Species Identification of Clinical Veillonella Isolates by MALDI-TOF Mass Spectrometry and Evaluation of Their Antimicrobial Susceptibility

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Abstract: We investigated the possibilities of the MALDI-TOF MS for species identification of anaerobic gram-negative cocci isolated from clinical specimens of cancer patients. A total 70 Veillonella clinical isolates and one Acidaminococcus intestini isolate were analysed by the Bruker Microflex MALDI-TOF instrument with the Biotyper 3.0 software. All isolates were identified to the species level with a scores greater than 1.9. The most common species were V. parvula (37 strains), then followed by decreasing the frequency V. dispar (16), V. atypica (16) and V. denticariosi (1). Susceptibilities of the isolates were determined by the E-test methodology. All Veillonella isolates were susceptible to imipenem, whereas a high resistance rates were observed for penicillin G, amoxicillin/clavulanate and metronidazole. The proportion of intermediate/resistant isolates of V. parvula, V. dispar and V. atypica to penicillin (MIC ≥ 1 µg/ml) was 86%, 85% and 100%, respectively. The resistance to amoxicillin/clavulanate (MIC 16 - 32 µg/ml) was observed in about 28,6% V. parvula isolates, 23,1% V. dispar isolates and 6,7% V. atypica isolates. According to EUCAST criteria, resistance to metronidazole (MIC ≥ 8 µg/ml) of V. parvula, V. dispar and V. atypica was 88,6%, 53,8% and 40%, respectively.

Keywords: Veillonella Clinical Isolates, Anaerobic Infections, MALDI-TOF MS, Antimicrobial Susceptibility, Resistance Rates, Cancer Patients

1. Introduction

The genus Veillonella and Acidaminococcus consists of strictly anaerobic, non-fermentative, gram-negative cocci, which is part of the normally microflora inhabiting the oral cavity, gastrointestinal, respiratory and genitourinary tracts. Although Veillonella spp. is considered to be of low virulence, they may cause serious infections such as bacteremia [1-3], endocarditis [4], discitis [5], meningitis [6] and pneumonitis [7], mainly in immunocompromised patients. Among the genus Veillonella three species, V. parvula, V. atypica and V. dispar are most frequently isolated from clinical specimens in polymicrobial cultures. Rarely, they are reported as the sole pathogen.

Identification of Veillonella isolates to the species level is not possible on a routine basis, because conventional phenotypic and biochemical testing does not provide an adequate discrimination between species. The isolation of Acidaminococcus spp. from clinical samples is relatively infrequent and usually do not distinguish them from Veillonella spp. Direct sequencing of the 16S rRNA gene has been described as the best method for identification of Veillonella strains at the species level [8, 9].

In recent years, another technique is increasingly used for the identification of bacteria: matrix-assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-TOF MS). The introduction of this technology to microbiology has been a major success and in the last decade
is widely used for routine diagnostic of the pathogens of infections [10]. The ability to cost-effectively and rapidly identify microorganisms by MALDI-TOF MS is replacing a more arduous and time consuming biochemical and antigen-based identification methods, as well as some genetic-sequence-based methodologies. Several reports show that MALDI-TOF MS can be successfully applied for identification of anaerobic bacteria [11-16].

Veillonella spp. are generally susceptible to most antimicrobials drugs used for treatment of anaerobic infections, including β-lactam antibiotics and metronidazole [17]. β-lactam antibiotics therapy is often administered for treatment of these infections, but there are several reports which describe penicillin resistance of veillonellae [18-20].

The aim of this study is to evaluate the ability of the Bruker Biotyper MALDI-TOF MS system for species identification of Veillonella spp. and determine their susceptibility to metronidazole, penicillin, amoxicillin/clavulanate and imipenem.

2. Methods

2.1. Specimen Collection and Growth Conditions

The study was performed in a 1500-bed N. N. Blokhin Cancer Research Center for adults and children in Moscow (Russian Federation), of the Department of Healthcare. All clinical specimens included in this study were collected between August 2004 and November 2016 from patients with various malignancies. Clinical samples were transported within 2 h of collection without using special anaerobic transport systems and inoculated onto Schaedler agar, supplemented with hemin, menadione and 5% blood and also in enrichment Thioglycollate broth. All plates were incubated anaerobically using the GasPak jar and AnaeroGen system (Oxoid, UK) at 37°C for 48-72 h. Each of morphotype growing colony was subcultured on Schaedler agar plates and simultaneously on blood agar plates. First plates were again incubated anaerobically and second plates were incubated under aerobic conditions to eliminate bacteria which were not strictly anaerobic. After anaerobic incubation, preliminary identification was based on Gram staining and the results of Rapid ID 32A, Vitek-2 (bioMerieux, France) or MicroScan WalkAway (Siemens, UK). Strains were collected and stored dry at room temperature. Escherichia coli ribosomal proteins (bacterial test standard, Bruker Daltonik, Germany) were used as a positive control and calibration standard. Mass spectra were obtained using MicroFlex LT mass spectrometer (Bruker Daltonik GmbH) and analyzed by Biotyper 3.0 software in the FlexControl program. For bacterial identification, the peak list for an unknown isolate is compared to the reference library of spectrum. A library of 5,629 standard spectra (version 4.0.0.1) was used. The MALDI-TOF Biotyper output is a log (score) within the range 0 to 3.0. According to the criteria proposed by the manufacturer, a log (score) between 1.7 and 1.99 indicates a genus level identification, and a log (score) ≥ 2 indicates a species level identification.

2.2. MALDI-TOF Identification of Isolates

Pure cultures of each frozen strain were twice subcultured on blood agar anaerobically and were analyzed by the direct transfer method. Few colonies were smeared directly on the stainless-steel target in a thin film using inoculation needle, and immediately all spots were overlaid with 1 µl of α-cyano-4-hydroxy-cinnamic acid matrix solution in organic solvent (50% acetonitrile and 2,5% trifluoroacetic acid). Each strain was smeared in three spots. The target plate was then left to
Table 1. Distribution and source of isolation of different Veillonella species.

| Source of isolation | No. of clinical Veillonella isolates | Total |
|---------------------|-------------------------------------|--------|
|                     | V. parvula | V. dispar | V. atypica | V. denticariosi |
| Gastrointestinal tract | 11 | 4 | 3 | 18 |
| Biliary tract | 8 | 1 | 6 | 15 |
| Lung | 6 | 4 | 4 | 14 |
| Head and neck | 6 | 3 | 2 | 11 |
| Soft tissue | 4 | 2 | 1 | 6 |
| Urogenital tract | 1 | 1 | 1 | 2 |
| Bone and joint | 1 | 1 | 1 | 3 |
| Blood | 1 | 1 | 1 | 1 |
| Total | 37 | 16 | 16 | 1 | 70 |

The Veillonella isolates were mainly obtained from body fluids (36, 6%), bile (22, 5%), surgical wounds (16, 9%) and pus samples (15, 5%) (Table 2). V. parvula and V. atypica are most frequently isolated from bile samples of patients with cholangitis, whereas no strain of V. dispar was isolated from bile. However, V. parvula and V. dispar compared to V. atypica are more frequently isolated from surgical wounds and abscesses. All Veillonella isolates were isolated in combination with other bacteria and none in pure culture, including a blood sample.

Table 2. Veillonella isolates from various clinical specimens.

| Clinical specimens | Veillonella strains (n) | Total |
|--------------------|------------------------|--------|
|                    | V. parvula | V. dispar | V. atypica | V. denticariosi |
| Abdomen fluid | 5 | 4 | 3 | 12 |
| Pleural fluid | 6 | 4 | 4 | 14 |
| Bile | 10 | 6 | 16 |
| Surgical wounds | 6 | 3 | 1 | 11 |
| Pus samples | 8 | 2 | 11 |
| Operating material | 2 | 2 | 1 | 5 |
| Blood | 1 | 1 | 1 |
| Total | 37 | 16 | 16 | 1 | 70 |

The results of MALDI-TOF MS identification show that for all strains mass spectra of good quality were obtained. A total of 68 isolates (95, 8%) were correctly identified to the species level and had scores higher than 2.0, among which 17 isolates (23, 9%) were identified with scores above 2.300 (Table 3). Only one V. dispar isolate and two V. parvula isolates were identified with a score between 1.9 and 1.999. Species dispar/parvula of the genus Veillonella have very similar patterns and their differentiation is difficult. V. denticariosi generated a score of 2.323 and Acidaminococcus intestine was identified with a score of 2.346.

Table 3. Allocation of 71 isolates of anaerobic gram-negative cocci based on obtained score values.

| Anaerobic gram-negative cocci (No. of strains) | No. of strains with MALDI spectrum score of: | Total |
|---------------------------------------------|------------------------------------------|--------|
|                                             | 2.300-2.500 | 2.000-2.299 | 1.900-1.999 |
| Veillonella parvula (37) | 4 | 31 | 2 |
| Veillonella dispar (16) | 4 | 11 | 1 |
| Veillonella atypica (16) | 7 | 9 |
| Veillonella denticariosi (1) | 1 |
| Acidaminococcus intestine (1) | 1 |
| Total (%) | 17 (23.9) | 51 (71.8) | 3 (4.2) |

The MICs distributions of antimicrobial agents tested against 64 Veillonella isolates are presented in Table 4. All isolates were susceptible to imipenem. The MIC of penicillin G varied between 0.25 – 32 µg/ml. Only 5 of 35 V. parvula isolates (14, 3%) were susceptible to penicillin (MIC ≤ 0.5 µg/ml). Among 13 V. dispar isolates only two (15, 4%) was susceptible to penicillin and none of 15 V. atypica isolates was susceptible to penicillin. MIC value exceeding 1 µg/ml indicates a decreased susceptibility to penicillin. Thus, we found that Veillonella isolates have a very high resistance to penicillin, which ranges from 85 to 100% depending on species.

Table 4. MICs of antimicrobial agents tested against 64 Veillonella isolates.

| Antibiotic | V. parvula (35) | V. dispar (13) | V. atypica (15) |
|------------|------------------|----------------|-----------------|
| Penicillin G | 0.5 - 32 µg/ml | 0.5 - 32 µg/ml | 16 - 2 µg/ml |
| Piperacillin | 1.25 - 16 µg/ml | 1.25 - 16 µg/ml | 16 - 2 µg/ml |
| Amoxicillin / Clavulanate | 0.125 - 8 µg/ml | 0.125 - 8 µg/ml | 1 - 0.125 µg/ml |
| Metronidazole | 0.25 - 16 µg/ml | 0.25 - 16 µg/ml | 16 - 0.25 µg/ml |

Among the Veillonella isolates, resistance to amoxicillin/clavulanate was also observed. For V. parvula and V. dispar isolates, the MIC ranges for amoxicillin/clavulanate were 0, 12 - 32 µg/ml, and for V. atypica isolates – narrower (1-16 µg/ml). 25 (71.4%) V. parvula isolates, 21 (76.9%) V. dispar isolates and 14 (93.3%) V. atypica isolates were susceptible to amoxicillin/clavulanate. Interestingly, all V. atypica strains were resistant to high doses of penicillin (MIC 2-32 µg/ml), but the resistance to amoxicillin/clavulanate was the lowest in comparison to other species.

Unexpected results were obtained for Veillonella susceptibility to metronidazole. Based on EUCAST
breakpoints of metronidazole, MIC ≤ 4 µg/ml indicates susceptibility and > 4 µg/ml – resistance. Sensitivity to metronidazole in V. dispar and V. atypica isolates was 46, 2% (6/13) and 60% (9/15), respectively. The lowest susceptibility to metronidazole was found among V. parvula isolates and was 11.4% (4/35). For the two V. parvula isolates, the MIC of metronidazole was higher than 256 µg/ml. CLSI breakpoints of metronidazole are significantly different from EUCAST breakpoints and MIC ≤ 8 µg/ml suggest susceptible, 16 µg/ml – intermediate and ≥32 µg/ml – resistance. According to CLSI criteria, the proportion of intermediate/resistant isolates of V. parvula, V. dispar and V. atypica to metronidazole (MIC ≥ 16 µg/ml) was 54.3%, 46.2% and 26.7%, respectively.

Only strain of V. denticae had a low sensitivity to penicillin (1 µg/ml), but it is highly sensitive to other antibiotics.

### Table 4. Distribution of 64 Veillonella isolates according to MIC of antimicrobial agents.

| Antibiotic             | Species                  | No. of isolates for which the MIC (µg/ml) was as follows: | 0.03 | 0.06 | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 |
|------------------------|--------------------------|----------------------------------------------------------|------|------|------|------|-----|---|---|---|---|---|----|----|-----|-----|
| Imipenem               | V. parvula               |                                                          | 3    | 2    | 1    | 8    | 18  | 3 |   |   |   |   |    |    |     |     |
|                        | V. dispar                |                                                          | 2    | 7    | 4    | 1    | 11  | 2  | 4 |   |   |   |    |    |     |     |
|                        | V. atypica               |                                                          | 3    | 11   | 1    | 1    | 2   | 1 | 1 |   |   |   |    |    |     |     |
|                        | V. denticaeae            |                                                          | 1    | 1    | 4    | 3    | 1   | 2  | 2 | 1 | 21 |    |    |    |     |     |
| Penicillin G           | V. parvula               |                                                          | 1    | 4    | 3    | 1    | 2   | 2  | 1 | 2 |   |   |    |    |     |     |
|                        | V. dispar                |                                                          | 2    | 1    | 3    | 1    | 1   | 5 |   |   |   |   |    |    |     |     |
|                        | V. atypica               |                                                          | 5    | 4    | 2    | 4    | 2   |   |   |   |   |   |    |    |     |     |
|                        | V. denticaeae            |                                                          | 1    | 1    | 4    | 3    | 1   |   |   |   |   |   |    |    |     |     |
| Amoxicillin/clavulanate| V. parvula               |                                                          | 1    | 3    | 3    | 4    | 4   | 5  | 5 | 3 | 7 |   |    |    |     |     |
|                        | V. dispar                |                                                          | 1    | 2    | 1    | 2    | 4   | 2 | 1 |   |   |   |    |    |     |     |
|                        | V. atypica               |                                                          | 4    | 3    | 5    | 1    | 1   |   |   |   |   |   |    |    |     |     |
|                        | V. denticaeae            |                                                          | 1    | 1    | 5    | 1    | 1   |   |   |   |   |   |    |    |     |     |
| Metronidazole          | V. parvula               |                                                          | 1    | 1    | 3    | 2    | 12  | 7  | 8 | 1 | 2 |   |    |    |     |     |
|                        | V. dispar                |                                                          | 1    | 5    | 1    | 5    | 1   |   |   |   |   |   |    |    |     |     |
|                        | V. atypica               |                                                          | 9    | 2    | 3    | 1    |     |   |   |   |   |   |    |    |     |     |
|                        | V. denticaeae            |                                                          | 1    | 1    | 7    | 8    | 1   |   |   |   |   |   |    |    |     |     |

4. Discussion

Malignancy is a major predisposing factor for invasive infection. This study demonstrated the prevalence of Veillonella spp. in various infections in immunocompromised patients which underwent surgery and received chemoradiation therapy. The largest numbers of Veillonella isolates were recovered from patients with abscesses, surgical wounds, peritonitis, cholangitis, empyema and disintegrating tumor. V. parvula was the dominant species and most frequently isolated from gastrointestinal and biliary tract. V. dispar was often isolated from gastrointestinal tract and lung, but never from bile. V. atypica strains were most frequently isolated from biliary tract and lung.

Since Veillonella are rarely isolated from clinical material and are not paid enough attention, as they are considered contaminants rather than etiological agents of infection, there are few data in the literature on the frequency and sites of their allocation. Usually veillonellae are isolated from mouth and lungs. In our study, Veillonella spp. were mainly allocated from gastrointestinal and biliary tracts (47% of total). The isolation of Acidaminococcus from clinical samples was very rarely reported and this anaerobe was often not distinguished from Veillonella.

Since Veillonella species have very similar patterns, phenotypic and biochemical testing does not provide discrimination between species. Most Veillonella spp. and species of other anaerobic gram-negative cocci were not included in the databases of the commercial phenotypic systems like the Rapid ID 32A or Vitek 2 ANC card. MicroScan WalkAway system database contain only one species - V. parvula. As these microorganisms can cause serious infections, their identification at the species level is necessary. Many authors have shown high reliability of identification of Veillonella spp. at the species level by MALDI-TOF MS through direct smear. Veloo et al. identified 10 strains of V. parvula with a score between 2.182 and 2.416 [16]. Barreau et al. identified 28 V. parvula isolates and one V. ratti isolate to the species level with a score above 1.9 [13].

The present study demonstrates that MALDI-TOF MS is an excellent tool for reliable, accurate, rapid and easy differentiation of anaerobic gram-negative cocci. All isolates were identified to genus level and 95, 8% of isolates were identified to the species level. The use of this device allows you to save time by performing species identification in a few minutes. This is very important, especially in the case of cancer patients which need immediate treatment. The accuracy and reliability of the results are also required, as these patients often undergo chemotherapy and radiation therapy which may change the biochemical profile of pathogens, and their identification by phenotypic methods becomes problematic.

Due to the lack of adequate numbers of reports on Veillonella as a pathogen, there are little data in the literature on antimicrobial susceptibility of this organism and treatment strategies of infections caused by Veillonella spp. During the 70’s, penicillin has been suggested as the drug of choice for the treatment of Veillonella-associative infections. Recently,
it was found that Veillonella species demonstrated a high level of resistance to penicillin G (up to 85%), but were susceptible to the combination of amoxicillin and clavulanate [18, 19]. Ready et al. investigated the sensitivity to penicillin of three main species: V. parvula, V. dispar and V. atypica, isolated from supragingival region of children. The highest rate of penicillin resistance was exhibited by V. dispers (73, 4%) [20]. In our study, most resistant species was V. atypica (100%), followed by V. parvula (85,7%) and V. dispers (84,6%). Previous studies suggested that all penicillin-resistant isolates were negative for \( \beta \)-lactamase production. It is proved that the presence of penicillin-binding proteins with low \( \beta \)-lactam affinity causes poor activity of penicillin against Veillonella species. 

Unlike some authors, 28,6% of V. parvula isolates, 23,1% of V. dispers isolates and 6,7% of V. atypica isolates in our study were resistant to amoxicillin/clavulanate (MIC 16 - 32 \( \mu \)g/ml). The fact that all V. atypica strains were resistant to penicillin, but only 6,7% of isolates were resistant to amoxicillin/clavulanate also confirms that the mechanism of penicillin-resistance in veillonellae is not associated with the production of \( \beta \)-lactamases. 

Limited data exist on susceptibility of Veillonella species to metronidazole. There are some references to sensitive strains of Veillonella. Our studies have shown a high level of resistance to metronidazole, especially of V. parvula isolates (88,6%). Metronidazole is considered to be the "golden standard" in the treatment of anaerobic infection, its use rarely causes resistance among anaerobes. Veillonella probably is an exception, since according to CLSI criteria resistance to metronidazole (MIC \( \geq \) 16 \( \mu \)g/ml) of V. parvula, V. dispers and V. atypica was 54,3%, 46,2% and 26,7%, respectively. Based on EUCAST criteria, resistance to metronidazole (MIC \( \geq \) 8 \( \mu \)g/ml) of V. parvula, V. dispers and V. atypica was 88,6%, 53,8% and 40%, respectively. As far as we know, nobody has investigated the mechanism of resistance of Veillonella to metronidazole. Such a high level of resistance should worry clinicians, because in many clinics metronidazole is the drug of choice for treatment of anaerobic infections, including Veillonella-associative infections.

5. Conclusion

Anaerobic gram-negative cocci, mainly Veillonella, can be very dangerous, causing infectious complications of different locations, especially in immunocompromised patients. Their isolation from the clinical material can not be considered only as contaminants. Moreover, Veillonella species exhibit a high level of resistance to \( \beta \)-lactam antibiotics and their combination with inhibitors of \( \beta \)-lactamase as well as to metronidazole. The highest level of resistance to penicillin was observed in V. atypica, but this species was the most sensitive to amoxicillin/clavulanate and metronidazole. V. parvula had the highest resistance to metronidazole and the level of resistance to penicillin and amoxicillin/clavulanate comparable to V. dispers.

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