Penicillin-binding proteins involved in high-level piperacillin resistance in *Veillonella* spp.

Maria M. Theron¹*, Marais N. Janse van Rensburg¹ and Lynda J. Chalkley²

¹Department of Medical Microbiology (G4), Faculty of Health Sciences, University of the Free State, Bloemfontein; ²Medical Research Council, Cape Town, South Africa

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Objectives: To investigate high-level piperacillin resistance in *Veillonella* spp. in the absence of β-lactamase activity.

Methods: Penicillin-binding protein (PBP) competition studies were conducted in *Veillonella* strains, with piperacillin MICs ranging from 0.5 to >128 mg/L and ampicillin MICs from 0.125 to 4 mg/L. Whole cell lysates were pre-incubated with piperacillin or ampicillin and post-labelled with [³H]benzylpenicillin.

Results: PBP competition studies showed that the PBP with greatest affinity for penicillin and ampicillin had a molecular weight of ∼66 kDa, and exhibited reduced binding of piperacillin in resistant strains.

Conclusions: This unusual focusing of different penicillins on one PBP may be the cause of selective mutants resulting from piperacillin MICs > 128 mg/L. In the absence of β-lactamases, alterations in penicillin-binding were seen to be major contributors to high-level piperacillin resistance development.

Keywords: anaerobic bacteria, resistance, penicillins, Gram-negative bacteria, β-lactams

Introduction

*Veillonella* spp. are Gram-negative anaerobic cocci and are part of the normal flora of the mouth, gastrointestinal tract and vagina of humans. Although clinically isolated, *Veillonella* spp. are often regarded as contaminants; they are often associated with oral infections, bite wounds, head, neck and various soft tissue infections, and have recently been implicated as pathogens in infections of the sinuses, lungs, heart, bone and central nervous system.¹² Recent reports have also indicated their isolation in pure culture in septic arthritis and meningitis.²³ β-Lactam antibiotics are used frequently, and for many years have been the first choice of treatment and prophylaxis for anaerobic infections.⁴⁵ However, susceptibility varies depending on the β-lactam and bacterial species to be targeted.⁵ Piperacillin has maintained efficacy, although it may be inactivated by chromosomal class A β-lactamases produced by anaerobes, and is invariably given in combination with a β-lactamase inhibitor, tazobactam.⁶ Resistance to piperacillin/tazobactam has been reported in *Veillonella* spp., and the occurrence of high-level resistance to piperacillin in β-lactamase-negative *Veillonella* spp. has been noted.⁸–¹⁰ The current study investigates alterations in penicillin-binding protein (PBP) involved in piperacillin resistance development in *Veillonella* spp.

Materials and methods

Veillonella isolates

Thirty-one *Veillonella* spp. were isolated from clinically significant infections from 1996–1997 from the Universitas and Pelonomi Hospitals, Bloemfontein.⁹

MIC determination

MICs of ampicillin (8 mg/L), piperacillin (32 mg/L), cefoxitin (16 mg/L) and imipenem (4 mg/L) were determined by the NCCLS agar dilution methods. Susceptibility breakpoints used (indicated in parentheses) were those suggested by the NCCLS (mg/L).¹¹ Wilkins Chalgren agar was supplemented with 5% laked horse blood to enhance growth of fastidious bacteria, such as *Veillonella*. Eleven *Veillonella* spp. were selected for PBP analysis (Table 1).

PBP labelling of whole cells

Cells were harvested from cultures grown overnight on BHI agar (Oxoid, Unipath, Basingstoke, UK) supplemented with vitamin K (10 mg/L), haemin (500 mg/L) and yeast extract (5000 mg/L) under an anaerobic atmosphere. The cells were suspended in Brucella broth (turbidity ≥5 McFarland standard); 100 µL aliquots were centrifuged at 16 000g and

*Corresponding author. Tel: +27-51-4053648; Fax: +27-51-4443437; E-mail: gnmbml@med.uovs.ac.za

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Penicillin-binding proteins in *Veillonella* spp.

Table 1. Details of selected β-lactamase-negative *Veillonella* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year</th>
<th>Site</th>
<th>ampicillin</th>
<th>piperacillin</th>
<th>cefoxitin</th>
<th>imipenem</th>
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<tr>
<td>V7</td>
<td>1996</td>
<td>unknown</td>
<td>≤0.06</td>
<td>0.25</td>
<td>0.25</td>
<td>≤0.06</td>
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<tr>
<td>V2</td>
<td>1996</td>
<td>unknown</td>
<td>0.125</td>
<td>0.5</td>
<td>0.5</td>
<td>≤0.06</td>
</tr>
<tr>
<td>V8</td>
<td>1996</td>
<td>bronchus</td>
<td>0.5</td>
<td>8</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>V11</td>
<td>1996</td>
<td>colon</td>
<td>0.25</td>
<td>16</td>
<td>0.5</td>
<td>0.125</td>
</tr>
<tr>
<td>V19</td>
<td>1996</td>
<td>bone</td>
<td>0.25</td>
<td>16</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>V31</td>
<td>1997</td>
<td>abdomen</td>
<td>1</td>
<td>64</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>V25</td>
<td>1997</td>
<td>lung (sputum)</td>
<td>4</td>
<td>32</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>V1</td>
<td>1996</td>
<td>lung abscess</td>
<td>4</td>
<td>&gt;128</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>V4</td>
<td>1996</td>
<td>lung abscess</td>
<td>2</td>
<td>&gt;128</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>V18</td>
<td>1996</td>
<td>open fracture</td>
<td>0.5</td>
<td>&gt;128</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>V28</td>
<td>1997</td>
<td>lung (sputum)</td>
<td>0.5</td>
<td>&gt;128</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

The pellets stored at −20°C. Pellets were resuspended in 25 µL lysis buffer (0.02 M sodium phosphate buffer, pH 7 and 0.2% Triton X-100) to which 10 µL lysozyme (1 mg/mL) was added. For PBP competition studies, concentrations of piperacillin 10, 5, 2 and 1 mg/L, and ampicillin 5, 2, 1 and 0.5 mg/L were added to whole cell preparations of *Veillonella* spp. Cell preparations were incubated at 37°C for 10 min, and post-labelled with 2 µCi [3H]penicillin, no piperacillin or ampicillin added. Lanes 2–5, pre-incubation with piperacillin: lane 2, 10 mg/L; 3, 5 mg/L; 4, 2 mg/L; 5, 1 mg/L. Lanes 6–9, pre-incubation with ampicillin: lane 6, 5 mg/L; 7, 2 mg/L; 8, 1 mg/L; 9, 0.5 mg/L.

Figure 1. Piperacillin and ampicillin competition studies on *Veillonella* strains (a) V2 (ampicillin MIC 0.125 mg/L, piperacillin MIC 0.5 mg/L), (b) V31 (ampicillin MIC 1 mg/L, piperacillin MIC 64 mg/L) and (c) V18 (ampicillin MIC 0.5 mg/L, piperacillin MIC > 128 mg/L). (a) Lane 1, post-labelled with 2 µCi [3H]penicillin, no piperacillin or ampicillin added. Lanes 2–5, pre-incubation with piperacillin: lane 2, 10 mg/L; 3, 5 mg/L; 4, 2 mg/L; 5, 1 mg/L. Lanes 6–9, pre-incubation with ampicillin: lane 6, 5 mg/L; 7, 2 mg/L; 8, 1 mg/L; 9, 0.5 mg/L.

Protein molecular-weight marker (Rainbow, [14C]-labelled, Amersham) was included in each gel run.

β-Lactamase production

To screen for β-lactamase production, bacterial cells were mixed with nitrocefin (Oxoid) and observed extensively for 1 h for any change in colour. A change from red to yellow would indicate the production of β-lactamase.

Results and discussion

Selective resistance to piperacillin (MIC ≥ 64 mg/L) was found in the absence of β-lactamase activity in 21/31 (68%) *Veillonella* isolates. Alteration of PBP prompted investigations into resistance development. Results from PBP ampicillin/piperacillin competition studies performed on *Veillonella* strains with piperacillin MICs ranging from 0.5 to >128 mg/L are illustrated in Figure 1(a–c). Ampicillin was seen to bind strongly (as with penicillin) to the high-molecular-weight PBP (∼66 kDa), as only a faint band was noted in lanes 8 and 9 of Figure 1(b and c). In contrast, affinity of piperacillin for the ∼66 kDa PBP was considerably lower in piperacillin-resistant strain V18 (MIC > 128 mg/L), as reduced binding to this PBP was noted, indicated by the strong binding of [3H]benzylpenicillin in lanes 2–5 (Figure 1c). Other resistant strains that showed the same results were V1, V4 and V28 (MICs > 128 mg/L). The PBP competition profile of strain V31 (Figure 1b) with intermediate resistance to piperacillin (MIC 64 mg/L), showed a reduction in piperacillin binding to this PBP similar to that found with V18 (Figure 1c), when compared with the binding to piperacillin in susceptible strain V2 (MIC 0.5 mg/L) (Figure 1a). Two other susceptible strains, V11 and V19 (MIC 16 mg/L), showed PBP binding similar to that of V2. It was evident that the PBP (∼66 kDa) revealing the greatest affinity for penicillin and ampicillin was seen to exhibit the lowest affinity for piperacillin in piperacillin-resistant strains (Figure 1a–c).

For many bacterial genera, it is unusual for the affinities of different penicillins to be focused on one high affinity PBP, yet for piperacillin, specific mutants exhibiting high-level resistance to piperacillin appear to have evolved. In 1996, Wren found that some strains of *Veillonella* were susceptible to carboxypenicillins and ureidopenicillins, and in 1997 Mendes, Gordon & Mitchell reported...
strains of *Veillonella* from Australia that were resistant to piperacillin/tazobactam. It is also known that a target PBP can alter affinity selectively with respect to one particular β-lactam. The findings of the present study and those of other researchers demonstrate the importance of further investigations into the involvement of PBPs in piperacillin resistance development in *Veillonella* spp.

References