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Group A Streptococci Are Protected from Amoxicillin-Mediated Killing by Vesicles Containing β-lactamase Derived from *Haemophilus influenzae*

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RUNNING TITLE: VESICLES CONTAIN β-LACTAMASE AND PROTECT GAS

Key words: betalactamase, group A streptococci, non-typeable *Haemophilus influenzae,* outer membrane vesicles, *Streptococcus pyogenes*

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

Methods: Antibiotic susceptibility of isolates was determined with Etests. The resistance genes $bla_{\text{TEM-1}}$ (encoding for NTHi β-lactamase), *bro-1* (*M. catarrhalis* β-lactamase), and *ftsI* (NTHi penicillin binding protein 3) were searched for by PCR followed by sequencing. OMV were isolated by ultracentrifugation, and western blots including specific rabbit polyclonal antibodies were used to detect β-lactamase in OMV. The chromogenic substrate nitrocefin was used for quantification and comparison of β-lactamase enzyme activity in OMV. Hydrolysis of amoxicillin by β-lactamase was estimated by an agar diffusion method.

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

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

Introduction

Streptococcus pyogenes (also designated group A streptococci; GAS) are Gram-positive cocci that colonize the throat and skin, and cause amongst other infections pharyngitis and impetigo, respectively.[1] GAS are highly susceptible to β-lactam antibiotics, but despite this there have been reports of increased treatment failures in patients with pharyngotonsillitis.[2-4] Interestingly, GAS colonize with the respiratory tract pathogens *Moraxella catarrhalis* and non-typeable *Haemophilus influenzae* (NTHi). In fact, Brook *et al.* showed that in a study of 548 children with acute pharyngotonsillitis, NTHi and *M. catarrhalis* carriage was correlated to the presence of GAS. There was a significantly higher number of patients that carried NTHi and GAS (29 %), or both *M. catarrhalis* and GAS (22 %), compared to 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, for example, acute otitis media in children and exacerbations in patients with chronic obstructive pulmonary disease (COPD). More than 90 % of *M. catarrhalis* and 2 to >50 % of *H. influenzae*, depending on their geographical origin, are β-lactamase positive.[5, 6]

NTHi and *Moraxella* release outer membrane vesicles (OMV; diameter 50-250 nm), which are formed as the outer membrane bulges out and pinches off. Several studies have shown that OMV act as vehicles for protein transfer and interact with the host immune system.[7-9] We have previously demonstrated that *M. catarrhalis* OMV contain β-lactamase, and that secreted OMV protect NTHi and *S. pneumoniae* from amoxicillin-induced killing.[10] In the present study we investigated whether OMV derived from β-lactam resistant NTHi carry β-lactamase, and if β-lactamase-containing OMV from NTHi or *M. catarrhalis* protect GAS from amoxicillin.

Material and methods

M. catarrhalis Bc5 has been described earlier[10], and NTHi and GAS isolates were from our Clinical Microbiology Laboratory (Table 1). Bacteria were grown on chocolate agar plates and in brain heart infusion (BHI) broth. NTHi was cultured in BHI supplemented with NAD and hemin (10 mg/L each). Isolates were analysed by PCR for $bla_{\text{TEM-1}}$ and *ftsI* genes encoding for β-lactamase and penicillin-binding protein (PBP)-3, respectively.[6, 11] OMV were isolated by ultracentrifugation according to a standard protocol.[12] Western blots, nitrocefin testing, determination of amoxicillin concentrations, and inactivation of amoxicillin were as described.[10] A rabbit anti-TEM-1 peptide (49LNSGKILESFRPE62) polyclonal antibody (pAb) (Genscript, Piscataway, NJ) and a previously described rabbit anti-β-lactamase pAb[10] were used to detect NTHi and *M. catarrhalis* β-lactamases, respectively. Bacterial growth was measured as a change in absorbance at OD_{600}.

Results and Discussion

We randomly selected ten clinical NTHi isolates and analyzed them for the presence of β-lactamase with nitrocefin followed by determination of amoxicillin MIC. Three out of ten NTHi were β-lactamase positive, which corresponded to previous epidemiological studies.[6] Resistant isolates were further analyzed for $bla_{\text{TEM-1}}$, 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.[11, 13] The substitutions Met377→Ile and Asn526→Lys were identified in PBP-3 of NTHi KR672 [6, 14], which also was resistant to amoxicillin/clavulanate and cefaclor (Table 1). The amoxicillin MIC for KR672 (256 mg/L) was significantly higher compared to KR664 (8 mg/L). It has previously been reported that BLPACR (β-lactamase positive amoxicillin/clavulanate resistant) isolates such as KR672, which have both a chromosomal (*ftsI* gene mutation) and enzymatic resistance, also have higher amoxicillin MIC.[11, 15] The two amoxicillin-resistant isolates, NTHi KR672 and KR664, with a high and low MIC, respectively, were selected for further experiments.

To confirm that OMV 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, OMV from the β-lactamase positive *M. catarrhalis* KR526 (amoxicillin MIC 1.0 mg/L)[10] was also analysed. The amoxicillin-susceptible strains NTHi KR665 and *M. catarrhalis* Bc5 were included as representative negative controls. These experiments confirmed that OMV from the resistant bacteria contained β-lactamase, whereas no enzyme was detected with the amoxicillin-susceptible strains. To further compare the β-lactamase activity between *Moraxella* and NTHi, we did a nitrocefin test.[10] As can be seen in Figure 1b, no significant difference was found regarding enzymatic activity between the OMV from β-lactamase positive NTHi or *M. catarrhalis* isolates albeit the observed differences in MIC (Table 1).

To investigate whether the β-lactamase in OMV hydrolyze amoxicillin, an agar diffusion assay was done with antibiotic concentrations as a function of the zones of growth inhibition by the amoxicillin-susceptible species *Micrococcus luteus* (previously known as *Sarcina lutea*)[10]. The β-lactamases residing in OMV were active and hydrolyzed amoxicillin up to 10 mg/L with OMV derived from NTHi KR664, NTHi KR672 and *M. catarrhalis* KR526 (Figure 1c). In contrast, OMV from the β-lactamase negative isolate NTHi KR665 did not hydrolyze amoxicillin (Figure 1c; Control).

After characterization of the β-lactamase-containing OMV (Figures 1a-c), we proceeded and analyzed whether OMV derived from β-lactamase-positive NTHi and *M. catarrhalis* protected GAS from amoxicillin-induced killing. OMV were pre-incubated with amoxicillin and bacterial growth was measured as a function of absorbance at OD_{600} (Figure 1d). Interestingly, OMV (25 mg/L) from NTHi KR664 and KR672 fully protected GAS at all amoxicillin concentrations tested (2-128 mg/L). In contrast, *M. catarrhalis* KR526 OMV rescued GAS up to 32 mg/L amoxicillin only. GAS was fully susceptible to amoxicillin in the presence of OMV from β-lactamase-negative NTHi KR665. These results suggested that OMV from NTHi as compared to *M. catarrhalis* OMV 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, that considerably exceeded peak plasma concentrations (8-10 mg/L). Various virulence factors can be enriched in OMV[16], and the potency of β-lactamase-containing OMV to inactivate amoxicillin may reflect this fact.

This is to our knowledge the first report delineating that OMV derived from NTHi contain active β-lactamase. OMV have unique functions since these vehicles transport proteins that may result in a long distance delivery that is potentially beneficial for bacteria in host/cell interactions. In the present study, we show that OMV-derived β-lactamases protect GAS from amoxicillin-induced killing also at high amoxicillin concentrations. In light of recent reports of treatment failures of GAS pharyngotonsillitis[2-4], and the fact that NTHi and *Moraxella* are associated with GAS in the upper respiratory tract of children with pharyngotonsillitis[17], our results suggest that OMV containing β-lactamase may play a role in the persistence of certain clinical conditions. A few years ago Casey and Pichichero conducted a meta-analysis of nine randomised controlled trials comparing cephalosporins to penicillin in the treatment of patients with GAS-associated pharyngotonsillitis.[18] Interestingly, the meta-analysis revealed that the failure rate for treatment of GAS tonsillitis with penicillin was twice as high compared to when cephalosporin was administered. Since β-lactamase-positive *H. influenzae* and *M. catarrhalis* are susceptible to the majority of third-generation and various second-generation cephalosporins[19], the hypothesis that NTHi and *M. catarrhalis* protect GAS from β-lactam antibiotics in co-infections is strengthened. However, whether OMV carrying β-lactamases play a role in our infected patients remains to be proven.

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Transparency declarations

None to declare.

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TABLE 1. Characteristics of clinical isolates and reference strains used in this study. The presence of genes encoding for β-lactamase in addition to PBP-3 mutations are indicated.

Clinical isolate/ strain	Site of isolation	Amoxicillin susceptibility	β-lactamase genotype[a]	PBP-3[b]	Amoxicillin MIC[c] (mg/L)	Amox/clav MIC[d] (mg/L)	Cefaclor inhibition zone[e] (mm)
NTHi							
KR664	Nasopharynx	Resistant	$bla_{\text{TEM-1}}$[f]	Wild type	8.0	0.75	23
KR665	Nasal cavity	Susceptible			0.50		
KR672	Tympanic cavity	Resistant	$bla_{\text{TEM-1}}$	M377I[g] N526K	256	4.0	12
M. catarrhalis							
KR526	Nasopharynx	Resistant	*bro-1*[h]		1.0		
Bc5	Nasopharynx	Susceptible			0.032		
S. pyogenes							
KR696	Nasopharynx	Susceptible			<0.016		

[a] The β-lactamase genotype was determined by sequencing after amplification of DNA by PCR.

[b] Penicillin binding protein (PBP)-3 is encoded by the *ftsI* gene.

[c] Minimal inhibitory concentrations (MIC) were determined by Etests. Amoxicillin resistance was defined as MIC ≥ 1 mg/L for NTHi and MIC > 0.125 mg/L for *M. catarrhalis*.

[d] For NTHi, amoxicillin/clavulanate (amox/clav) resistance was defined as MIC > 2 mg/L.

[e] Cefaclor resistance was defined as an inhibition zone < 23 mm in a disk diffusion assay (39 μg cefaclor).

[f] $bla_{\text{TEM-1}}$ is the most common genotype that encodes for NTHi β-lactamase.

[g] Two mutations were found in PBP-3 of NTHi KR672.

[h] *bro-1* encodes for the most common *M. catarrhalis* β-lactamase.

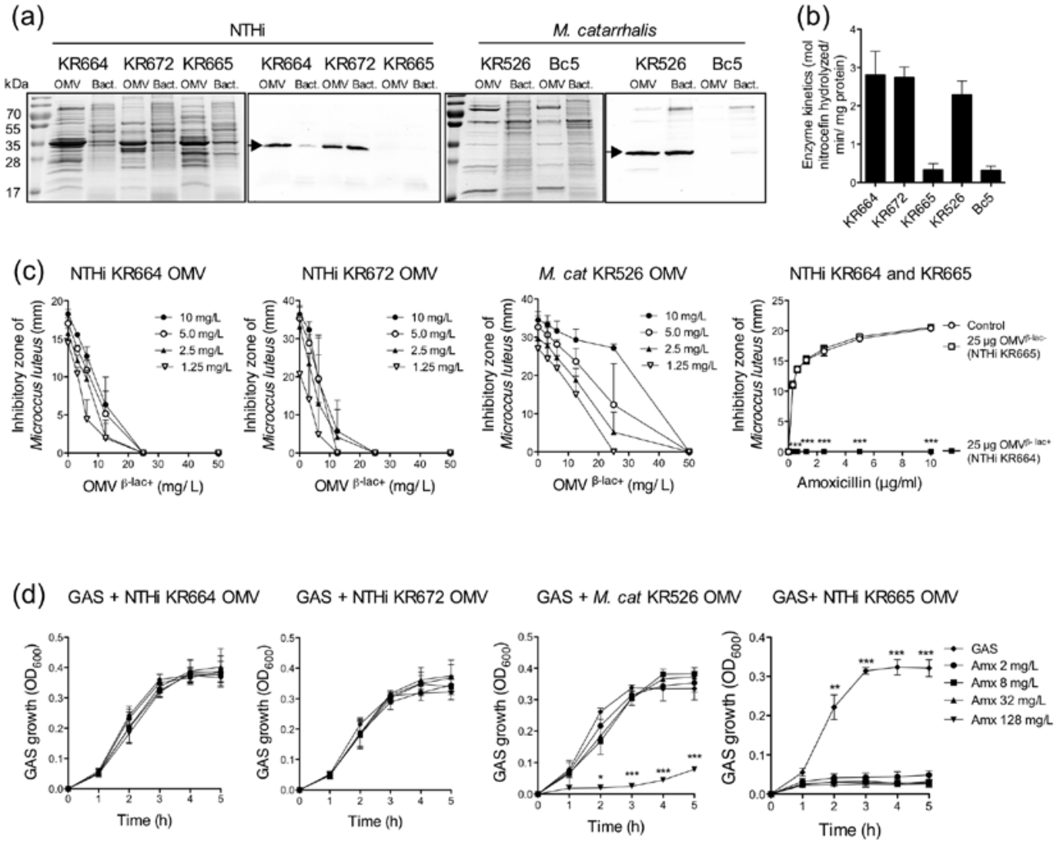
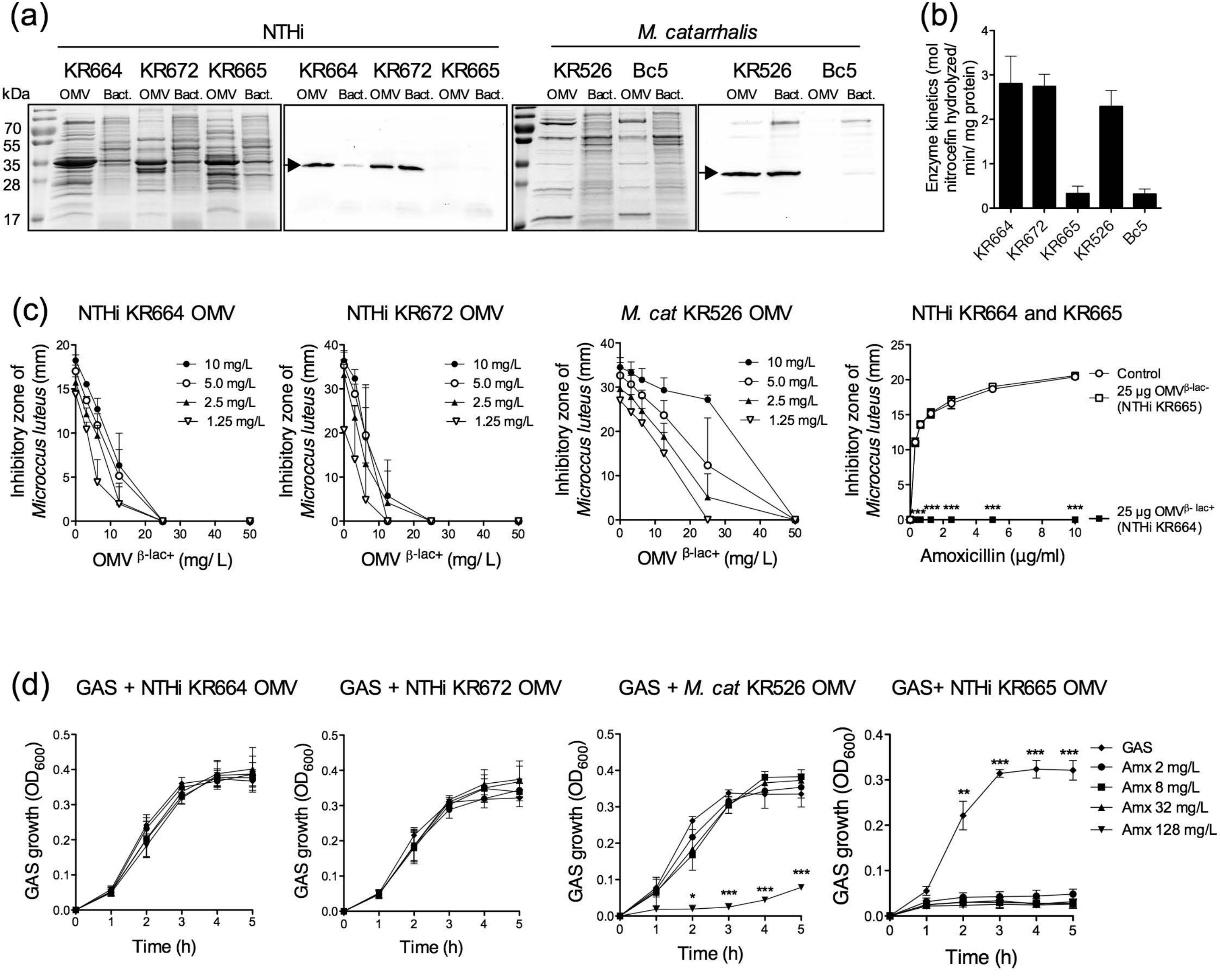


Figure 1. Outer membrane vesicles (OMV) from β-lactamase-positive non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* contain enzymatically active β-lactamase that rescue group A streptococci (GAS) from amoxicillin-induced killing. (a) The NTHi β-lactamase (arrow) was detected in OMV and bacterial cell lysate (15 μg and 30 μg, respectively) of strains KR664 and KR672 after analysis on SDS-PAGE (left) and Western blot (right). The corresponding *M. catarrhalis* β-lactamase was present in KR526 OMV 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 pAb were used for detection of NTHi and *M. catarrhalis* β-lactamases. In (b), the β-lactamase activities of NTHi and *M. catarrhalis* OMV were quantified by the capacity of the enzymes to hydrolyze the chromogenic substrate nitrocefin. (c) OMV derived from β-lactamase positive NTHi KR664 and NTHi KR672 were compared to *M. catarrhalis* KR526. OMV from the β-

lactamase negative and positive strains NTHi KR665 and KR664, respectively, were compared to a negative control (amoxicillin only). Amoxicillin concentrations were determined by measuring the inhibitory growth zones of the antibiotic-susceptible bacterium *Micrococcus luteus*. (d) GAS survived after preincubation of amoxicillin with OMV 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 to starting concentrations measured as a change in absorbance (OD_{600}). Amoxicillin was pre-incubated with OMV (25 μg) for 1 h before addition to bacteria. The data presented are mean values from three separate experiments and error bars represent SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



(a)
NTHi
M. catarrhalis
KR664 KR672 KR665 KR664 KR672 KR665
KR526 Bc5 KR526 Bc5
kDa
OMV Bact. OMV Bact. OMV Bact.
70
55
35
28
17
(b)
Enzyme kinetics (mol nitrocefin hydrolyzed/ min/ mg protein)
KR664 KR672 KR665 KR526 Bc5
(c)
NTHi KR664 OMV
NTHi KR672 OMV
M. cat KR526 OMV
NTHi KR664 and KR665
Inhibitory zone of Micrococcus luteus (mm)
10 mg/L
5.0 mg/L
2.5 mg/L
1.25 mg/L
OMV β-lac+ (mg/ L)
Control
25 µg OMVβ-lac- (NTHi KR665)
25 µg OMVβ- lac+ (NTHi KR664)
Amoxicillin (µg/ml)
***
(d)
GAS + NTHi KR664 OMV
GAS + NTHi KR672 OMV
GAS + M. cat KR526 OMV
GAS+ NTHi KR665 OMV
GAS growth (OD600)
Time (h)
GAS
Amx 2 mg/L
Amx 8 mg/L
Amx 32 mg/L
Amx 128 mg/L
*
**
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