Purification and Characterization of a High-Molecular-Weight Outer Membrane Protein of Moraxella (Branhamella) catarrhalis

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Moraxella (Branhamella) catarrhalis is an important bacterial cause of otitis media in children and lower respiratory tract infections in adults. In this study, we describe the presence of a novel high-molecular-weight outer membrane protein (HMW-OMP). This protein varies from 350 to 720 kDa in apparent molecular mass among strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was detected on SDS-PAGE in 13 of 14 strains tested. We developed a monoclonal antibody and polyclonal antisera to this protein. In immunoblot assays, the protein was present in all 14 strains tested. The immunoblot assays suggest that the protein has at least one epitope that is conserved among strains. A purification method using anion-exchange chromatography is described. Treatment of outer membrane preparations and purified protein by heat and reducing agents did not change the apparent molecular mass of the HMW-OMP. Formic acid treatment of outer membrane preparations and purified HMW-OMP produced a single band with an apparent molecular mass of 120 to 140 kDa. We postulate that this may be the monomer of an oligomeric protein. The HMW-OMP, which varies in molecular mass among strains and is antigenically conserved, will be studied further to determine its role in the human immune response and may be useful as a marker in studying strain acquisition in patients.

Moraxella (Branhamella) catarrhalis, formerly Neisseria catarrhalis, is a gram-negative aerobic bacterium previously thought to be a harmless commensal organism. Over the last decade, it has become clear that this bacterium is pathogenic. The reasons for its emergence as a pathogen are unclear. In adults, this organism is an important cause of lower respiratory tract infections, particularly in the setting of chronic obstructive pulmonary disease (6, 16). M. catarrhalis causes acute exacerbations of chronic obstructive pulmonary disease, pneumonia, and laryngotracheobronchitis in this population. It is also a rare cause of sepsisemia, meningitis, and endocarditis in immunocompromised adults (3, 5).

M. catarrhalis is the third most common cause of bacterial otitis media in children, after Streptococcus pneumoniae and nontypeable Haemophilus influenzae. Otitis media is a major cause of morbidity in the pediatric population in developed countries and is the most frequent diagnosis made by health care providers in this age group in the acute health care setting (2, 22). An effective otitis media vaccine will need to provide immunity to all three organisms. M. catarrhalis is also a frequent cause of sinusitis in this age group.

Development of a vaccine requires an understanding of the host immune response and the pathogenesis of infection. Prior work on S. pneumoniae and nontypeable H. influenzae has focused on antigens on the bacterial surface as being important determinants in bacterial virulence and the host immune response and as potential vaccine candidates. Little work has been done to explore the outer membrane surface-exposed components of M. catarrhalis and their role in the pathogenesis of infection and the host immune response.

Previous studies (14) have shown that the outer membrane of M. catarrhalis contains 10 to 20 proteins. The number of outer membrane proteins (OMPs) and their apparent sizes on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (20 to 100 kDa) are similar to those of other gram-negative organisms. Unlike the case for nontypeable H. influenzae and Neisseria gonorrhoeae (which are also primarily mucosal pathogens), there is only minor variation of the electrophoretic pattern of the OMPs among strains of M. catarrhalis (12). The structures and functions of these proteins have not been well described. In addition to these eight major OMPs, immunoblot assays using rabbit polyclonal serum immunized with whole organism lysate of M. catarrhalis show a large macromolecular complex that remains near the top of 15% polyacrylamide gels (unpublished data).

The goal of this study is to describe some characteristics of this high-molecular-weight OMP (HMW-OMP) of M. catarrhalis. In addition, a method for purification of this protein is presented.

MATERIALS AND METHODS

Bacteria. Fifteen strains of M. catarrhalis were studied. One strain (25240) was an American Type Culture Collection strain, and 14 strains were clinical isolates from diverse clinical and geographic sources. Ten (four middle ear isolates, three conjunctival isolates, and three sputum isolates), were from Buffalo, N.Y., one was a sputum isolate from Houston, Tex., and three were sputum isolates from Johnson City, Tenn.

Bacteria were grown overnight on Mueller-Hinton agar or chocolate agar at 37°C in 5% CO2. The strains grew equally well on both media, and no difference in outer membrane preparations of the same strains grown on the two different media was noted. Strains were stored at about −70°C in Mueller-Hinton broth with 10% glycerol.

Purification of outer membranes. Outer membranes were prepared by a Zwittergent extraction method as previously described (13). Zwittergent OMP preparations were stored at −70°C. Storage of Zwittergent OMP preparations for longer...
than a week at −20°C resulted in the disappearance of the HMW-OMP band on SDS-polyacrylamide gels. The HMW-OMP was more stable at −20°C with use of a sarcosyl-insoluble outer membrane preparation, although outer membranes prepared in this fashion contain cytoplasmic membrane components (12). Sarcosyl-insoluble outer membrane preparations were prepared as described previously (11). Bacteria were grown overnight at 37°C in 5% CO₂ on 30 150-mm-diameter Mueller-Hinton agar plates. The bacterial lawn was scraped off the plates and suspended in 0.01 M N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), collected by centrifugation at 10,000 × g for 20 min, and resuspended in HEPES buffer. The whole cells were disrupted by sonication for 1 min at 100 W on ice. The lysed cells were centrifuged twice at 10,000 × g at 4°C for 20 min to remove unbroken cells. After the first spin, the pellet was resuspended in HEPES buffer and sonicated again to improve the yield of the final product. This second extract was then centrifuged twice at 10,000 × g at 4°C for 20 min. The supernatants were centrifuged at 100,000 × g for 1 h at 4°C. The pellet was suspended in 1% sarcosyl in HEPES buffer for 30 min at room temperature. The suspension was subjected to centrifugation again at 100,000 × g. Approximately 2 ml (4 mg of total protein) of sarcosyl-insoluble outer membranes was produced from the original 30 150-mm-diameter agar plates of bacteria. The sarcosyl-insoluble fraction was used as the starting material for the purification method and in the affinity purification of the polyclonal sera.

**Proteinase K digestion.** Some samples of whole organisms or outer membrane preparations were subjected to proteinase K digestion. Whole organisms or outer membrane preparations were suspended in sterile phosphate-buffered saline buffer to an optical density at 660 nm of 0.3. The sample was pelleted by centrifugation at 3,000 rpm for 10 min, suspended in lysing buffer (1 M Tris [pH 6.8], 4% β-mercaptoethanol [β-ME], 10% glycerol, 2% SDS), and boiled for 10 min. The sample was allowed to cool, 200 μg of proteinase K in lysing buffer was added, and the mixture was incubated at 65°C for 90 min. The mixture was pelleted by centrifugation at 3,000 rpm for 10 min. The supernatant was the proteinase K digest.

**SDS-PAGE and immunoblot assays.** Samples were subjected to SDS-PAGE on 11 or 3 to 20% gradient gels, and immunoblot assays were performed as previously described (11), using a Mighty Small (Bio-Rad Laboratories, Richmond, Calif.) vertical unit. Gradient gels were also made, using a 16-cm-long Hoefer SE 600 unit. Some of the samples of outer membrane preparations and purified HMW-OMP were treated with up to 100 mM dithiothreitol (DTT) or 10% β-ME, heated for 30 min to 100°C, to attempt to disaggregate the HMW-OMP. Some samples were also subjected to treatment with DTT and iodoacetamide as described by Newhall et al. (15). Gels were stained with Coomassie brilliant blue (11) or silver stain (19).

For immunoblot assays, samples subjected to SDS-PAGE were electrophoresed and transferred onto nitrocellulose. The nitrocellulose was blocked in 3% skim milk at room temperature for 30 min. The blot was probed with antiserum diluted in buffer A (0.01 M Tris, 0.15 M NaCl [pH 7.4]). After an overnight incubation at room temperature, the nitrocellulose was incubated with protein A-peroxidase at a 1:3,000 dilution or goat anti-mouse immunoglobulin M at a dilution of 1:1,000. These preparations were developed either with horseradish peroxidase color developer solution (Bio-Rad) or with a chemiluminescence kit (Hoefer Scientific Instruments, San Francisco, Calif.).

**Development of polyclonal antisera.** Zwittergent extracts of strains 4223 and 25240 were subjected to SDS-PAGE on 3 to 20% laneless gradient gels. The gels were stained with Coomassie brilliant blue, and the HMW-OMP band was cut from the gel. The cut band was emulsified with 1 ml of incomplete Freund’s adjuvant and injected subcutaneously into a rabbit. The rabbit was immunized on days 0, 14, and 28, and antiserum was harvested on day 30.

**Affinity purification of rabbit anti-HMW-OMP sera.** Because both the rabbit pre- and postimmunization sera contained antibodies to multiple bands on immunoblot assays, antibody to the HMW-OMP was affinity purified by using a modification of a procedure described by Olmsted (17). A Zwittergent extract of the homologous strain was subjected to SDS-PAGE on a 3 to 20% gradient gel and transferred to nitrocellulose. The nitrocellulose was blocked in 3% skim milk for 30 min at room temperature and was incubated overnight at room temperature with the antiserum at a 1:500 dilution in buffer A. Vertical strips from either end of the blot were cut and developed by using protein A-peroxidase and horseradish peroxidase color developer solution, and the location of the HMW-OMP band was determined. A horizontal strip, corresponding to the location of the band, was cut from the undeveloped blot. Antibodies were eluted from the nitrocellulose by incubation in 0.2 M glycine (pH 2.5) for 2 min. The solution was neutralized with 2 M Tris (pH 8.5) and dialyzed against buffer A overnight at 4°C.

**Development of monoclonal antibodies.** In an effort to develop monoclonal antibodies to potentially serotype-specific determinants, mice were immunized by using a schedule modeled after that of Hockfield (8). Outer membranes of *M. catarrhalis* 8184 and 56 were purified by the Zwittergent extraction method (13). Neonatal BALB/c mice were immunized intraperitoneally with 250 μg of purified outer membrane of strain 8184 on days 1, 3, 5, and 7 following birth. One mouse was then immunized with 250 μg of purified outer membrane of strain 56 on days 28, 33, 37, and 41. On day 44 of life, the splenocytes were harvested and fused with Sp2/0-Ag14 plasmacytoma cells by previously described methods (10, 18). Tissue culture supernatants from hybridomas were screened initially by immunodot assays using whole cell lysates of four strains of *M. catarrhalis*.

**Formic acid denaturation of the HMW-OMP.** Sarcosyl-insoluble outer membrane preparations were subjected to electrophoresis on laneless 9% polyacrylamide gels and stained overnight with Coomassie blue. After destaining, the HMW-OMP was cut from the gel and lyophilized overnight. The gel strips were then placed in 1 ml of 70% formic acid and incubated overnight at room temperature in darkness. The next morning, the liquid was removed from the tubes containing the gel strips and diluted with 1 ml of distilled H₂O. These samples were lyophilized overnight. SDS-PAGE sample buffer was added to the resulting powder, heated for 5 min to 100°C, and subjected to electrophoresis (9). Purified HMW-OMP produced by the method described below was also subjected to formic acid denaturation.

**Purification of the HMW-OMP.** Organisms were grown overnight at 37°C with 5% CO₂ on 30 150-mm-diameter Mueller-Hinton plates. Sarcosyl-insoluble outer membrane preparations were made as described above. One milliliter of
sarcosyl-insoluble pellet was solubilized in 1.5% sodium deoxycholate-0.05 M Tris-0.2 M NaCl-0.05 M EDTA (pH 9.0) (1.5% deoxycholate buffer) for 10 min at room temperature. This mixture was centrifuged at 10,000 × g for 20 min at 4°C; the resulting pellet was suspended in 5% Zwittergent in 0.75 M Tris (pH 8.0). This mixture was applied to a 30-ml DEAE-Sephacl (Pharmacia, Piscataway, N.J.) column equilibrated with buffer Z (0.05% Zwittergent, 0.05 M Tris, 0.01 M EDTA [pH 8.0]). After washing of the column with 2 column volumes of buffer Z, proteins were eluted from the column by using a 0 to 1 M NaCl linear gradient in buffer Z (total gradient volume of 100 ml) followed by a washout with 2 column volumes of buffer Z containing 1 M NaCl. The column fractions were monitored at an optical density of 280 nm, and the peak fractions were pooled, ethanol precipitated, and subjected to SDS-PAGE. All buffers for this procedure contained 1 mM phenylmethylsulfonyl fluoride.

Protein concentrations were determined by using the bichinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, Ill.).

RESULTS

Presence of the HMW-OMP among strains. Fourteen strains were studied by SDS-PAGE and immunoblot assay. Zwittergent-extracted outer membranes from 13 of the 14 strains had evidence of a high-molecular-weight band when gels were stained with either silver or Coomassie blue stain. The apparent molecular masses varied among strains from approximately 350 to 720 kDa (Fig. 1). The Mr for the HMW-OMP of a particular strain was repeatedly the same when different preparations of the strain were compared. Samples boiled in sample buffer containing 1% SDS and up to 100 mM DTT or 10% β-ME for up to 30 min were subjected to SDS-PAGE. There was no apparent loss of the HMW-OMP band, nor was a new band present, nor did a band become more prominent when samples subjected to boiling and reducing agents were compared with unheated, unreduced samples. Treatment with 40 mM DTT and 100 mM iodosacetamide as described by Newhall et al. (15) produced inconsistent results that were not reproducible, usually because of nonspecific proteolysis. One strain (238) had no apparent band on Coomassie blue- or silver-stained gels but did have a reactive band upon immunoblotting with affinity-purified anti-4223 HMW-OMP and monoclonal antibody 9E9 (Fig. 2).

To determine whether the HMW-OMP is a protein, whole organism lysates and sarcosyl-insoluble outer membrane preparations were treated with proteinase K. The HMW-OMP band disappeared from proteinase K-treated samples but not from controls on SDS-PAGE and gels stained with Coomassie blue or silver stain. This series of experiments showed that since the HMW-OMP band is proteinase K sensitive, the HMW-OMP band seen on SDS-PAGE was a protein (Fig. 3).

Characterization of monoclonal antibody 9E9. Mice were immunized as described above, a fusion was performed, and hybridoma supernatants were tested by immunodot assays for the presence of antibodies to whole organism lysates of four strains of M. catarrhalis, including 8184 and 56. Initial screening identified a hybridoma, labeled 9E9, which produced positive immunodot assays with all four strains. Antibody 9E9 was identified as an immunoglobulin M isotype.

To identify the antigen which antibody 9E9 recognizes, an immunoblot assay using a whole organism lysate of strain 56 was performed. The antibody bound to a high-molecular-weight band. The outer membrane of several strains of M. catarrhalis was purified (13) and subjected to immunoblot assay. Antibody 9E9 recognized a high-molecular-weight band in the outer membrane of each strain (Fig. 2). This band corresponded to the HMW-OMP. Indeed, antibody 9E9 recognizes purified HMW-OMP in immunoblot assays. Sometimes when sarcosyl-insoluble outer membrane preparations were subjected to electrophoresis on gradient gels, immunoblotted, and probed with 9E9, a broad low-molecular-weight band was also noted along the bottom edge of the nitrocellulose paper. This band disappeared with treatment of the sarcosyl-insoluble outer membrane preparation with proteinase K and may represent a breakdown product of the HMW-OMP or another OMP with a shared epitope.

FIG. 1. Zwittergent outer membrane preparations of seven strains of M. catarrhalis on a 3 to 20% gradient gel stained with Coomassie blue. All specimens were heated to 100°C for 5 min in sample buffer containing 1% β-ME. Approximately 50 µg of protein was loaded in each lane. The HMW-OMP is noted with arrowheads. Strains: lane A, M7; lane B, 48; lane C, 238; lane D, 8184; lane E, 10205; lane F, 10277; lane G, 25240; lane H, sarcosyl-insoluble outer membrane preparation of strain 4223. Molecular mass standards in kilodaltons are noted at the left.

FIG. 2. Immunoblot assay of Zwittergent outer membrane preparations of nine strains of M. catarrhalis. Samples containing ~50 µg of protein were heated to 100°C for 5 min in sample buffer containing 1% β-ME and subjected to SDS-PAGE on a 3 to 20% minigel. The immunoblot assay mixture was incubated overnight with 9E9 tissue culture supernatant and developed with protein A-peroxidase. Strains: lane A, 10632; lane B, 10630; lane C, 9528; lane D, 9790; lane E, 238; lane F, 135; lane G, 48; lane H, 32; lane I, 21. The HMW-OMP is noted near the top of the gel with small arrowheads. The band visible in some lanes marked with the large arrowhead above the 94-kDa marker may be the monomer form or a partial cleavage product. The less distinct band at the bottom of the gel is either a partial cleavage product or another protein with a similar epitope. It disappears with proteinase K digestion of the samples. Molecular mass standards in kilodaltons are at the right.
To determine the strain specificity of the epitope recognized by antibody 9E9, immunodot assays of 51 clinical isolates of *M. catarrhalis* of diverse clinical and geographic sources were performed. Antibody 9E9 was reactive in immunodot assays in 31 of 51 strains tested.

**Characterization of the HMW-OMP with affinity-purified polyclonal sera.** Rabbit polyclonal antibody (Fig. 4) which was eluted from the HMW-OMP of the homologous strain was studied in immunoblot assays to determine whether the HMW-OMP from different strains shared antigenic cross-reactivity. The affinity-purified anti-25240 HMW-OMP recognized 8 of 11 strains tested in immunoblot assays. Affinity-purified anti-4223 recognized 9 of 11 strains tested. These data and the results of immunosassays with 9E9 show that antigenic determinants on the HMW-OMP are shared among strains.

**Purification of the HMW-OMP.** The sarcosyl-insoluble fraction of an *M. catarrhalis* whole cell lysate was used as the starting material for this method. These preparations were preferable to Zwittergent extracts of whole cell lysates because a significant portion of the HMW-OMP was not solubilized. The protein also underwent proteolytic breakdown more readily in Zwittergent OMP preparations than in sarcosyl-insoluble preparations. The 1.5% sodium deoxycholate buffer solubilized other contaminating proteins and lipooligosaccharides present in the sarcosyl-insoluble fraction; the HMW-OMP was insoluble. Solubilization of the HMW-OMP was a major obstacle in its purification. Several detergents, including Triton X-100, Brij-35, *n*-octyl-β-d-glucoside, 3-[(3-cholamidopropyldimethyl-ammonio)-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)dimentyl-ammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), Eulgent (Calbiochem, San Diego, Calif.), and SDS at various pHs and concentrations, were tried to either solubilize the HMW-OMP or to remove other contaminating proteins. Most of the HMW-OMP was not solubilized during the sarcosyl outer membrane preparation, and most of the protein was discarded after the sonication step. Once the sarcosyl preparation had been extracted with sodium deoxycholate buffer, 5% Zwittergent 3-14 in a Tris solution (pH 8.0) reliably solubilized the protein. When this solution was applied to a DEAE-Sephas column, the other contaminating proteins were eluted from the column either in the buffer Z wash or at low-molarity salt concentrations. The HMW-OMP eluted as a single peak at approximately 1 M NaCl concentrations (Fig. 5 and 6). Typically, from 2 mg of sarcosyl-insoluble outer membrane preparation, 80 to 90 µg of purified HMW-OMP was recovered.

**Formic acid denaturation of the HMW-OMP.** Since reducing agents and heat failed to disaggregate the HMW-OMP, formic acid was used to denature the protein (4). Formic acid treatment of either the HMW-OMP purified by chromatography or the HMW-OMP band cut from a gel resulted in the appearance of one band with an apparent molecular mass of 120 to 140 kDa (Fig. 7). This band may represent the monomeric form of the HMW-OMP.
DISCUSSION

This study reports presence of a novel high-molecular-weight complex found on the outer membrane of all strains of *M. catarrhalis* tested. We have determined that it is a protein which varies in apparent *M* from strain to strain and is not disrupted into smaller subunits by using typical methods such as heat and reducing agents. Formic acid treatment of the purified protein reveals the presence of a single band with an apparent molecular mass of 120 to 140 kDa on SDS-PAGE. We have also developed a purification method for this protein.

Other organisms, such as *N. gonorrhoeae*, have a high-molecular-weight protein. The high-molecular-weight complex of *N. gonorrhoeae* has been named the OMP-macromolecular complex (15, 21). This 800-kDa complex is composed of 10 to 12 identical subunits of a peptide with an apparent molecular mass of 76 kDa on SDS-PAGE. The OMP-macromolecular complex of *N. gonorrhoeae* is surface exposed and present in all strains tested. Patients with disseminated gonococcal infections mount an immune response to this protein, and at least one antigenic determinant is conserved among the strains that have been tested (1, 7, 20).

Two lines of evidence indicate that the HMW-OMP is a protein: (i) its SDS-PAGE staining characteristics with use of Coomassie blue and (ii) the disappearance of the band after proteinase K digestion.

Incubation of the purified HMW-OMP of *M. catarrhalis* in formic acid causes the protein to separate as a single band of approximately 120 kDa in SDS-PAGE. It is likely that formic acid disrupts oligomers and that the band represents the monomer of the HMW-OMP. Formic acid has been used previously in this manner to disaggregate large protein complexes, such as fimbrial proteins (4). Alternatively, formic acid may cause breaking of covalent bonds and cleavage into peptides. However, this is less likely because a single band is observed following formic acid treatment. In addition, polyclonal rabbit antiserum eluted from the fully assembled HMW-OMP recognizes the band at approximately 120 kDa in formic acid-treated samples. Finally, antibody 9E9 recognizes a faint band at approximately 120 kDa in addition to the HMW-OMP in immunoblots of outer membrane preparations. This faint band likely represents a small amount of monomer. Therefore, we conclude that the monomer size of the HMW-OMP is likely 120 to 140 kDa. Definitive proof will await the cloning of the gene which encodes the protein.

The apparent *M* by SDS-PAGE of the HMW-OMP varies from strain to strain. It is unlikely that this heterogeneity in size is due to nonspecific aggregation of smaller proteins. Nonspecific aggregates would probably not survive as a single protein band on SDS-PAGE or immunoblot assay after treatment with SDS, β-ME, heat, or formic acid. The observation that the HMW-OMP of each strain has a reproducible size could be due to variation of the *M* of the monomeric protein; alternatively, each strain may have a different number of monomers making up an oligomeric HMW-OMP. It is difficult to determine from the present data whether the variation in the HMW-OMP size among strains is due to differences in monomer subunit size or because of a strain-to-strain variation in the number of monomeric protein molecules which form the HMW-OMP seen by SDS-PAGE. The ability of the affinity-purified anti-HMW-OMP antiserum and monoclonal antibody 9E9 to react to most of the strains in immunoblot assays is supportive of the HMW-OMP being antigenically conserved if not also the monomeric protein being conserved among strains.

Bacterial proteins with molecular masses of 200 kDa or greater are generally oligomers of one or more subunits. Another explanation for a large molecular size as determined on SDS-PAGE is aberrant movement through the gel system.
due to charge and conformation. Denaturation of secondary and tertiary structure of the protein by formic acid may lead to a change in apparent molecular mass, but this is usually an apparent increase of the mass due to linearization of the protein. Also, the shift in apparent molecular mass seen is not usually this large (from 350 to 720 to 120 to 140 kDa). Additionally, glycosylated proteins move aberrantly through gel systems. This explanation cannot be entirely ruled out for the HMW-OMP. Glycosylated proteins are uncommon in bacteria and are not a likely explanation of the large size of the HMW-OMP on SDS-PAGE. Further experiments will need to be performed to determine whether the HMW-OMP is glycosylated.

The purification method described here has yielded a peak of approximately 4% of the total protein in the sarcosyl preparation that is used as the starting material for the anion-exchange chromatography method. Most of the protein is lost in the initial steps of the sarcosyl extraction method. Better solubilization of the protein will be required, as larger quantities of the protein will be necessary to perform additional structural and functional studies.

The HMW-OMP is a novel outer membrane protein of M. catarrhalis that is antigenically conserved among the 14 strains tested. It is one of the few OMPs of M. catarrhalis that varies in its apparent Mr among strains, although this variation may be due only to variation in monomer number comprising the complex or monomer size. A protein that varies among strains may be useful as a marker to track acquisition of strains in patients. A protein which is antigenically conserved among strains, such as the HMW-OMP, requires further study to determine whether it is surface exposed and to characterize its role as an antigen in the human immune response. Work in these areas will determine whether this protein will be useful as a vaccine component to prevent infections due to M. catarrhalis.

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