Down-regulation of porin M35 in Moraxella catarrhalis by aminopenicillins and environmental factors and its potential contribution to the mechanism of resistance to aminopenicillins

Marion Jetter1, Violeta Spaniol1, Rolf Troller1 and Christoph Aebi1,2*

1Institute for Infectious Diseases, University of Bern, CH-3010 Bern, Switzerland; 2Department of Pediatrics, University of Bern, Inselspital, CH-3010 Bern, Switzerland

*Corresponding author. Tel: +41-31-632-9487; Fax: +41-31-632-9484; E-mail: christoph.aebi@insel.ch

Received 23 March 2010; returned 17 May 2010; revised 21 July 2010; accepted 21 July 2010

Objectives: The outer membrane protein M35 of Moraxella catarrhalis is an antigenically conserved porin. Knocking out M35 significantly increases the MICs of aminopenicillins. The aim of this study was to determine the biological mechanism of this potentially new antimicrobial resistance mechanism of M. catarrhalis and the behaviour of M35 in general stress situations.

Methods: PCR using m35-specific primers was used to detect the m35 gene in clinical isolates. The m35 mRNA expression of strains 300, O35E and 415 after exposure to amoxicillin and different stress conditions was measured by real-time PCR and normalized in relation to their 16S rRNA expression. The expression of M35 protein was analysed by SDS-PAGE and western blotting.

Results: Screening of 52 middle ear isolates resulted in positive PCR products for all tested strains. The analysis of m35 mRNA expression after amoxicillin treatment showed 24%–85% down-regulation compared with the respective amoxicillin-free controls in all three strains tested. Also, analysis of protein concentrations revealed lower M35 expression after growth with amoxicillin. Investigation of M35 during general stress responses showed down-regulation of the porin with growth at 26°C and 42°C, under hyperosmolar stress and under iron restriction.

Conclusions: The reduced expression of M35 after aminopenicillin exposure indicates a novel resistance mechanism against aminopenicillins in M. catarrhalis, which may be relevant in vivo. The differences in expression after different stress treatments demonstrate that M35 is involved in general stress responses.

Keywords: acute otitis media, amoxicillin, antimicrobial susceptibility, stress response

Introduction

Moraxella catarrhalis is a Gram-negative diplococcus and an exclusively human pathogen, mainly involved in exacerbations of chronic obstructive pulmonary diseases (COPD) in adults and acute otitis media in young children.1–5 The proportion of cases of acute otitis media caused by M. catarrhalis varies between 5% and 20%, with recent studies showing an increase of M. catarrhalis-caused otitis media since the introduction of routine infant immunization with pneumococcal conjugate vaccine.2–4,6,7 Acute otitis media treated with standard or high-dose amoxicillin is still the recommended therapeutic standard in Europe as well as in the USA.8–11 Treatment failures after the use of amoxicillin are documented in different studies and are usually related to infections with β-lactamase-producing strains of Haemophilus influenzae or M. catarrhalis or a drug-resistant strain of Streptococcus pneumoniae.2,11–15 More than 90% of M. catarrhalis isolated worldwide are resistant to penicillin and until now the only known resistance mechanism has been the production of one of two broβ-lactamases (BRO-1 and BRO-2).2,16–19 We previously demonstrated that M. catarrhalis strains lacking the outer membrane protein (OMP) M35 display an MIC of aminopenicillins up to 3-fold higher in comparison with their respective wild-types, indicating that M35 is involved in the susceptibility of the organism to these antimicrobials.20 M35 is a highly conserved porin in type 1 strains of M. catarrhalis; it is involved in nutrient uptake, appears essential for nasal colonization in mice and results in a mucosal immune response manifested as specific IgA in human saliva.20–22 Porins are water-filled open channels in the outer membrane of bacteria and allow the passive penetration of hydrophilic molecules. They can be differentiated by their activity...
from New England Biolabs (Beverly, MA, USA). Electrocompetent M. catarrhalis was prepared and DNA was electrophorated as described. DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer (PE Biosystems, Rotkreuz, Switzerland) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). M. catarrhalis strains 1–52 (middle ear isolates provided by Prof. Ron Dagan, Beer-Sheva, Israel) were analysed for the presence of m35 by PCR using forward primer M35F3 (5′-CTTGGCTTGCAACGGCAG-3′) and reverse primer M35_R_MJ (5′-CGTAGCATGGTTTTCATCACCAC-3′) and visualized by 1% agarose gel electrophoresis.

**β-Lactamase production testing and bro gene typing**

Isolates were investigated for β-lactamase production by the nitrocefin disc test (BD, Basel, Switzerland). β-Lactamase BRO typing was performed by sequencing the putative promoter region of the bro gene, described elsewhere, which shows clear sequence differences between BRO-1 and BRO-2. DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer (PE Biosystems) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequences were analysed with Lasergene software (DNASTAR, Madison, WI, USA). To sequence the relevant bro gene region, DNA was amplified using the primers _bro_F_MJ_ (5′-TATCCACCCCTAGAGGACAA-3′) and _bro_R_MJ_ (5′-GTAAGAATTGTTTTCGCTATC-3′).

**RNA methods**

RNA for _m35_ mRNA expression analysis was isolated and used for cDNA synthesis as described elsewhere. Quantitative real-time PCR was performed in triplicate for both target (_m35_) and normalizer [16S ribosomal RNA (rRNA)] genes. No-template controls and reverse transcriptase-negative controls were included in each run. Primers and probes for _m35_ were purchased from Applied Biosystems (Rotkreuz, Switzerland). The forward primer was M35ANYF (5′-GCCCTTTGCTCATGATCCCTG-3′), the reverse primer was M35ANYR (5′-GCATTGATAGGGCCTGTGCTA-3′) and the TaqMan probe was 5′-(FAM)CACCCACACCAAACTG(TAMRA)-3′. Primers and probes for 16S rRNA were used as described elsewhere. Relative quantification of gene expression was performed using the comparative threshold method. The ratios obtained after normalization were expressed as fold changes compared with untreated samples.

**Preparation of OMPs**

OMPs were prepared by the EDTA buffer method as described. Bacteria were harvested after amoxicillin or cold shock treatment, respectively, as described, resuspended in EDTA buffer (0.05 M Na2HPO4, 0.15 M NaCl, 0.01 M EDTA, pH 7.4), homogenized and incubated at 55°C at 300 rpm

---

**Materials and methods**

**Bacterial strains and culture conditions**

The _M. catarrhalis_ strains and their isogenic _m35_ mutants used in this study are listed in Table 1. All strains were cultured at 37°C and 150–200 rpm in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) or on BHI agar plates in an atmosphere containing 5% CO2. Media were supplemented with kanamycin (20 mg/L) for culturing of the mutants. _Escherichia coli_ DH5α was grown on Luria-Bertani (LB) agar plates or in LB broth. For analysis of the effects of amoxicillin, bacteria were cultured in BHI broth to an optical density (600 nm) (OD600) of 0.18. Afterwards, 60 mg/L amoxicillin (Sigma-Aldrich, Steinheim, Germany) was added and bacteria were cultured for an additional 4 h. To quantify viable _M. catarrhalis_ at various amoxicillin concentrations, bacteria were cultured at different concentrations (0, 6, 18, 60 mg/L) of the antibiotic for 4 h, and both the OD600 and cfu were determined at different time-points (Figure 1). For temperature experiments, bacteria were cultured to an OD600 of 0.3 before exposing them to 26°C, 42°C or 37°C, for 3 h. _M35_ expression under hyperosmolar stress and iron depletion conditions was analysed by adding 0.5 mol/L NaCl or 50 μM desferrioxamine to the BHI medium at a culture density (OD600) of 0.3.

**DNA methods**

Plasmids were isolated using the Wizard Plus SV Miniprep DNA purification system (Promega, Madison, WI, USA). _E. coli_ DH5α was transformed as described previously. Restriction enzymes were purchased

| Strain/Description | MIC (mg/L)<sup>20</sup> | β-Lactamase production | BRO type | Reference or source |
|--------------------|-------------------------|------------------------|----------|---------------------|
| Isolates 1–52       | not done                | not done               | not done | Beer-Sheva, Israel  |
| 035E                | 3                       | +                      | BRO-1    | 71                  |
| 035E+53             | 6                       | +                      | BRO-1    | this study          |
| 300                 | 6                       | +                      | BRO-1    | 72,73               |
| 300+53              | 16                      | +                      | BRO-1    | this study          |
| 415                 | 2                       | +                      | BRO-1    | 72,73               |
| 415+53              | 6                       | +                      | BRO-1    | this study          |
| DH5α (E. coli)      | not done                | not done               | not done | 74                  |

---
for 1 h. Cells and cell debris were eliminated by centrifugation at 10000 g for 15 min at 4 °C. Finally, OMPs were collected by ultracentrifugation at 100000 g for 2 h at 4 °C.

**SDS-PAGE and immunoblot**

Samples were resolved by SDS-PAGE using a 10% polyacrylamide gel. To compare protein expression between treated and untreated samples, the protein concentration loaded on the SDS-PAGE was always 1 mg/L. Band intensity was quantified using the AlphaEaseFC program (Inotech, San Leandro, CA, USA). Antibody detection was performed by western blot analysis as described elsewhere. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). IgA binding was detected using human saliva samples as the primary antibody source and goat anti-human IgA labelled with horseradish peroxidase (Sigma) as the secondary antibody. SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL, USA) was used for detection of antibody binding. Unstimulated human saliva was collected from healthy volunteers using Salivette sponges (Sarstedt, Nuembrecht, Germany), centrifuged for 5 min at 2000 rpm and stored at -20 °C. All volunteers were laboratory researchers and provided oral informed consent. Sampling of saliva from healthy volunteers was approved by the local ethics committee. OMPs of m35 knockout mutants were used as negative controls for their respective wild-type strains. Construction of the isogenic mutants O35E, m35, 300.m35 and 415.m35 has been described elsewhere.

**Results**

**PCR analysis of 52 clinical isolates**

Our previous findings of increased amoxicillin MIC for m35 knockout mutants compared with their respective wild-type parent strains prompted us to investigate the possibility of naturally occurring clinical middle ear isolates lacking an m35 gene. We studied 52 middle ear isolates obtained from Prof. Ron Dagan (Beer-Sheva, Israel). PCR resulted in positive products for all 52 strains.

**Down-regulation of m35 mRNA expression after amoxicillin treatment**

Reduced membrane permeability—one of the main strategies used by bacteria for protection against antibiotics—is generally regulated by altered porin expression. Consequently, we investigated the m35 mRNA expression of strains 300, O35E and 415 during amoxicillin treatment by quantitative real-time PCR. The breakpoint of bactericidal amoxicillin concentration was evaluated by a time–kill curve assay and was found to be at 60 mg/L (Figure 1), which correlates with the MIC for the wild-type strain 300 depending on the inoculum, which is more than 10 times higher in this experimental setting than in antimicrobial resistance testing using Etests. Growth curves with 60 mg/L amoxicillin demonstrated that this concentration seems not to be completely bactericidal but inhibits growth for a period of ~4 h before proliferation resumes (data not shown). Compared with bacteria grown without amoxicillin, strain 300 grown with 60 mg/L amoxicillin showed a decrease of 85 ± 2% in its m35 mRNA expression (Figure 2). Strain O35E demonstrated a similar effect, i.e. 73.7 ± 24% down-regulation of m35 mRNA after amoxicillin treatment (data not shown). In strain 415, down-regulation was only 24 ± 9%. We also observed down-regulation of m35 mRNA transcription in strain 300 after treatment with a subinhibitory concentration of amoxicillin (15 mg/L). This effect, however, was less pronounced (33 ± 5.6%) than with an amoxicillin concentration at the MIC (data not shown).
M35 protein expression is also affected by amoxicillin

To determine that a reduction of mRNA copy number translates into less M35 protein in the outer membrane, OMPs from bacteria grown with and without amoxicillin were isolated and protein expression was analysed by SDS-PAGE and western blotting. Strain 300 (Figure 3) as well as strains O35E and 415 (data not shown) expressed less M35 protein both on the SDS gel (Figure 3) and in western blot analysis (Figure 3). These data demonstrate that *M. catarrhalis* senses the presence of amoxicillin in the medium and subsequently down-regulates M35 porin expression.

β-Lactamase production and BRO typing

The investigation of the β-lactamase production of all three strains and its m35 knockout mutants showed that all strains produced β-lactamases. The sequences of the putative promoter region of the *bro* gene were identical to those published by Bootsma *et al.* and identified all our strains as BRO-1 β-lactamase producers.

Cold shock induces down-regulation of m35 mRNA

Porins involved in antimicrobial resistance have often been described to take part in general stress responses. One of the best known stress responses of *M. catarrhalis* is the cold shock response, which alters the expression of specific OMPs after exposure to 26°C. In the course of our further studies, and emphasizing that the expression of M35 appeared to be particularly stress-sensitive, we thus investigated the response of m35 induced by general stress stimuli such as cold shock treatment. Indeed, all three strains—O35E, 300 and 415—showed an effect reminiscent of amoxicillin exposure in that expression was down-regulated after growth at 26°C in comparison with 37°C. Strains O35E (Figure 4), 300 and 415 demonstrated down-regulation of 76 ± 6%, 57.6 ± 3% (data not shown) and 52.2 ± 19%, respectively.

M35 protein expression is involved in the cold shock response

To prove that down-regulation induced by cold shock is not only a transcriptional event, OMPs from cold shock- and non-cold shock-treated bacteria were isolated and analysed by SDS-PAGE and western blotting. Indeed, M35 protein expression was clearly down-regulated in strain O35E (Figure 5) as well as in strains 300 and 415 (data not shown).

m35 mRNA expression is affected by general stress conditions

Cold shock is not the only stress condition that affects *M. catarrhalis*. Amongst others, heat shock, altered osmolarity and iron depletion occur in their mucosal habitat. Thus, we analysed m35 mRNA expression under these conditions. The analysis revealed m35 down-regulation after growth at 42°C (66 ± 29%), as well as in response to hyperosmolar stress (86 ± 14%) and iron depletion (56.3 ± 22%) (Figure 6). Taken together, these data indicate that the level of m35 is influenced by various environmental variables, which may consequently affect susceptibility to amoxicillin in the mucosal habitat of the respiratory tract.

Figure 3. SDS-PAGE (a) and western blot (c) of *M. catarrhalis* OMPs (1 mg/L) from strain 300 after growth with and without 60 mg/L amoxicillin together with its respective m35 knockout mutant as the negative control. The rectangle shows the position of the M35 protein band at 36 kDa. (b) The spectra display the intensity of each SDS-PAGE protein band from strain 300 grown without amoxicillin and with 60 mg/L amoxicillin proportional to the total protein intensity.
Antimicrobial resistance is based on three major strategies: detoxifying enzymes to degrade or modify antibiotics; target protection to impair target recognition and thus antimicrobial activity; and the membrane barrier to limit intracellular access of antimicrobials. For M. catarrhalis, until now just one of these three strategies has been described—the production of two different chromosomal b-lactamases. More than 95% of clinical isolates are resistant to penicillin and it was shown recently that 80% of all strains tested in the UK and Ireland were resistant to cefaclor and 5% to cefuroxime. Drug resistance to b-lactam antibiotics is often associated with a second major resistance strategy—reduced outer membrane permeability. Reduced membrane permeability can be the result of altered porin expression or the presence of a mutated porin.

Our analysis of 52 clinical middle ear isolates using PCR amplification with conserved primers failed to identify strains lacking an m35 gene on their chromosome. This finding corroborates previous data indicating that m35 is a highly conserved porin gene and indicates that in vitro aminopenicillin susceptibility must be mediated by means other than lack of porin expression. Easton et al. demonstrated that M35 is structurally homologous to classical Gram-negative porins, such as OmpC from E. coli and OmpK36 (OmpC homologue) from Klebsiella pneumoniae. Both porin types are involved in antimicrobial resistance mechanisms and are described in detail elsewhere.

Several investigators have shown altered porin expression during antibiotic therapy, resulting in specific antimicrobial resistances, dependent on the ability of the bacteria to produce b-lactamase, their general porin composition and the antimicrobial substance they are confronted with. Our findings of the down-regulation of M35 expression at the transcriptional level as well as at the protein level in all three b-lactamase-producing strains tested, together with our previous observations of the significantly higher aminopenicillin MICs for the M35 knockout mutants, indicate that we have found a potentially novel resistance mechanism against aminopenicillins in M. catarrhalis. The MICs of ampicillin and amoxicillin for the M35 knockout strains were up to 3-fold greater than for their respective wild-type strains, and even amoxicillin/clavulanate displayed the same effect.

The mechanism seems to be similar to those described for K. pneumoniae OmpK36 and Omp36 porin (OmpC homologue) from Enterobacter aerogenes. Down-regulation of OmpK36 leads, depending on which b-lactamase the isolate is producing, to resistance to oxyimino and zwitterionic cephalosporins or carbapenems. In E. aerogenes, loss of Omp36 leads mainly to imipenem resistance. This demonstrates an additional analogy to our findings. The specificity for aminopenicillins—which are also zwitterionic molecules—is reminiscent of the physical characteristics of OmpK36 and Omp36. Nevertheless, the differences between the down-regulation of M35

![Figure 4. Down-regulation of m35 mRNA expression after cold shock exposure. Quantitative real-time PCR was performed after 3 h of incubation at 26°C or 37°C. Up- or down-regulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate. Data are presented as means ± 1 SD (n = 3).](image)

**Figure 4.** Down-regulation of m35 mRNA expression after cold shock exposure. Quantitative real-time PCR was performed after 3 h of incubation at 26°C or 37°C. Up- or down-regulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate. Data are presented as means ± 1 SD (n = 3).

**Discussion**

Antimicrobial resistance is based on three major strategies: detoxifying enzymes to degrade or modify antibiotics; target protection to impair target recognition and thus antimicrobial activity; and the membrane barrier to limit intracellular access of antimicrobials. For M. catarrhalis, until now just one of these three strategies has been described—the production of two different chromosomal b-lactamases. More than 95% of clinical isolates are resistant to penicillin and it was shown recently that 80% of all strains tested in the UK and Ireland were resistant to cefaclor and 5% to cefuroxime. Drug resistance to b-lactam antibiotics is often associated with a second major resistance strategy—reduced outer membrane permeability. Reduced membrane permeability can be the result of altered porin expression or the presence of a mutated porin.

Our analysis of 52 clinical middle ear isolates using PCR amplification with conserved primers failed to identify strains lacking an m35 gene on their chromosome. This finding corroborates previous data indicating that m35 is a highly conserved porin gene and indicates that in vitro aminopenicillin susceptibility must be mediated by means other than lack of porin expression. Easton et al. demonstrated that M35 is structurally homologous to classical Gram-negative porins, such as OmpC from E. coli and OmpK36 (OmpC homologue) from Klebsiella pneumoniae. Both porin types are involved in antimicrobial resistance mechanisms and are described in detail elsewhere.

Several investigators have shown altered porin expression during antibiotic therapy, resulting in specific antimicrobial resistances, dependent on the ability of the bacteria to produce b-lactamase, their general porin composition and the antimicrobial substance they are confronted with. Our findings of the down-regulation of M35 expression at the transcriptional level as well as at the protein level in all three b-lactamase-producing strains tested, together with our previous observations of the significantly higher aminopenicillin MICs for the M35 knockout mutants, indicate that we have found a potentially novel resistance mechanism against aminopenicillins in M. catarrhalis. The MICs of ampicillin and amoxicillin for the M35 knockout strains were up to 3-fold greater than for their respective wild-type strains, and even amoxicillin/clavulanate displayed the same effect.

The mechanism seems to be similar to those described for K. pneumoniae OmpK36 and Omp36 porin (OmpC homologue) from Enterobacter aerogenes. Down-regulation of OmpK36 leads, depending on which b-lactamase the isolate is producing, to resistance to oxyimino and zwitterionic cephalosporins or carbapenems. In E. aerogenes, loss of Omp36 leads mainly to imipenem resistance. This demonstrates an additional analogy to our findings. The specificity for aminopenicillins—which are also zwitterionic molecules—is reminiscent of the physical characteristics of OmpK36 and Omp36. Nevertheless, the differences between the down-regulation of M35
after amoxicillin treatment between strain O35E and 415 with the same MICs for wild-type and mutants, respectively, indicate that there must be a regulation mechanism whose complexity exceeds β-lactamase production and protein down-regulation.

The bacterial stress response is based on a complex network of regulatory systems—a cascade of alterations in gene expression and protein activity that favour survival under extreme and rapidly changing conditions. Porins, especially those involved in antimicrobial resistance, such as those of the OmpF and OmpC-like porin families, are often reported to be associated with general stress responses. Cold shock as well as heat shock responses are well known mechanisms and have been researched intensively in E. coli and Bacillus subtilis. We have shown previously that the cold shock response is obviously an important mechanism for M. catarrhalis as an adaptation and survival mechanism in the nasopharyngeal habitat, but also regarding its virulence and colonization abilities. Begic and Worobec showed that temperature is also a predominant effector of expression regulation of OmpF and OmpC in Serratia marcescens. This organism is a β-lactam-resistant Gram-negative bacterium, whose porins—OmpF and OmpC—show high similarities to those of E. coli and are also involved in β-lactam resistance by decreasing outer membrane permeability. By analysing the regulation of these porins, these authors showed that OmpF is up-regulated and OmpC is down-regulated after growth at 28°C, but the opposite way round after growth at 42°C. The same effect of OmpC regulation, comparable to our findings in M35 after cold shock treatment (Figures 4 and 5), has been described in E. coli several times. M35 down-regulation after exposure to 42°C has not been described until now. The general effect described in all Gram-negative bacteria is up-regulation of the OmpC-like protein after heat shock treatment, whereas OmpF is down-regulated. However, antagonistic regulation of OmpF and OmpC is apparently not the general mechanism in all E. coli strains. Allen et al. observed down-regulation of both OmpF and OmpC after cold shock treatment in E. coli O157:H7. Certainly, the physiological basis for porin regulation under temperature stress conditions is still unclear and needs further investigation. Likewise, our findings concerning the down-regulation of M35 under high osmolarity conditions need further scrutiny. Generally, the OmpC-like porin is down-regulated at low osmolarities and up-regulated at high osmolarity conditions—similar to our findings under temperature stress conditions. However, it is currently not known whether M. catarrhalis possesses an OmpF-like porin acting as an M35 antagonist. It is conceivable that M. catarrhalis expresses only the OmpF-like porin M35 comparable to K. pneumoniae strains expressing extended-spectrum β-lactamase (ESBL). These ESBL-expressing strains produce only the OmpC-like porin OmpK36. This could explain—together with the high specificity of molecule transport—why the role of M35 in stress response regulation is clearly different from that of OmpC-like porins associated with an antagonist. The expression of an OmpC-like porin in response to iron depletion has, to our knowledge, also never been described before, but substantiates the notion that the regulation of M35 is an important mechanism allowing the survival of M. catarrhalis under changing environmental conditions.

In summary, we describe here a new antimicrobial resistance mechanism in M. catarrhalis against amoxicillin, which obviously could lead to significantly higher MICs that would affect the use of amoxicillin in the therapy of acute otitis media caused by M. catarrhalis in a critical manner. To verify these findings in vivo and clarify its clinical relevance regarding amoxicillin therapy of acute otitis media caused by M. catarrhalis, further analyses will be needed. In particular, it will be necessary to elucidate whether down-regulation of M35 during treatment may contribute to treatment failure caused by isolates that have been shown to be susceptible in vitro.

Acknowledgements
Professor George Syrogiannopoulos (Larissa, Greece) provided the nasopharyngeal M. catarrhalis isolates 300 and 415.

Funding
This work was supported by the Swiss National Science Foundation (SNF) grants 3100A0-102246 and 3100A0-116053 (to C. A.).

Transparency declarations
None to declare.

References
1 Murphy TF, Brauer AL, Grant BJ et al. Moraxella catarrhalis in chronic obstructive pulmonary disease: burden of disease and immune response. Am J Respir Crit Care Med 2005; 172: 195–9.
2 Murphy TF, Parameswaran GI. Moraxella catarrhalis, a human respiratory tract pathogen. Clin Infect Dis 2009; 49: 124–31.
3 Marchant CD. Spectrum of disease due to Branhamella catarrhalis in children with particular reference to acute otitis media. Am J Med 1990; 88: 155–95.
Down-regulation of porin M35 in M. catarrhalis

4. Palmu A, Herva E, Savolainen H et al. Association of clinical signs and symptoms with bacterial findings in acute otitis media. Clin Infect Dis 2004; 38: 234–42.

5. Verduin CM, Hol C, Fleer A et al. Moraxella catarrhalis: from emerging to established pathogen. Clin Microbiol Rev 2002; 15: 125–44.

6. Escola J, Kilpi T, Palmu A et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344: 403–9.

7. Revi K, McCormick DP, Potel J et al. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics 2006; 117: 1823–9.

8. UpToDateOnline. Acute Otitis Media in Children: Treatment. 2010; 17.3. http://www.uptodate.com/online/content/topic.do?topickey=pedi_id/10593&selectedTitle=5%7E3E150&source=search_result (9 June 2010, date last accessed).

9. Gilbert DN, Moellering RC Jr, Eliopoulos GM et al. The Sanford Guide to Antimicrobial Therapy 2009. Sperryville, USA: Antimicrobial Therapy, Inc. 2009; 212.

10. Dowell SF, Butler JC, Giebink GS et al. Acute otitis media: management and surveillance in an era of pneumococcal resistance—a report from the Drug-resistant Streptococcus pneumoniae Therapeutic Working Group. Pediatr Infect Dis J 1999; 18: 1–9.

11. Pelton SI, Leibovitz E. Recent advances in otitis media. Pediatr Infect Dis J 2009; 28: S133–7.

12. Al-Shawwa BA, Wegner D. Trimethoprim-sulfamethoxazole plus topical antibiotics as therapy for acute otitis media with otorhea caused by community-acquired methicillin-resistant Staphylococcus aureus in children. Arch Otolaryngol Head Neck Surg 2005; 131: 782–4.

13. Santos F, Mankarious LA, Eavey RD. Methicillin-resistant Staphylococcus aureus: pediatric otitis. Arch Otolaryngol Head Neck Surg 2000; 126: 1383–5.

14. Pichichero ME, Casey JR. Emergence of a multiresistant serotype 19A pneumococcal strain not included in the 7-valent conjugate vaccine as an otopathogen in children. JAMA 2007; 298: 1772–8.

15. Leibovitz E, Broides A, Greenberg D et al. Association of clinical signs and symptoms with bacterial findings in acute otitis media. Clin Infect Dis 2004; 38: 234–42.

16. Verduin CM, Hol C, Fleer A et al. Moraxella catarrhalis: from emerging to established pathogen. Clin Microbiol Rev 2002; 15: 125–44.

17. Escola J, Kilpi T, Palmu A et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344: 403–9.

18. Revi K, McCormick DP, Potel J et al. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics 2006; 117: 1823–9.

19. UpToDateOnline. Acute Otitis Media in Children: Treatment. 2010; 17.3. http://www.uptodate.com/online/content/topic.do?topickey=pedi_id/10593&selectedTitle=5%7E3E150&source=search_result (9 June 2010, date last accessed).

20. Dowell SF, Butler JC, Giebink GS et al. Acute otitis media: management and surveillance in an era of pneumococcal resistance—a report from the Drug-resistant Streptococcus pneumoniae Therapeutic Working Group. Pediatr Infect Dis J 1999; 18: 1–9.

21. Pelton SI, Leibovitz E. Recent advances in otitis media. Pediatr Infect Dis J 2009; 28: S133–7.

22. Al-Shawwa BA, Wegner D. Trimethoprim-sulfamethoxazole plus topical antibiotics as therapy for acute otitis media with otorhea caused by community-acquired methicillin-resistant Staphylococcus aureus in children. Arch Otolaryngol Head Neck Surg 2005; 131: 782–4.

23. Santos F, Mankarious LA, Eavey RD. Methicillin-resistant Staphylococcus aureus: pediatric otitis. Arch Otolaryngol Head Neck Surg 2000; 126: 1383–5.

24. Pichichero ME, Casey JR. Emergence of a multiresistant serotype 19A pneumococcal strain not included in the 7-valent conjugate vaccine as an otopathogen in children. JAMA 2007; 298: 1772–8.

25. Leibovitz E, Broides A, Greenberg D et al. Association of clinical signs and symptoms with bacterial findings in acute otitis media. Clin Infect Dis 2004; 38: 234–42.

26. Verduin CM, Hol C, Fleer A et al. Moraxella catarrhalis: from emerging to established pathogen. Clin Microbiol Rev 2002; 15: 125–44.

27. Escola J, Kilpi T, Palmu A et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344: 403–9.

28. Revi K, McCormick DP, Potel J et al. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics 2006; 117: 1823–9.

29. UpToDateOnline. Acute Otitis Media in Children: Treatment. 2010; 17.3. http://www.uptodate.com/online/content/topic.do?topickey=pedi_id/10593&selectedTitle=5%7E3E150&source=search_result (9 June 2010, date last accessed).

30. Dowell SF, Butler JC, Giebink GS et al. Acute otitis media: management and surveillance in an era of pneumococcal resistance—a report from the Drug-resistant Streptococcus pneumoniae Therapeutic Working Group. Pediatr Infect Dis J 1999; 18: 1–9.

31. Pelton SI, Leibovitz E. Recent advances in otitis media. Pediatr Infect Dis J 2009; 28: S133–7.

32. Al-Shawwa BA, Wegner D. Trimethoprim-sulfamethoxazole plus topical antibiotics as therapy for acute otitis media with otorhea caused by community-acquired methicillin-resistant Staphylococcus aureus in children. Arch Otolaryngol Head Neck Surg 2005; 131: 782–4.

33. Santos F, Mankarious LA, Eavey RD. Methicillin-resistant Staphylococcus aureus: pediatric otitis. Arch Otolaryngol Head Neck Surg 2000; 126: 1383–5.

34. Pichichero ME, Casey JR. Emergence of a multiresistant serotype 19A pneumococcal strain not included in the 7-valent conjugate vaccine as an otopathogen in children. JAMA 2007; 298: 1772–8.

35. Leibovitz E, Broides A, Greenberg D et al. Association of clinical signs and symptoms with bacterial findings in acute otitis media. Clin Infect Dis 2004; 38: 234–42.

36. Verduin CM, Hol C, Fleer A et al. Moraxella catarrhalis: from emerging to established pathogen. Clin Microbiol Rev 2002; 15: 125–44.

37. Escola J, Kilpi T, Palmu A et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344: 403–9.

38. Revi K, McCormick DP, Potel J et al. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics 2006; 117: 1823–9.

39. UpToDateOnline. Acute Otitis Media in Children: Treatment. 2010; 17.3. http://www.uptodate.com/online/content/topic.do?topickey=pedi_id/10593&selectedTitle=5%7E3E150&source=search_result (9 June 2010, date last accessed).

40. Dowell SF, Butler JC, Giebink GS et al. Acute otitis media: management and surveillance in an era of pneumococcal resistance—a report from the Drug-resistant Streptococcus pneumoniae Therapeutic Working Group. Pediatr Infect Dis J 1999; 18: 1–9.

41. Pelton SI, Leibovitz E. Recent advances in otitis media. Pediatr Infect Dis J 2009; 28: S133–7.
52 Helminen ME, Maciver I, Paris M et al. A mutation affecting expression of a major outer membrane protein of Moraxella catarrhalis alters serum resistance and survival in vivo. J Infect Dis 1993; 168: 1194–201.

45 Heiniger N, Troller R, Meier PS et al. Cold shock response of the UspA1 outer membrane adhesin of Moraxella catarrhalis. Infect Immun 2005; 73: 8247–55.

46 Heiniger N, Spaniol V, Troller R et al. A reservoir of Moraxella catarrhalis in human pharyngeal lymphoid tissue. J Infect Dis 2007; 196: 1080–7.

47 Murphy TF, Loeb MR. Isolation of the outer membrane of Branhamella catarrhalis. Microb Pathog 1989; 6: 159–74.

48 Spaniol V, Troller R, Aebi C. Physiologic cold shock increases adherence of Moraxella catarrhalis to and secretion of interleukin 8 in human upper respiratory tract epithelial cells. J Infect Dis 2009; 200: 1593–601.

49 Davin-Regli A, Bolla JM, James CE et al. Membrane permeability and regulation of drug ‘influx and efflux’ in enterobacterial pathogens. Curr Drug Targets 2008; 9: 750–9.

50 Malmwall BE, Brarsson JE, Johnsson J. In vitro sensitivity to penicillin V and β-lactamase production of Branhamella catarrhalis. J Antimicrob Chemother 1977; 3: 374–5.

51 Sader HS, Jones RN, Dowzicky MJ et al. Antimicrobial activity of ticarcillin tested against nosocomial bacterial pathogens from patients hospitalized in the intensive care unit. Diagn Microbiol Infect Dis 2005; 52: 203–8.

52 Johnson DM, Sader HS, Fritsche TR et al. Susceptibility trends of Haemophilus influenzae and Moraxella catarrhalis against orally administered antimicrobial agents: five-year report from the SENTRY Antimicrobial Surveillance Program. Diagn Microbiol Infect Dis 2003; 47: 373–6.

53 Deshpande LM, Sader HS, Fritsche TR et al. Contemporary prevalence of BRO β-lactamases in Moraxella catarrhalis: report from the SENTRY antimicrobial surveillance program (North America, 1997 to 2004). J Clin Microbiol 2006; 44: 3775–7.

54 Morrissey I, Maher K, Williams L et al. Non-susceptibility trends among Haemophilus influenzae and Moraxella catarrhalis from community-acquired respiratory tract infections in the UK and Ireland, 1999–2007. J Antimicrob Chemother 2008; 62(Suppl 2): i97–103.

55 Nikaido H. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob Agents Chemother 1989; 33: 1831–6.

56 Bornef C, Davin-Regli A, Bosi C et al. Imipenem resistance of Enterobacter aerogenes mediated by outer membrane permeability. J Clin Microbiol 2000; 38: 1048–52.

57 Thiolas A, Bornef C, Davin-Regli A et al. Resistance to imipenem, cefepime, and cefpirome associated with mutation in Omp36 osmopin of Enterobacter aerogenes. Biochem Biophys Res Commun 2004; 317: 851–6.

58 Hernandez-Allens S, Alberti S, Alvarez D et al. Porin expression in clinical isolates of Klebsiella pneumoniae. Microbiology 1999; 145: 673–9.

59 Martinez-Martinez L, Hernandez-Allens S, Alberti S et al. In vivo selection of porin-deficient mutants of Klebsiella pneumoniae with increased resistance to cefoxitin and expanded-spectrum-cephalosporins. Antimicrob Agents Chemother 1996; 40: 342–8.

60 Martinez-Martinez L, Pascual A, Hernandez-Allens S et al. Roles of β-lactamases and porins in activities of carbapenems and cephalosporins against Klebsiella pneumoniae. Antimicrob Agents Chemother 1999; 43: 1669–73.

61 Bradford PA, Urban C, Mariano N et al. Imipenem resistance in Klebsiella pneumoniae is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. Antimicrob Agents Chemother 1997; 41: 563–9.

62 Ardany C, Linares J, Dominguez MA et al. Outer membrane profiles of clonally related Klebsiella pneumoniae isolates from clinical samples and activities of cephalosporins and carbapenems. Antimicrob Agents Chemother 1998; 42: 1636–40.

63 Danelon C, Nestorovich EM, Winterhalter M et al. Interaction of zwitterionic penicillins with the OmpF channel facilitates their translocation. Biophys J 2006; 90: 1617–27.

64 Nestorovich EM, Danelon C, Winterhalter M et al. Designed to penetrate: time–resolved interaction of single antibiotic molecules with bacterial pores. Proc Natl Acad Sci USA 2002; 99: 9789–94.

65 Hengge-Aronis R. Recent insights into the general stress response regulatory network in Escherichia coli. J Mol Microbiol Biotechnol 2002; 4: 341–6.

66 Weber MH, Marahiel MA. Bacterial cold shock responses. Sci Prog 2003; 86: 9–75.

67 Anderson KL, Roberts C, Disz T et al. Characterization of the Staphylococcus aureus heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. J Bacteriol 2006; 188: 6739–56.

68 Rouadi P, Baroody FM, Abbott D et al. A technique to measure the ability of the human nose to warm and humidify air. J Appl Physiol 1999; 87: 400–6.

69 Gutmann L, Williamson R, Moreau N et al. Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of Klebsiella, Enterobacter, and Serratia. J Infect Dis 1985; 151: 501–7.

70 Sanders CC, Sanders WE Jr. β-Lactam resistance in gram-negative bacteria: global trends and clinical impact. Clin Infect Dis 1992; 15: 824–39.

71 Helminen ME, Maciver I, Latimer JL et al. A major outer membrane protein of Moraxella catarrhalis is a target for antibodies that enhance pulmonary clearance of the pathogen in an animal model. Infect Immun 1993; 61: 2003–10.

72 Stutzmann Meier PS, Heiniger N, Troller R et al. Salivary antibodies directed against outer membrane proteins of Moraxella catarrhalis in healthy adults. Infect Immun 2003; 71: 6793–8.

73 Meier PS, Troller R, Heiniger N et al. Moraxella catarrhalis strains with reduced expression of the UspA outer membrane proteins belong to a distinct subpopulation. Vaccine 2005; 23: 2000–8.

74 Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.