Outer membrane vesicles shield *Moraxella catarrhalis* β-lactamase from neutralization by serum IgG

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Objectives: The aim of this study was to detect the presence of IgG against *Moraxella catarrhalis* β-lactamase in healthy adults, and to determine whether outer membrane vesicles (OMVs) could protect the enzyme from inhibition by anti-β-lactamase IgG.

Methods: Transmission electron microscopy was used to detect the presence of β-lactamase in OMVs. Sera were examined by ELISA for specific IgG directed against recombinant *M. catarrhalis* β-lactamase in addition to the outer membrane adhesins MID/Hag, UspA1 and A2. Binding of anti-β-lactamase IgG from serum to OMVs was analysed by flow cytometry. The chromogenic substrate nitrocefin was used to quantify β-lactamase enzyme activity.

Results: The presence of β-lactamase was determined in OMVs from a 9-year-old child suffering from *M. catarrhalis* sinusitis. Furthermore, anti-β-lactamase IgG was detected in sera obtained from healthy adults. Out of 40 adult blood donors (aged 18–65 years) tested, 6 (15.0%) carried anti-β-lactamase IgG. No correlation between IgG titres against β-lactamase and the adhesins was found. Flow cytometry analyses revealed that anti-β-lactamase IgG from serum bound to β-lactamase-positive OMVs. By comparing the β-lactamase activity of intact OMV with OMV that were permeabilized with saponin we found that OMVs shielded active β-lactamase from the anti-β-lactamase IgG.

Conclusions: *Moraxella catarrhalis* β-lactamase is found in, or associated with, OMVs, providing clinical relevance for the vesicles in the spread of antibiotic resistance. Furthermore, OMVs protect β-lactamase from specific IgG.

Keywords: amoxicillin, antibiotic resistance, sinusitis

Introduction

*Moraxella catarrhalis* is a Gram-negative human pathogen that causes respiratory tract infections. The species is found as a commensal in pre-school children and causes ~15%–20% of acute otitis media (AOM) cases. *M. catarrhalis* is also the third most common cause of exacerbations in patients with chronic obstructive pulmonary disease (COPD) after *Streptococcus pneumoniae* and *Haemophilus influenzae*.¹ *M. catarrhalis* adheres to and infects pulmonary epithelial cells, and has been found to hide in the palatine tonsils.²,³ Since the 1980s >97% of *M. catarrhalis* strains have been β-lactamase positive.⁴

In parallel with most other Gram-negative species, *M. catarrhalis* releases outer membrane vesicles (OMVs), which are small spheres released from the outer membrane as the membrane bulges out and pinches off. The OMVs thus reflect the composition of the outer membrane, carrying mainly lipids and outer membrane proteins.⁵–⁸ As the release of OMVs is an energy-demanding process, it has been suggested that this is an essential virulence mechanism for Gram-negative bacteria. Evidence from various studies supports this notion, as OMVs not only have a role in pathogenesis, but also in biofilm formation, nutrient acquisition and horizontal gene transfer.⁷,⁹–¹¹ OMVs interact both with host cells and with other bacteria residing in a mutual niche. Moreover, OMVs act as vehicles for secretion whereby vesicle components are protected from destruction and delivered to target cells at a distance.⁵,⁸,¹²

We recently showed that OMVs from β-lactamase-positive *M. catarrhalis* also contain the enzyme and consequently can absorb and hydrolyse amoxicillin.¹³ β-Lactamase is known as a
periplasmic enzyme, and therefore its storage in OMVs and subsequent secretion would potentially be an important virulence mechanism. Since *M. catarrhalis* is often found with other bacterial species, we hypothesized that conferring antibiotic resistance on susceptible bacteria might make co-infection a highly advantageous mechanism also for other species. Intriguingly, we found that *β-lactamase*-positive *M. catarrhalis* OMVs confer resistance on amoxicillin-susceptible *H. influenzae* and *S. pneumoniae* in addition to the now rare *β-lactamase*-negative *M. catarrhalis*.13

The antibody levels against major *Moraxella* outer membrane proteins, such as *Moraxella* IgG-binding protein (MID)/haemagglutinin (Hag) and ubiquitous surface proteins (Usp) A1 and UspA2, have previously been studied in both adults and children.16–18 IgG levels are generally lower in children compared with adults, leading to a higher incidence of infections in children. However, the human antibody response to *M. catarrhalis* *β-lactamase* is currently unknown. In this study, we determined IgG levels against *Moraxella* *β-lactamase* in healthy adults using a recombinant protein. A group of individuals that had significant anti-*β-lactamase* IgG titres was identified, and this polyclonal antibody (pAb) recognized *β-lactamase*-positive OMVs. Interestingly, we discovered that the hydrolysing *β-lactamase* activity was partially protected within the OMV. This suggests that *Moraxella* OMVs not only play a role in polymicrobial infections but also act as protective reservoirs for *β-lactamase*,13 avoiding neutralization by the host adaptive immune system.

**Materials and methods**

**Bacterial strains and growth conditions**

*M. catarrhalis* clinical and reference strains KRS26 and BcS, respectively, were cultured on chocolate agar plates. Bacteria were grown at 37°C in 5% CO2. To determine amoxicillin MICs, both Etests (Biodisk, Solna, Sweden) and colony counting after growth in liquid media with varying antibiotic concentrations were used.

**Production of recombinant *β-lactamase*, UspA1, A2 and MID**

The manufacture of full-length recombinant *β-lactamase*26–318 from *M. catarrhalis* strain RH4 was done as described previously.13 Briefly, the *β-lactamase* bro gene was cloned into the vector pET26b(+) and, after selection in *Escherichia coli* DH5α, the protein was produced in *E. coli* BL21(DE3) by induction with IPTG. Bacteria were sonicated and proteins were purified using affinity chromatography. Recombinant full-length UspA150–770 and UspA230–539 in addition to the truncated protein MID62–1200, which was selected due to a size approximately similar to that of the *β-lactamase*26–318, were all from *M. catarrhalis* BcS. Recombinant proteins were produced as previously described.17

**Isolation of *M. catarrhalis* OMVs**

OMVs were isolated according to the method described by Rosen et al.19 Briefly, bacteria were grown in brain heart infusion broth overnight at 37°C with shaking, and after centrifugation the resulting supernatant was filtered through 0.2 μm pore filters (Sartorius, Goettingen, Germany) to obtain a cell-free solution. The flow-through was filtered with 100 kDa Vivaspin centrifugal concentrators (Vivasience, Hannover, Germany). The remaining concentrate was further concentrated by ultracentrifugation at 100,000 g and washed with PBS followed by centrifugation. The protein concentration was measured using NanoDrop (NanoDrop Technologies, Wilmington, DE), and plated on chocolate agar plates in order to confirm that the preparations were free from bacteria.

**Transmission electron microscopy (TEM)**

A fresh nasal discharge from a 9-year-old child with *M. catarrhalis* sinusitis (pure growth of *M. catarrhalis* from a nasal aspirate) was examined. The sample was prepared by suspending a drop of the purulent nasal discharge in 1 mL of PBS with 4% paraformaldehyde. The cellular fraction was obtained by centrifuging the specimen at 14,000 rpm. Following fixation of samples, ultrathin sections of specimens were mounted on gold grids and subjected to antigen retrieval with metaperiodate. The grids were floated on drops of immune reagents displayed on Parafilm, and 50 mM glycine was used to block free aldehyde groups. Grids were subsequently blocked with 5% (vol/vol) goat serum diluted in incubation buffer (0.2% BSA–C19 in PBS, pH 7.6) (Aurion, Wageningen, Netherlands) for 15 min. OMVs were incubated with primary antibodies (dilution 1:50 and 1:100) overnight at 4°C. The grids were washed in incubation buffer and floated on drops of gold conjugate reagents of sizes 10 and 5 nm, diluted 1:10 to 1:20 in incubation buffer, for 1 h at room temperature. After washes in incubation buffer, the sections were fixed in 2% glutaraldehyde. The sections were then washed in distilled water and post-stained with uranyl acetate and lead citrate. Sections were examined with an electron microscope (JEM 1230; Jeol, Tokyo, Japan) operated at a 60 kV accelerating voltage. The images were recorded with a Gatan Multiscan 791 charge-coupled device camera (Gatan, Pleasanton, CA).

**ELISA**

To analyse antibody concentrations in serum, ELISA was done as described previously.17 Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 1 μg recombinant protein (UspA1/A2, MID or *β-lactamase*) per well in Tris–HCl buffer (pH 9.0) overnight at 4°C. After washing and blocking steps, human sera from healthy blood donors (n=40; aged 18–65 years) were added in duplicate for 1 h at room temperature. Horseradish peroxidase (HRP)-labelled anti-human IgG polyclonal antibody (pAb) (1:6000) (Dako, Glostrup, Denmark) was added as a secondary layer to plates for 20 min, and after subsequent washing steps the plates were developed and measured at OD405. Each sample was tested in duplicate.

**Purification of human anti-*β-lactamase* IgG**

Human sera were purified against a recombinant *β-lactamase* from *M. catarrhalis* strain RH4 on a CNBr-Sepharose coupled column (VWR International, Leicestershire, UK) as previously described.13 Sera were diluted 1:5 in PBS. The *β-lactamase* binding fraction was eluted with 0.1 M glycine (pH 2.4) and immediately mixed with 3 M Tris–HCl (pH 8.8) and 5 M NaCl. The flow-through after purification was used as a negative control serum devoid of specific anti-*β-lactamase* IgG. Both fractions were absorbed against *β-lactamase*-negative *M. catarrhalis* strain BcS for 1 h at room temperature in order to remove non-specific antibodies.

**Flow cytometry analysis**

To analyse the recognition by IgG of *β-lactamase*-positive OMVs from *M. catarrhalis*, OMVs (2 μg) were fixed with 3.5% formaldehyde for 15 min at room temperature. After a subsequent wash (100,000 g for 30 min), the OMVs were incubated with purified anti-*β-lactamase* IgG. After another washing step, FITC-labelled rabbit anti-human pAb...
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(Dako) was added as a secondary step in PBS/BSA (1%) for 20 min at room temperature. Samples were analysed in an EPICS XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL) and a gate was set to exclude signals <2.0%.

**SDS-PAGE and western blotting**

To check the binding of purified anti-β-lactamase IgG to the recombinant RH4 β-lactamase, SDS-PAGE (12%) and western blotting were performed. Proteins were transferred from gels to Immobilon-P membranes (Millipore, Bedford, MA) at 20 V overnight, and following transfer the membranes were blocked with 5% milk in PBS containing 0.1% Tween (PBS-Tween). After subsequent washing with PBS-Tween, the membranes were incubated with anti-β-lactamase pAb purified from normal human or rabbit serum for 1 h at room temperature as described previously. After several washing steps, membranes were incubated for 1 h with HRP-conjugated secondary rabbit anti-human or swine anti-rabbit pAb (Dako) respectively, which were diluted 1:1000. Membranes were washed and developed using enhanced chemiluminescence western blot detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

**Generation of the inhibitory effect of serum anti-β-lactamase antibodies blocking β-lactamase activity in M. catarrhalis OMV**

The enzyme activity in OMVs was quantified with a nitrocefin assay as previously described. The chromogenic cephalosporin nitrocefin (Oxoid, Thermo Scientific, Cambridge, UK) was used. Briefly, OMVs (0.3 μg/mL) were pre-incubated with saponin (0.2%) for 5 min at room temperature to lyse them, and incubated with purified anti-β-lactamase pAb isolated from normal human serum or the flow-through control serum (dilution 1:10) for 1 h at room temperature. OMVs were incubated with nitrocefin (500 μg/mL) for 30 min at 37 °C in the dark, followed by centrifugation at 13000 g for 3 min. After chromogenic hydrolysis the subsequent colour change was determined using NanoDrop at OD485 nm. The enzyme activity of the OMV preparations was estimated using a standardized curve from a recombinant β-lactamase (VWR International, Leicester, UK). The activity was quantified as the number of moles of nitrocefin hydrolysed per minute per milligram of protein.

**Statistical analysis**

The statistical analyses were performed with GraphPad Prism 5 software (San Diego, CA). The unpaired Student’s t-test was used to determine the statistical differences between control and treated samples. All data are expressed as the mean±SEM, and n represents the number of experiments performed.

**Results**

**Outer membrane vesicles from M. catarrhalis carry β-lactamase in vivo**

We have recently shown that Moraxella releases OMVs that are loaded with β-lactamase. To determine whether β-lactamase is also associated with OMVs in vivo, a specimen obtained via a nasopharyngeal swab from a 9-year-old child with Moraxella sinusitis was analysed by TEM. As can be seen in Figure 1(a), M. catarrhalis readily released OMVs in the nasopharynx.

To manufacture specific detection antibodies for M. catarrhalis β-lactamase, we produced a full-length recombinant β-lactamase, in E. coli followed by immunization of rabbits. The resulting anti-β-lactamase pAb was conjugated with gold granules and used with our clinical samples for detection in TEM (Figure 1a). The presence of β-lactamase was clearly seen in OMVs or in the close vicinity as seen in several sections (Figure 1b–e).

**Sera obtained from healthy adults contain IgG directed against M. catarrhalis β-lactamase**

In order to determine whether adults carry IgG against β-lactamase, sera were collected from 40 healthy individuals (aged 18–65 years). To quantify the IgG concentration, recombinant β-lactamase was immobilized on microtitre plates followed by ELISA (Figure 2a). In addition, the well-defined adhesins MID26–1200, UspA150–770 and UspA230–539 were included for comparison. Both UspA150–770 and UspA230–539 are full-length recombinant proteins, whereas MID26–1200 is a 238 amino acid truncated fragment of the native MID protein (mol. wt. ~200 KDa). The IgG antibody titres against β-lactamase showed large variation between individuals, and titres were found to be significantly lower than the average anti-UspA1/A2 IgG titres, but higher than the mean anti-MID26–1200 IgG titres. The high antibody titres for UspA1 and A2 were in agreement with results previously obtained in our laboratory.

To confirm the specificity of anti-β-lactamase IgG, binding of antibodies against our recombinant β-lactamase was analysed by ELISA. An example can be seen in Figure 2(b), where we show a serum that bound to β-lactamase in a dose-dependent manner when compared with a non-binding serum. Intriguingly, out of sera from 40 patients, only 6 (15.0%) were detected positive for anti-β-lactamase IgG as verified by the binding specificity curve of the antibodies. When the human sera were compared in detail, we found that sera with either high or low titres of antibodies against β-lactamase (n = 3 of each) had equal levels of IgG against MID and UspA1/A2 in both groups (Figure 2c). In conclusion, no correlation was found between anti-β-lactamase and anti-UspA1/A2 IgG antibody titres.

**OMVs protect β-lactamase from specific anti-β-lactamase IgG**

Since we have previously found that β-lactamase is associated with OMVs, we wanted to investigate whether human anti-β-lactamase IgG could inhibit the enzyme activity. Specific antibodies were affinity purified on a Sepharose column containing recombinant β-lactamase. The resulting anti-β-lactamase IgG efficiently detected recombinant β-lactamase and was comparable to the rabbit anti-β-lactamase pAb, as shown by western blotting (Figure 3a).

To analyse whether the purified human IgG has the capacity to recognize β-lactamase-loaded OMVs, we included flow cytometry in our analysis. Anti-β-lactamase IgG significantly
detected OMVs isolated from the β-lactamase-positive M. catarrhalis KR526 (Figure 3b). A 5.6-fold increase in mean fluorescence intensity was observed with strain KR526 OMVs compared with the control with the FITC-conjugated secondary detection antibody only (Figure 3c). In contrast, no binding was seen with the β-lactamase-negative strain Bc5.

Figure 1. OMVs from M. catarrhalis contain β-lactamase in vivo. (a) Arrows show vesicles secreted from β-lactamase-positive Moraxella in a 9-year-old patient. A sample taken with a nasopharyngeal swab was analysed by TEM. The M. catarrhalis produced β-lactamase as confirmed by gold-labelled pAb in TEM. (b–e) The presence of β-lactamase inside or close to OMVs could clearly be seen in several TEM sections.
To investigate whether the anti-β-lactamase antibodies inhibit the enzymatic activity of β-lactamase, OMVs were incubated with purified anti-β-lactamase IgG for 1 h to allow binding. Thereafter, β-lactamase activity was measured using the chromogenic substrate nitrocefin. When OMVs were incubated with anti-β-lactamase IgG, the enzyme activity decreased by 60.8% ± 19.6%, confirming that antibodies partially inhibited β-lactamase activity in the OMVs (Figure 3d). However, since there was still significant enzymatic activity in the OMV preparation, vesicles were treated with saponin in order to lyse them. Interestingly, this opening of the vesicles increased the enzymatic activity of β-lactamase-positive OMVs by 32.8% ± 16.8%, albeit not statistically significant, it suggests the presence of β-lactamase inside OMVs. The inhibitory effect by the anti-β-lactamase IgG was more prominent, resulting in a decrease of enzymatic activity by 94.6% ± 3.0%. Taken together, our results suggest that β-lactamase was located both inside and on the surface of the OMVs.

Discussion

OMVs were first observed in the 1970s, and since then increasing evidence has emerged showing that these small spheres play an important role in both bacterial survival and pathogenesis. In this study we show that OMVs not only act as protective vesicles, whereby proteins can be delivered in complex with other cellular material, but are also important factors in the interplay between bacteria and the host’s humoral immunity. We have previously demonstrated the presence of β-lactamase in M. catarrhalis OMVs, and showed that these
OMVs confer antibiotic resistance on amoxicillin-susceptible M. catarrhalis, S. pneumoniae and H. influenzae. The last two species are important pathogens causing AOM and exacerbations in COPD patients. M. catarrhalis is often isolated as a co-pathogen in infections with S. pneumoniae and H. influenzae. We suggest that Moraxella has an important role in protecting its co-pathogens in the upper respiratory tract, by helping them resist antibiotic treatment. Interestingly, electron microscopy analysis of a nasopharyngeal sample from a child with sinusitis caused by M. catarrhalis revealed the enzyme β-lactamase. This further proves that β-lactamase is also found in or associated with OMVs in vivo, providing a clinical relevance for the vesicles in the spread of antibiotic resistance.

We also show that there is an immunological response against β-lactamase, as revealed by serum analysis. When IgG levels in sera obtained from healthy adults were compared, it was found that 15% carried anti-β-lactamase IgG. We analysed specific anti-β-lactamase IgG levels using recombinant β-lactamase produced in E. coli, and observed higher antibody titres against β-lactamase compared with titres against MID. In contrast, significantly lower antibody titres existed against β-lactamase when compared with anti-UspA1/A2 IgG titres. This suggests that UspA1, in contrast to MID, contains immunogenic epitope(s), generating a strong serological response. It has to be kept in mind, however, that the truncated MID 962–1200 is considerably shorter than the UspAs and represents only a small portion of the large MID molecule. Nevertheless, it seems that β-lactamase is significantly less immunogenic compared with UspA1/A2. To our knowledge, this is the first report on antibodies against M. catarrhalis β-lactamase in serum. Taking into consideration that M. catarrhalis β-lactamase is a unique enzyme that is highly conserved within the species, we assume that these antibodies were specific for Moraxella β-lactamase.

Figure 3. Purified anti-β-lactamase IgG from human serum does not inhibit β-lactamase enzymatic activity inside OMVs. (a) Western blotting showed that purified human anti-β-lactamase IgG detected recombinant β-lactamase (35 kDa). Recombinant RH4 β-lactamase26–318 (1 µg) was run in each lane. Rabbit anti-β-lactamase IgG was used as a positive control. (b) Flow cytometry analysis confirmed that purified anti-β-lactamase bound OMVs from the β-lactamase-positive M. catarrhalis KR526, illustrated by a positive shift (arrow), but not the β-lactamase-negative strain Bc5. OMVs (2 µg) without IgG were compared with OMVs incubated with purified anti-β-lactamase IgG (arrow). (c) Bar graph representing the ratio of mean fluorescence intensity between control and IgG-treated KR526 and Bc5 OMVs. Flow cytometry results represent three independent experiments. (d) A nitrocefin assay determined that β-lactamase enzyme activity of OMVs from KR526 was inhibited when OMVs were lysed with saponin (0.2%) and incubated with anti-β-lactamase IgG purified from human serum. In (d), 250 µg/mL OMVs was used and serum was diluted 1:100. β-Lactamase activity was quantified as a function of the change in absorbance from OD458 to OD585 as determined by spectrophotometry. Data in (d) are mean and standard error of the mean (SEM) of at least three independent experiments. **P ≤ 0.01; ***P ≤ 0.001.
In a paper by Giwercman et al., the levels of β-lactamase in cystic fibrosis (CF) patients with Pseudomonas aeruginosa infections were analysed. These authors found that the β-lactamase activity in sputum was high in CF patients and that levels significantly increased in patients treated with certain antibiotics. In a more recent paper the presence of IgG against chlamydosomal β-lactamase in serum and sputum samples of CF patients was demonstrated. In healthy controls, no anti-β-lactamase IgG was detected. The authors speculated that antibodies could potentially increase the efficiency of treatment with β-lactam antibiotics by inhibiting the β-lactamase present in serum, and thereby contributing some degree of protection against infection.

In our study, anti-β-lactamase IgG from sera obtained from healthy adults was found to bind to OMVs carrying β-lactamase. However, significant enzyme activity still remained in vesicles after incubation with antibodies, indicating that OMVs protected the enzyme from inhibition. In the light of the observations made by Giwercman et al., this could give the bacterium an advantage against removal of the serum anti-β-lactamase IgG. Since we have recently shown that vesicles confer antibiotic resistance in cultures with other bacterial species, it is interesting to speculate that protection from antibody neutralization of β-lactamase inside OMV might to some level also play a role in the interplay with other bacteria. However, considering that ~97% of M. catarrhalis strains are β-lactamase positive, it is worth noting that only 15% of healthy adults carry β-lactamase antibodies. Since OMVs function as a storage pool for β-lactamase, it is suggested that proteins located inside the OMV perhaps are not properly taken care of by the adaptive immune system. Although speculative, this perhaps may result in less efficient antibody production against β-lactamase compared with the adhesins UspA1 and A2.

M. catarrhalis was considered a harmless commensal for a long time. Although invasive disease may occur, evidence shows that Moraxella is mainly involved in AOM as well as in exacerbations in COPD patients, rendering it a genuine respiratory pathogen. With the introduction of a pneumococcal conjugate vaccine the relative frequency of isolating Moraxella as a pathogen has also increased, suggesting a shift in the bacterial niche. We suggest that another important role for Moraxella is, by means of OMVs, to provide an advantageous environment for other more severe disease-causing bacteria. Our results clearly show that specific IgG exists against β-lactamase in healthy donors, but also that OMVs protect the periplasmic β-lactamase residing inside OMVs from inhibition by antibodies.

Bacterial resistance against antimicrobial agents is an emerging problem, and it is of the greatest importance to take into account all aspects of the field. The present study may be an impetus for further studies on the role of OMVs in infections caused by antibiotic-resistant bacteria. It is highly relevant to examine in detail these mechanisms considering the long-term goal of finding new approaches to antimicrobial therapy.

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**Transparency declarations**

None to declare.

**References**


