibiotics beta-lactam. Strains of *E. faecalis* also have natural resistance to quinupristin/dalfopristin which can be used for infections of *E. faecium*. Thus, as indicated by the results of the analysis, it should be clear that the proper identification of species, genus *Enterococcus* allows to determine a correct targeted therapy, which is very important from a clinical point of view.

CONCLUSIONS

The gold standard in the differentiation of species between *E. faecium* and *E. faecalis* is still the amplification of a *ddl* gene fragment encoding the D-Ala-D-Ala ligase. What is more, identification of the fragment of the gene encoding superoxide dismutase allows for distinguishing more than 20 species of enterococci. Due to the high cost and the time-consuming nature of molecular methods, they are not used in routine microbiological diagnosis. Therefore, it appears that the more sensitive, faster and cheaper mass spectroscopy gains an advantage over methods of biochemical identification.

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