Group A streptococci are protected from amoxicillin-mediated killing by vesicles containing β-lactamase derived from *Haemophilus influenzae*

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**Objectives:** Group A streptococci (GAS) cause, among other infections, pharyngotonsillitis in children. The species is frequently localized with the Gram-negative respiratory pathogens non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis*, which both produce outer membrane vesicles (OMVs). The aim of this study was to investigate whether OMVs isolated from NTHi contain functional β-lactamase and whether the OMVs hydrolyse amoxicillin and thus protect GAS from killing by the antibiotic.

**Methods:** The antibiotic susceptibility of isolates was determined using the Etest. The resistance genes *bla*TEM-1 (encoding NTHi β-lactamase), *bro*-1 (encoding *M. catarrhalis* β-lactamase) and *ftsI* (encoding NTHi penicillin-binding protein 3) were searched for by PCR, followed by sequencing. OMVs were isolated by ultracentrifugation and the presence of β-lactamase was detected by western blots including specific rabbit polyclonal antibodies. The chromogenic substrate nitrocefin was used to quantify and compare the β-lactamase enzyme activity in the OMVs. The hydrolysis of amoxicillin by β-lactamase was estimated by an agar diffusion method.

**Results:** We showed that OMVs released from β-lactam-resistant *M. catarrhalis* and NTHi contain functional β-lactamase that hydrolyses amoxicillin and protects GAS from killing by amoxicillin.

**Conclusions:** This is the first report of the presence of β-lactamase in NTHi OMVs. We suggest that OMV-derived β-lactamase from coinfecting pathogens such as NTHi and *M. catarrhalis* may contribute to the occasional treatment failures seen in GAS tonsillitis.

**Keywords:** non-typeable *Haemophilus influenzae*, outer membrane vesicles, *Streptococcus pyogenes*

**Introduction**

*Streptococcus pyogenes* (also designated group A streptococci; GAS) are Gram-positive cocci that colonize the throat and skin and cause (among other infections) pharyngitis and impetigo, respectively. GAS are highly susceptible to β-lactam antibiotics, but despite this there have been reports of increased treatment failures in patients with pharyngotonsillitis. Interestingly, GAS colonize with the respiratory tract pathogens *Moraxella catarrhalis* and non-typeable *Haemophilus influenzae* (NTHi). In fact, Brook & Gober showed that in a study of 548 children with acute pharyngotonsillitis, NTHi and *M. catarrhalis* carriage was correlated with the presence of GAS. There was a significantly higher number of patients who carried NTHi and GAS (29%), or both M. catarrhalis and GAS (22%), compared with NTHi (19%) or M. catarrhalis (10%) as single pathogens. No such correlation in carriage was found between GAS and *Staphylococcus aureus* or *Streptococcus pneumoniae*.

*M. catarrhalis* and NTHi are Gram-negative species occasionally causing, e.g., acute otitis media in children and exacerbations in patients with chronic obstructive pulmonary disease (COPD). More than 90% of *M. catarrhalis* and between 2% and >50% of *H. influenzae*, depending on their geographical origin, are β-lactamase positive. NTHi and *Moraxella catarrhalis* release outer membrane vesicles (OMVs; diameter 50–250 nm), which are formed as the outer membrane bulges out and pinches off. Several studies have shown that OMVs act as vehicles for protein transfer and interact with the host immune system. We have previously demonstrated that *M. catarrhalis* OMVs contain β-lactamase, and that secreted OMVs protect NTHi and *S. pneumoniae* from amoxicillin-induced killing. In the present study we investigated whether OMVs derived from β-lactam-resistant NTHi carry β-lactamase, and whether β-lactamase-containing OMVs from NTHi or *M. catarrhalis* protect GAS from amoxicillin.

**Materials and methods**

*M. catarrhalis* Bc5 has been described previously. NTHi and GAS isolates were from our Clinical Microbiology Laboratory (Table 1). Bacteria were grown on chocolate agar plates and in brain heart infusion broth. NTHi

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was cultured in brain heart infusion broth supplemented with NAD and haemin (10 mg/L each). Isolates were analysed by PCR for the blaTEM-1 and ftsI genes, which encode \( β \)-lactamase and penicillin-binding protein (PBP)-3, respectively.\(^a\) OMVs were isolated by ultracentrifugation according to a standard protocol.\(^b\) Western blots, nitrocefin testing, determination of amoxicillin concentrations and inactivation of amoxicillin were carried out as described previously.\(^b\) A rabbit anti-TEM-1 peptide (49LNSG KILESFRPE62) polyclonal antibody (pAb) (Genscript, Piscataway, NJ, USA) and a previously described rabbit anti-β-lactamase pAb\(^b\) were used to detect NTHi and \( M. \) catarrhalis β-lactamases, respectively. Bacterial growth was measured as a change in absorbance at OD\(_{600}\) and confirmed with cfu counts.

### Results and discussion

We randomly selected 10 clinical NTHi isolates and analysed them with nitrocefin for the presence of \( β \)-lactamase, followed by determination of the amoxicillin MIC. Three of the 10 NTHi were \( β \)-lactamase positive, which corresponded with previous epidemiological studies.\(^c\) Resistant isolates were further analysed for \( \text{bla}_{\text{TEM-1}}\), the most common \( β \)-lactamase gene (Table 1). To investigate whether bacterial isolates had an additional mechanism of penicillin resistance, mutations in PBP-3 were also determined.\(^d\,e\) The substitutions Met377 → Ile and Asn526 → Lys were identified in PBP-3 of NTHi KR672,\(^d\,e\) which was also resistant to amoxicillin/clavulanate and cefaclor (Table 1). The amoxicillin MIC for KR672 (256 mg/L) was significantly higher compared with that for KR664 (8 mg/L). It has previously been reported that \( β \)-lactamase-positive amoxicillin/clavulanate-resistant isolates such as KR672, which have both chromosomal (ftsI gene mutation) and enzymatic resistance, also have higher amoxicillin MICs.\(^d\,e\) The two amoxicillin-resistant isolates, NTHi KR672 and KR664, with a high and a low MIC, respectively, were selected for further experiments.

To confirm that OMVs derived from the amoxicillin-resistant NTHi isolates contained \( β \)-lactamase, western blots were performed (Figure 1a) using a specific rabbit anti-\( β \)-lactamase pAb. For comparison, OMVs from the \( β \)-lactamase-positive \( M. \) catarrhalis KR526 (amoxicillin MIC 1.0 mg/L)\(^c\) were also analysed. The amoxicillin-susceptible strains NTHi KR665 and \( M. \) catarrhalis Bc5 were included as representative negative controls. These experiments confirmed that OMVs from the resistant bacteria contained \( β \)-lactamase, whereas those from the amoxicillin-susceptible strains did not. To further compare the \( β \)-lactamase activity between \( M. \) catarrhalis and NTHi, we performed a nitrocefin test.\(^\text{11,12}\) As can be seen in Figure 1(b), no significant difference was found regarding enzymatic activity between the OMVs from \( β \)-lactamase-positive NTHi or \( M. \) catarrhalis isolates, despite the observed differences in MICs (Table 1).

To investigate whether the \( β \)-lactamases in OMVs hydrolyse amoxicillin, an agar diffusion assay was performed with antibiotic concentration as a function of the zone of growth inhibition of the amoxicillin-susceptible species \( \text{Micrococcus luteus} \) (previously known as \( \text{Sarcina lutea} \)).\(^\text{13}\) The \( β \)-lactamases residing in OMVs derived from NTHi KR664, NTHi KR672 and \( M. \) catarrhalis KR526 were active and hydrolysed amoxicillin when present at concentrations up to 10 mg/L (Figure 1c). By contrast, OMVs from the \( β \)-lactamase-negative isolate NTHi KR665 did not hydrolyse amoxicillin (Figure 1c; open squares). After characterization of the \( β \)-lactamase-containing OMVs (Figure 1a–c), we analysed whether OMVs derived from \( β \)-lactamase-positive NTHi and \( M. \) catarrhalis protected GAS from amoxicillin-induced killing. OMVs were pre-incubated with amoxicillin and bacterial growth was measured as a function of absorbance at OD\(_{600}\) (Figure 1d). Interestingly, OMVs (25 mg/L) from NTHi KR664 and KR672 fully protected GAS at all amoxicillin concentrations tested (2 – 128 mg/L). By contrast, \( M. \) catarrhalis KR526 OMVs

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**Table 1.** Characteristics of clinical isolates and reference strains used in this study

<table>
<thead>
<tr>
<th>Clinical isolate/strain</th>
<th>Site of isolation</th>
<th>Amoxicillin susceptibility</th>
<th>β-Lactamase genotype(^a)</th>
<th>PBP-3(^b)</th>
<th>Amoxicillin MIC(^c) (mg/L)</th>
<th>Amoxicillin/clavulanate MIC(^d) (mg/L)</th>
<th>Cefaclor inhibition zone(^e) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTHi</td>
<td></td>
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<td>KR664</td>
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<td>resistant</td>
<td>( \text{bla}_{\text{TEM-1}}f )</td>
<td>wild-type</td>
<td>8.0</td>
<td>0.75</td>
<td>23</td>
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<tr>
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<td>nasal cavity</td>
<td>resistant</td>
<td>( \text{bla}_{\text{TEM-1}}f )</td>
<td>wild-type</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KR668</td>
<td>tonsil</td>
<td>resistant</td>
<td>( \text{bla}_{\text{TEM-1}}f )</td>
<td>wild-type</td>
<td>6.0</td>
<td>0.75</td>
<td>23</td>
</tr>
<tr>
<td>KR672</td>
<td>tympanic cavity</td>
<td>resistant</td>
<td>( \text{bla}_{\text{TEM-1}}f )</td>
<td>M377I, N526K(^g)</td>
<td>256</td>
<td>4.0</td>
<td>12</td>
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<td>( M. ) catarrhalis</td>
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<td></td>
</tr>
<tr>
<td>KR526</td>
<td>nasopharynx</td>
<td>resistant</td>
<td>( \text{bro-1}h )</td>
<td>1.0</td>
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<td></td>
<td></td>
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<tr>
<td>Bc5</td>
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<td></td>
<td>0.032</td>
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<td></td>
<td></td>
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<tr>
<td>( S. ) pyogenes</td>
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<tr>
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<td>&lt;0.016</td>
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</table>

\(^a\)The \( β \)-lactamase genotype was determined by sequencing after the amplification of DNA by PCR.  
\(^b\)PBP-3 is encoded by the \( ftsI \) gene.  
\(^c\)MICs were determined using the Etest. Amoxicillin resistance was defined as an MIC \( ≥1 \) mg/L for NTHi and \( >0.125 \) mg/L for \( M. \) catarrhalis.  
\(^d\)For NTHi, amoxicillin/clavulanate resistance was defined as an MIC \( >2 \) mg/L.  
\(^e\)Cefaclor resistance was defined as an inhibition zone \(<23 \) mm in a disc diffusion assay (39 \( \mu \)g of cefaclor).  
\(^f\)\( \text{bla}_{\text{TEM-1}} \) is the most common genotype that encodes NTHi \( β \)-lactamase.  
\(^g\)Two mutations were found in PBP-3 of NTHi KR672.  
\(^h\)\( \text{bro-1} \) encodes the most common \( M. \) catarrhalis \( β \)-lactamase.
Vesicles contain β-lactamase and protect GAS

**Figure 1.** OMVs from β-lactamase-positive NTHi and *M. catarrhalis* contain enzymatically active β-lactamases that rescue GAS from amoxicillin-induced killing. (a) The NTHi β-lactamase (arrow) was detected in OMVs and bacterial cell lysates (15 μg and 30 μg, respectively) of strains KR664 and KR672 after analysis by SDS-PAGE (left) and western blot (right). The corresponding *M. catarrhalis* β-lactamase was present in KR526 OMVs and whole bacterial cell lysate (arrow). The β-lactamase-negative bacteria NTHi KR665 and *M. catarrhalis* Bc5 were used as negative controls. Two different anti-β-lactamase pAbs were used for the detection of NTHi and *M. catarrhalis* β-lactamases. (b) β-Lactamase activities of NTHi and *M. catarrhalis* OMVs were quantified by the capacity of the enzymes to hydrolyse the chromogenic substrate nitrocefin. (c) OMVs derived from β-lactamase-positive NTHi KR664 and NTHi KR672 were compared with *M. catarrhalis* KR526. OMVs from the β-lactamase-negative and -positive strains NTHi KR665 and KR664, respectively, were compared with a negative control (amoxicillin only). Amoxicillin concentrations were determined by measuring the growth inhibitory zones of the antibiotic-susceptible bacterium *M. luteus*. (d) GAS survived after pre-incubation of amoxicillin with OMVs from NTHi KR664, NTHi KR672 or *M. catarrhalis* KR526, but not from the β-lactamase-negative strain NTHi KR665. Growth was expressed as relative growth compared with starting cultures. The data presented are mean values from three separate experiments and error bars represent the SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. AMX, amoxicillin.

rescued GAS only at amoxicillin concentrations ≤32 mg/L. GAS were fully susceptible to amoxicillin in the presence of OMVs from β-lactamase-negative NTHi KR665. These results suggest that OMVs from NTHI, as compared with *M. catarrhalis* OMVs, were slightly better in protecting GAS from amoxicillin-mediated killing. Vesicles from both NTHI and *M. catarrhalis* hydrolysed β-lactamase at experimental amoxicillin concentrations up to 32 and 128 mg/L, respectively, which considerably exceed peak plasma concentrations (8–10 mg/L). Various virulence factors can be enriched in OMVs, and the potency of β-lactamase-containing OMVs at inactivating amoxicillin may reflect this fact.

This is to our knowledge the first report showing that OMVs derived from NTHI contain active β-lactamase. OMVs have unique functions because these vehicles can transport proteins over long distances, which is potentially beneficial for bacteria in host–cell interactions. In the present study, we showed that OMV-derived β-lactamase protects GAS from amoxicillin-induced killing even at high amoxicillin concentrations. In light of recent reports of treatment failures of GAS pharyngotonsillitis,1–3 and the fact that NTHI and Moraxella catarrhalis are associated with GAS in the upper respiratory tract of children with pharyngotonsillitis,5 our results suggest that OMVs containing β-lactamase may play a role in the persistence of certain clinical conditions. A few years ago Casey and Pichichero18 conducted a meta-analysis of nine randomized controlled trials comparing cephalosporins with penicillin in the treatment of patients with GAS-associated
pharyngotonsillitis. Interestingly, the meta-analysis revealed that the failure rate for the treatment of GAS tonsillitis with penicillin was twice that for treatment with cephalosporins. Since β-lactamase-positive *H. influenzae* and *M. catarrhalis* are susceptible to the majority of third-generation and various second-generation cephalosporins, the hypothesis that NTHi and *M. catarrhalis* protect GAS from β-lactam antibiotics in coinfections is strengthened. However, whether OMVs carrying β-lactamase play a role in our infected patients remains to be proven.

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**Transparency declarations**

None to declare.

**References**