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Schaar, Viveka; Paulsson, Magnus; Mörgelin, Matthias; Riesbeck, Kristian

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

VIVEKA SCHAAR[1], MAGNUS PAULSSON[1], MATTHIAS MÖRGELIN[2], and KRISTIAN RIESBECK[1]*

[1]*Medical Microbiology, Dept. of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, Malmö, Sweden.* [2]*Section of Clinical and Experimental Infectious Medicine, Dept. of Clinical Sciences, Lund University, Lund, Sweden*

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* Corresponding author. Dr. Kristian Riesbeck, Medical Microbiology, Dept. of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden. Phone: 46-40-338494. Fax: 46-40-336234.

E-mail: kristian.riesbeck@med.lu.se

ABSTRACT

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

Methods: Transmission electron microscopy was used to analyse 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 OMV 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 OMV from a 9-year old child suffering from *M. catarrhalis* sinusitis. Furthermore, anti-β-lactamase IgG were detected in sera obtained from healthy adults. Out of 40 adult blood donors (ages 18-65 years) tested, six (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 detected β-lactamase positive OMV. Interestingly, when OMV were permeabilised by saponin followed by quantification of β-lactamase enzyme we found that OMV shielded active β-lactamase from the anti-β-lactamase IgG.

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

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 approximately 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 more than 97% of *M. catarrhalis* strains have been β-lactamase positive.[4]

In parallel with most other Gram-negative bacteria, *M. catarrhalis* releases outer membrane vesicles (OMV), which are small spheres secreted from the outer membrane as the membrane bulges out and pinches off. The OMV thus reflect the composition of the outer membrane; carrying mainly lipids, outer membrane proteins but also DNA.[5-8] As the secretion of OMV 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 OMV not only have a role in pathogenesis, but also in biofilm formation, nutrient acquisition as well as horizontal gene transfer.[7, 9-11] OMV interact both with host cells and with other bacteria residing in a mutual niche. Moreover, OMV act as vehicles for secretion of proteins and DNA, whereby these components are protected from destruction and delivered to target cells at a distance.[5, 8, 12]

We recently showed that OMV from β-lactamase positive *M. catarrhalis* also contain the enzyme and consequently absorb and hydrolyze amoxicillin.[13] β-lactamase is known as a periplasmic enzyme, and therefore the storage in OMV and subsequent secretion would potentially be an important virulence mechanism.[14] Since

M. catarrhalis often is found with other bacterial species,[15] we hypothesized that conferral of antibiotic resistance to 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 to amoxicillin-susceptible *H. influenzae* and *S. pneumoniae* in addition to the nowadays rare β-lactamase negative *M. catarrhalis*.[13]

The antibody levels against major *Moraxella* outer membrane proteins such as *Moraxella* IgD-binding protein (MID)/ hemagglutinin (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 to adults, leading to a higher incidence of infections in children. However, the human antibody response to *M. catarrhalis* β-lactamase is currently unknown. In the present study, we determined IgG levels against *Moraxella* β-lactamase in healthy adults using recombinant protein. A group of individuals that have significant anti-β-lactamase IgG titres was identified, and these polyclonal antibodies (pAb) recognized β-lactamase positive OMV. Interestingly, we discovered that the hydrolyzing β-lactamase activity was partially protected within the OMV. This suggests that *Moraxella* OMV 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.

MATERIAL AND METHODS

Bacterial strains and growth conditions. *M. catarrhalis* clinical and reference strains KR526 and Bc5, respectively, were cultured on chocolate agar plates. Bacteria were grown at 37 °C in 5% CO_2. To determine MIC for amoxicillin, both Etests (Biodisk, Solna, Sweden) and colony counting (colony forming units; cfu) 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$^{26\text{-}318}$ 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 *E. coli* DH5α, the protein was produced in *E. coli* BL21(DE3) by induction with isopropyl-1-thio-β-D-galactoside (IPTG). Bacteria were sonicated and proteins were purified using affinity chromatography. Recombinant full length UspA1$^{50\text{-}770}$ and UspA2$^{30\text{-}539}$ in addition to the truncated protein MID$^{962\text{-}1200}$, which was selected due to an approximately similar size as the β-lactamase$^{26\text{-}318}$, were all from *M. catarrhalis* Bc5. Recombinant proteins were produced as previously described.[17]

Isolation of *M. catarrhalis* outer membrane vesicles (OMV). OMV were isolated according to the method described by Rosen *et al.*[19] Briefly, bacteria were grown in brain heart infusion (BHI) broth overnight at 37°C while shaking, and after centrifugation the resulting supernatant was filtered through 0.2 μm pore-size filters (Sartorius, Goettingen, Germany) to obtain a cell-free solution. The flow through was filtered with 100 kDa Vivaspin centrifugal concentrators (Vivascience, Hannover, Germany). The remaining concentrate was further concentrated by ultracentrifugation at 100,000 x *g* and washed with phosphate-buffered saline (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% bovine serum albumin-C in PBS, pH 7.6) (Aurion, Wageningen, Netherlands) for 15 min. OMV 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 sizes 10 and 5 nm, diluted 1:10 to 1:20 in incubation buffer, for 1 h at room temperature (RT). After washes in incubation buffer, the sections were fixed in 2% glutaraldehyde. The sections were thereafter washed in distilled water, and post-stained with uranyl acetate and lead citrate. Sections were examined with an election 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).

Enzyme-linked immunosorbant assay (ELISA). To analyze 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; ages 18-65 years) were added in duplicates for 1 h at RT. Horse-radish peroxidase (HRP)-labelled anti-human IgG polyclonal antibodies (pAb) (1:6,000) (DAKO, Glosterup, Denmark) was added as a secondary layer to plates for 20 min, and after subsequent washing steps the plates were developed and measured at OD_{450}. 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 described.[13] Sera were diluted 1:5 in PBS. The β-lactamase binding fraction was eluted with 0.1 M glycine (pH 2.4), 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 Bc5 for 1 h at RT in order to remove non-specific antibodies.

Flow cytometry analysis. To analyze the recognition of IgG to β-lactamase-positive OMV from *M. catarrhalis*, OMV (2 μg) were fixed with 3.5% formaldehyde for 15 min at RT. After a subsequent wash (100,000x*g* for 30 min), the OMV were incubated with purified anti-β-lactamase IgG. After another washing step, FITC-labelled rabbit anti-human pAb (DAKO) were added as a secondary step in PBS-BSA (1%) for 20 min at RT. Samples were analyzed 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 blots were performed. Proteins were transferred from gels to Immobilon-P

membranes (Millipore, Bedford, MA) at 20V 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 serum or rabbit for 1 h at RT as described previously.[17] After several washing steps, membranes were incubated for 1 h with HRP-conjugated secondary rabbit anti-human or swine anti-rabbit pAb (DAKO) respectively, that were diluted 1:1,000. Membranes were washed and developed using enhanced chemiluminescence western blot detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

Determination of the inhibitory effect of serum anti-β-lactamase antibodies blocking β-lactamase activity in *M. catarrhalis* OMV. The enzyme activity in OMV was quantified through a nitrocefin assay as previously described.[13] The chromogenic cephalosporin nitrocefin (Oxoid, Thermo Scientific, Cambridge, UK) was used. Briefly, OMV (0.3 μg/mL) were pre-incubated with saponin (0.2%) for 5 min at RT to lyse OMV (20),[20] 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 RT. OMV were incubated with nitrocefin (500 μg/mL) for 30 min at 37°C in the dark, followed by centrifugation at 13,000x*g* for 3 min. After chromogenic hydrolysis the subsequent colour change was determined using NanoDrop at OD_{485}. The enzyme activity of the OMV preparations was estimated using a standardized curve from a recombinant β-lactamase (VWR International, Leicestershire, UK). The activity was quantified as the number of moles nitrocefin hydrolyzed per min per mg protein.

Statistical analysis. The statistical analyses were performed with the 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, where *n* is the number of experiments performed. Significant values were defined as *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.

RESULTS

***Outer membrane vesicles from* M. catarrhalis *carry β-lactamase* in vivo.** We have recently shown that *Moraxella* releases OMV that are loaded with β-lactamase.[13] To determine whether β-lactamase is also associated with OMV *in vivo*, a specimen obtained with a nasopharyngeal swab from a 9-year old child with *Moraxella* sinusitis was analyzed by transmission electron microscopy (TEM). As can be seen in Figure 1A, *M. catarrhalis* readily released OMV in the nasopharynx.

To manufacture specific detection antibodies for *M. catarrhalis* β-lactamase, we produced a full-length recombinant β-lactamase$^{26\text{-}318}$ in *E. coli* followed by immunization of rabbits. Resulting anti-β-lactamase pAb were 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 OMV or in the close vicinity as exemplified 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 (ages 18-65 years). To quantify the IgG concentration, recombinant β-lactamase$^{26\text{-}318}$ was immobilized in microtiter plates followed by ELISA (Figure 2a). In addition, the well-defined adhesins MID$^{962\text{-}1200}$, UspA1$^{50\text{-}770}$ and UspA2$^{30\text{-}539}$ were included for comparison. Both UspA1$^{50\text{-}770}$ and UspA2$^{30\text{-}539}$ are full-length recombinant proteins, whereas MID$^{962\text{-}1200}$ is a 238 amino acid long truncated fragment of the native MID protein (molecular weight app. 200 kDa). The IgG antibody titres against β-lactamase$^{26\text{-}318}$ showed a 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-

MID$^{962-1200}$ IgG titres. The high antibody titres for UspA1 and A2 were in parallel with results previosuly obtained in our laboratory.[17] Finally, sera from the healthy donors in general contained higher mean antibody levels against UspA1 as compared to UspA2 (>0.5-fold difference).

To confirm the specificity of anti-β-lactamase IgG, binding of antibodies against our recombinant β-lactamase$^{26-318}$ was analyzed by ELISA. An example can be seen in Figure 2B, where we show a serum that bound to β-lactamase$^{26-318}$ in a dose-dependent manner when compared to a non-binding serum. Intriguingly, out of the 40 patients, only six sera (15.0%) were detected positive for anti-β-lactamase IgG. 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.

OMV protect the β-lactamase from specific anti-β-lactamase IgG. Since we previously have found that β-lactamase is associated with OMV,[13] we wanted to investigate whether human anti-β-lactamase IgG may inhibit the enzyme activity. Specific antibodies were affinity purified on a Sepharose column containing recombinant β-lactamase$^{26-318}$. The resulting anti-β-lactamase$^{26-318}$ IgG efficiently detected recombinant β-lactamase and was comparable with the rabbit anti-β-lactamase pAb as shown in western blot (Figure 3a).

To analyze whether the purified human IgG has the capacity to recognize β-lactamase-loaded OMV, we included flow cytometry in our analysis. Anti-β-lactamase IgG significantly detected OMV isolated from the β-lactamase positive *M.*

catarrhalis KR526 (Figure 3b). A 5.6-fold increase in mean fluorescence intensity (MFI) was observed with strain KR526 OMV compared to 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.

To investigate whether the anti-β-lactamase antibodies inhibit the enzymatic activity of β-lactamase, OMV 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 OMV were incubated with anti-β-lactamase IgG, the enzyme activity decreased with 60.8%±19.6%, confirming that antibodies partially inhibited β-lactamase activity in the OMV (Figure 3d). However, since there still was a significant enzymatic activity in the OMV preparation, vesicles were treated with saponin in order to lyse the vesicles. Interestingly, this opening of the vesicles significantly increased the enzymatic activity of β-lactamase positive OMV with 32.8% ±16.8%, proving the presence of β-lactamase inside OMV. 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 OMV.

DISCUSSION

Outer membrane vesicles were first observed in the 1970s,[21] and since then increasing evidence has emerged showing that these small spheres play an important role in both bacterial survival and pathogenesis.[6] In the present study, we show that OMV 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 humoral immunity.

We have previously determined the presence of β-lactamase in *M. catarrhalis* OMV, and showed that these OMV confer antibiotic resistance to amoxicillin-susceptible *M. catarrhalis, S. pneumoniae* and *H. influenzae*. The latter 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*, contained the enzyme β-lactamase. This further proves that β-lactamase is also found in or associated with OMV *in vivo*, providing a clinical relevance for the vesicles in spreading 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 as compared to titres against MID. In contrast, significantly lower antibody titers existed against β-lactamase when compared to 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 to UspA1/A2. To our knowledge, this is the first report on β-lactamase antibodies in serum against *M. catarrhalis.* Taking into consideration that *M. catarrhalis* β-lactamase is a unique enzyme that is highly conserved within the species,[22] we assume that these antibodies were specific for *Moraxella* β-lactamase.

In a paper by Giwercman *et al.*,[23] the levels of β-lactamase in cystic fibrosis (CF) patients with *Pseudomonas aeruginosa* infections were analyzed. 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 chromosomal β-lactamase in serum and sputum samples of CF patients was demonstrated.[24] In healthy controls, no anti-β-lactamase IgG was detected. The authors speculate that antibodies could potentially affect the efficiency of the treatment 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 were found to bind to OMV carrying β-lactamase. However, a significant enzyme activity still remained in vesicles after incubation with antibodies, indicating that OMV protected the enzyme from inhibition. In the light of the observations made by Giwercman *et al*,[23] this could give the bacterium an advantage against removal of the serum anti-β-lactamase IgG. Since we recently have shown that vesicles confer antibiotic resistance in cultures with other bacterial species, it is interesting to observe that this mechanism seems to be to some level protected against antibody

neutralization. Considering that approximately 97% of *M. catarrhalis* strains are β-lactamase positive, it is worth noticing that merely 15% of healthy adults carry β-lactamase antibodies. Since OMV function as a storage pool for β-lactamase it is therefore 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 a less efficient antibody production against β-lactamase as compared to 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 of COPD patients, rendering it a genuine respiratory pathogen.[25, 26] 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.[1, 18] We suggest that another important role for *Moraxella* is by means of OMV, 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 OMV protect the periplasmic β-lactamase residing inside OMV from inhibition by antibodies.

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

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TRANSPARENCY DECLARATIONS

Nothing to declare.

FIGURE LEGENDS

FIG. 1. *OMV 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 was taken with a nasopharyngeal swab followed by TEM analysis. The *M. catarrhalis* produced β-lactamase as confirmed by gold-labelled pAb in TEM. (b-e). The presence of β-lactamase inside or within close vicinity to OMV could clearly be seen in several TEM sections. The horizontal bar represents (a) 500 nm or (b-e) 100 nm.

FIG. 2. *A minor portion of healthy adults have IgG antibodies directed against* M. catarrhalis *β-lactamase and MID as compared UspA1/A2.* (a) The IgG concentrations from healthy adults against β-lactamase$^{26\text{-}318}$, UspA1$^{50\text{-}770}$, UspA2$^{30\text{-}539}$ and MID$^{962\text{-}1200}$ were quantified by ELISA. (b) The specificity of a β-lactamase$^{26\text{-}318}$ binding serum was compared to a non-binding serum in ELISA. (c) The anti-β-lactamase$^{26\text{-}318}$, anti-UspA1$^{50\text{-}770}$, anti-UspA2$^{30\text{-}539}$ and anti-MID$^{962\text{-}1200}$ IgG in the weakest β-lactamase binding sera (*n*=3) were compared to the highest β-lactamase binding sera (*n*=3). Human sera were diluted 1:200. Binding was measured as a function of absorbance at 450 nm. In (a), the horizontal bars represent the mean values. In (b), mean values and SEM are shown. All results represent triplicate values from two separate experiments.

FIG. 3. *Purified anti-β-lactamase IgG from human serum does not inhibit β-lactamase enzymatic activity inside OMV.* (a) A Western blot showed that purified human anti-β-lactamase IgG detected recombinant β-lactamase (35kDa). (b) A flow

cytometry analysis confirmed that purified anti-β-lactamase bound OMV from the β-lactamase positive *M. catarrhalis* KR526, illustrated by a positive shift (arrow), but not the β-lactamase negative strain Bc5. (c) A bar graph representing the ratio of mfi between control and IgG-treated KR526 and Bc5 OMV. (d) A nitrocefin assay determined that the β-lactamase enzyme activity of OMV from KR526 was inhibited when lysed with saponin (0.2%) and incubated with anti-β-lactamase IgG purified from human serum. In (a), recombinant RH4 β-lactamase$^{26\text{-}318}$ (1 μg) was run in each lane. Rabbit anti-β-lactamase IgG was used as a positive control. In (b), OMV (2 μg) without IgG were compared to OMV incubated with purified anti-β-lactamase IgG (arrow). In (d), 250 μg/mL OMV were used and serum was diluted 1:100. The β-lactamase activity was quantified as a function of the change in absorbance from OD_{380} to OD_{485} as determined by spectrophotometry. Flow cytometry results are representative of three independent experiments. The data in (d) are presented as means and the standard error of means (SEM) of at least three independent experiments **, $p \leq 0.01$, ***, $p \leq 0.001$.

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Fig. 1, Schaar *et al.*

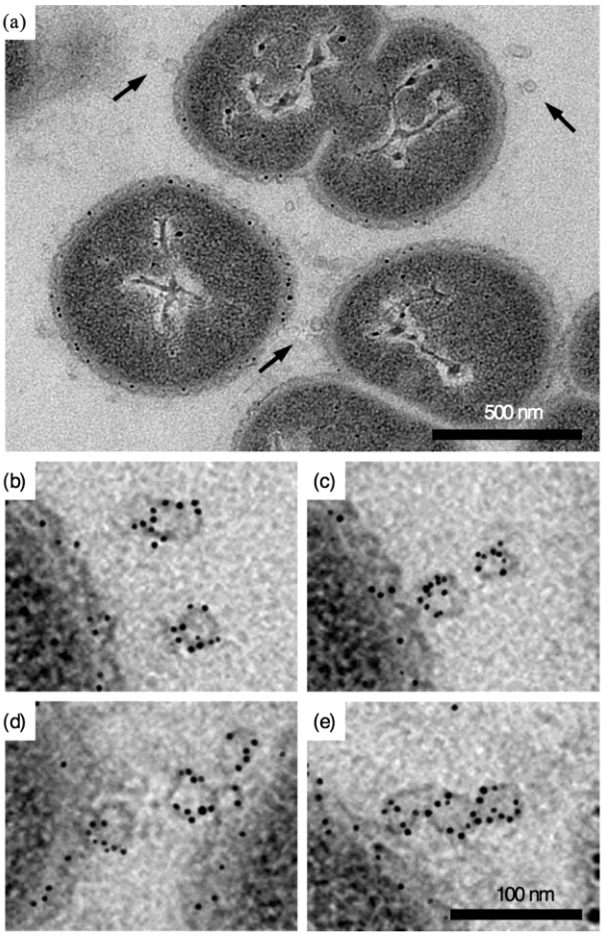

Fig. 2, Schaar *et al.*

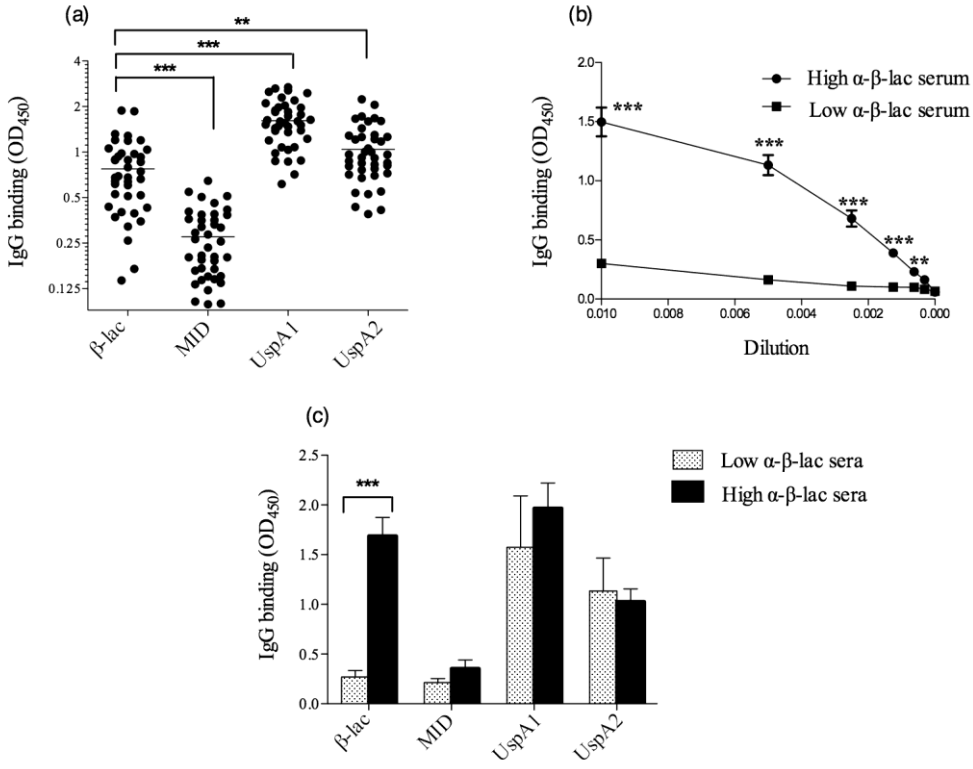

Fig. 3, Schaar *et al.*