Detection of Virulence Factors in a Corneal Isolate of *Klebsiella pneumoniae*

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Objective: To report on the microbiological findings of a *Klebsiella pneumoniae* strain isolated from a patient with keratitis.

Design: Interventional case report.

Intervention and Testing: Conjunctival swabs and corneal scrapings from the right eye were inoculated for culture. The isolate was analyzed for the presence of the mucoid phenotype and the ability to form biofilm. We also investigated whether the formation of biofilm by the corneal *Klebsiella* isolate is affected by *N*-acetylcysteine.

Main Outcome Measures: Culture results and biofilm production were analyzed.

Results: *K. pneumoniae* was grown from the conjunctiva and cornea. The isolate showed the mucoid phenotype and strong biofilm production. *N*-acetylcysteine had an inhibitory effect on both biofilm formation and preformed biofilm.

Conclusions: *K. pneumoniae* can cause severe keratitis. The presence of virulence factors, such as the mucoid phenotype and the ability to form biofilm, may be important in determining corneal infection. *N*-acetylcysteine is a potential candidate for use as an inhibitor of *Klebsiella* biofilm formation. *Ophthalmology 2005;112:xxx © 2005 by the American Academy of Ophthalmology.*

The genus *Klebsiella* belongs to the family *Enterobacteriaceae*. It consists of gram-negative, facultatively anaerobic, encapsulated, nonmotile rods or coccobacilli. Seven species can be identified: *Klebsiella pneumoniae*, *K. pneumoniae* subspecies *ozaenae*, *K. pneumoniae* subspecies *rhinoscleromatis*, *Klebsiella oxytoca*, *Klebsiella ornithinolytica*, *Klebsiella planticola*, and *Klebsiella terrigena*. *Klebsiella* is widespread throughout the environment and is commonly carried on human skin, in the nasopharynx, and in the bowel. It is a well-recognized opportunistic pathogen, causing nosocomial infections such as pneumonia, sepsis, and urinary tract and wound infections. Feces are the most significant source of infection. Approximately one third of patients carry *Klebsiella* in their stools, but carriage rates may increase as much as 3-fold with hospitalization and antibiotics use in adults.[1]

Klebsiella is an uncommon ocular pathogen. Devastating endogenous endophthalmitis from systemic sepsis is the most serious manifestation.[2,3] *K. pneumoniae* also has been reported to cause endophthalmitis from contaminated donor corneas after penetrating keratoplasty[4] and as a result of corneal ulcers,[5–8] neonatal conjunctivitis,[9,10] and chronic conjunctivitis.[11]

Disease-associated *Klebsiella* strains isolated from extraocular specimens possess important virulence factors, such as the toxic lipopolysaccharide (LPS), the capsular polysaccharide (K antigen), the mucoid phenotype, fimbriae, and biofilm production.[12–17] To our knowledge, there have been no studies on virulence factors in corneal *Klebsiella* isolates. We report herein a case of *K. pneumoniae* keratitis in a pseudophakic patient. The isolate was analyzed for the presence of virulence determinants, such as the mucoid phenotype and the ability to form biofilm. We also investigated whether the production of biofilm by the corneal *Klebsiella* isolate is affected by *N*-acetylcysteine, a drug with mucous-dissolving properties that recently has been shown to influence growth, extracellular polysaccharide production, and bacterial biofilm formation.[18]

Case Report

A 76-year-old pseudophakic man was referred in October 2003 with a 2-day history of pain, redness, watering, and blurred vision in the right eye. There was no history of ocular trauma. He had been experiencing chronic bronchitis since 1975 and systemic hypertension since 1992. He had undergone right lower lid entropion surgery in 1996 and uneventful cataract phacoemulsification with posterior chamber intraocular lens implantation in both eyes in 2000.

On examination, best-corrected visual acuity was hand motions in the right eye and 20/20 in the left eye. Slit-lamp examination of the right eye showed severe meibomian gland dysfunction with irregularities of the lid margins, conjunctival hyperemia, and purulent discharge. The right cornea had an upper paracentral ulcer approximately 3 mm in diameter, with underlying suppurative stromal infiltration also involving the deep layers. The anterior chamber showed 4+ cells and 3-mm hypopyon. A posterior chamber intraocular lens was disclosed in both eyes. A presumptive diagnosis of microbial keratitis was made.

Conjunctival swabs and corneal scrapings were used directly to inoculate fresh blood, chocolate, McConckey, and Sabouraud's

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agar plates and thioglycolate broth.[19] Gram stains of the corneal scrapings revealed gram-negative rods. Cultures from the conjunctiva and cornea yielded a heavy growth of gram-negative bacilli, later identified biochemically as *K. pneumoniae*. Antibiotic susceptibility testing determined by agar disk diffusion (Kirby-Bauer method) revealed that the isolates were susceptible to amikacin, gentamicin, tobramycin, netilmicin, ciprofloxacin, piperacillin, amoxicillin/clavulanic acid, ceftriaxone, cefotaxime, ceftazidime, cephalothin, aztreonam, imipenem, and trimethoprim-sulfamethoxazole.

Given the severe corneal involvement, soon after specimen collection, a regimen of oral ciprofloxacin (500 mg) and intramuscular piperacillin (2 g) twice daily, topical fortified tobramycin (1.5%) hourly, ciprofloxacin (0.3%) 6 times daily, and atropine (1%) twice daily was begun. Subconjunctival gentamicin (20 mg) also was given during the first 3 days. There was gradual clinical improvement over the next 3 weeks, with complete resolution of the hypopyon and healing of the corneal ulcer. One month after the initial examination, best-corrected visual acuity in the right eye was 20/25. A stromal scar was present in the area of the previous ulcer.

Detection of Virulence Factors and Influence of *N*-Acetylcysteine on Biofilm Formation

The *K. pneumoniae* isolate was plated on trypticase soy agar and was incubated at 37 °C for 24 hours.[16] After incubation, bacterial colonies with mucoid aspect were observed.

The ability of *K. pneumoniae* to form biofilm on an abiotic surface was assayed as described previously.[17,20] The isolate was grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) with 0.25% glucose at 37 °C. The culture was diluted 1:20 in fresh brain heart infusion broth with 0.25% glucose at 37 °C, and 200 μl of this suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Iwaki, Tokyo, Japan). After 24 hours at 37 °C, the wells were washed with phosphate-buffered saline solution, dried in an inverted position, and stained with 1% crystal violet for 15 minutes. The wells were rinsed again and the crystal violet was solubilized in 200 μl of ethanol-acetone (80:20, vol/vol). The optical density at 595 nm (OD_{595}) was determined using a microplate reader (Multiskan EX; Labsystem, Helsinki, Finland). The following values were used to assess biofilm production: $OD_{595} \leq 1$ (no biofilm production), $1 < OD_{595} \leq 2$ (weak biofilm production), $2 < OD_{595} \leq 3$ (medium biofilm production), and $OD_{595} > 3$ (strong biofilm production). Assays were performed in triplicate and repeated 3 times. The *K. pneumoniae* isolate showed strong biofilm production ($OD_{595} = 3.7$).

To evaluate the effect of *N*-acetylcysteine on biofilm synthesis, 2 assays were performed. In the first experiment, the procedure described above was used, with the exception that the growth medium was supplemented with different concentrations of *N*-acetylcysteine (0.06, 0.12, 0.25, 0.50, 1, 2, 4, or 8 mg/ml). Assays were performed in triplicate and repeated 3 times. The results of biofilm formation in the presence of different concentrations of *N*-acetylcysteine are shown in Figure 1. *N*-acetylcysteine was able to inhibit *K. pneumoniae* biofilm production, which decreased from "strong" to "weak." Overall, the decrease in the OD_{595} of the biofilm was directly proportional to *N*-acetylcysteine concentration, up to a concentration of 1 mg/ml.

In the second experiment, *N*-acetylcysteine's effect on preformed biofilm was evaluated. Different *N*-acetylcysteine concentrations (0.06, 0.12, 0.25, 0.50, 1, 2, 4, or 8 mg/ml) were added to the wells after bacteria were allowed to form biofilm for 5 hours. After 24 hours at 37 °C, the wells were washed with phosphate-buffered saline solution, dried in an inverted position, and stained with 1% crystal violet for 15 minutes. The wells were rinsed again and the crystal violet was solubilized in 200 μl of ethanol-acetone (80:20, vol/vol). Biofilm production was determined as described above. Assays were performed in triplicate and repeated 3 times. The results of *N*-acetylcysteine's effect on preformed biofilm are reported in Figure 2. *N*-acetylcysteine also had an inhibitory effect on preformed *K. pneumoniae* biofilm. Overall, the decrease in the OD_{595} of the biofilm was directly proportional to *N*-acetylcysteine concentration, up to a concentration of 2 mg/ml.

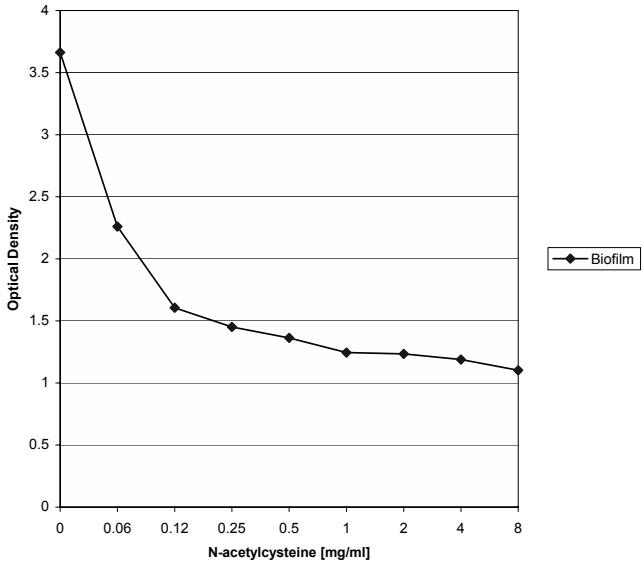

Figure 1. Effect of *N*-acetylcysteine on *Klebsiella pneumoniae* biofilm formation. Data are the means of 3 repeats in the biofilm assay.

Discussion

In this report, we describe *K. pneumoniae* corneal ulcer in a pseudophakic patient. Intensive antibiotic therapy was success-

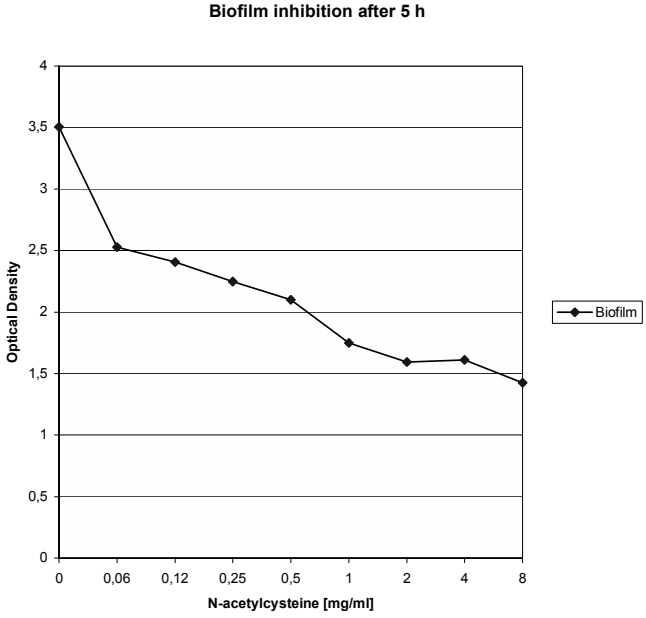

Figure 2. Effect of *N*-acetylcysteine on preformed *Klebsiella pneumoniae* biofilm. Data are the means of 3 repeats in the biofilm assay. h = hours.

ful, and the corneal lesion gradually healed over the next 3 weeks. The isolate showed the mucoid phenotype and was a strong biofilm producer. *N*-acetylcysteine had an inhibitory effect on both biofilm formation and preformed biofilm.

K. pneumoniae is an uncommon but serious cause of endogenous endophthalmitis, which is associated with a very poor visual prognosis, despite intensive antibiotic treatment.[2,3] The organism is a highly virulent intraocular pathogen, causing devastating metastatic endophthalmitis from systemic sepsis. The source of bacteremia is commonly from the urinary, hepatobiliary, and respiratory systems. Although clinical evidence indicates that the biochemical environment of the vitreous is optimal for a rapid multiplication of *K. pneumoniae*, very little is known about the presence of virulence factors in ocular *Klebsiella* isolates.

K. pneumoniae is an exceedingly rare cause of indolent corneal ulcers, usually in debilitated or immunocompromised patients or in corneas with an underlying pathologic condition.[5] This organism has been reported to cause contact lens-related keratitis in an 88-year-old patient with band keratopathy[8] and bilateral keratitis resulting in corneal perforation in an 83-year-old demented woman who received appropriate antibiotic treatment.[7] Both patients had risk factors predisposing to bacterial keratitis, including contact lens use, ocular surface disease, dementia, and old age.[6,21,22] In our report, *K. pneumoniae* caused a corneal ulcer with hypopyon in a 76-year-old patient who had risk factors for bacterial keratitis, such as chronic blepharitis and previous cataract and eyelid surgery. Overall, the clinical features of *K. pneumoniae* keratitis are nonspecific and do not permit diagnosis on clinical findings alone; however, keratitis may be severe and may result in corneal melting and perforation.

Disease-associated *Klebsiella* strains isolated from extraocular specimens possess a number of significant virulence determinants, including the gram-negative LPS, the capsular polysaccharide, mucoid phenotype, fimbriae, and biofilm production.[12–17]

In gram-negative bacteria, LPS is one of the major structural and immunodominant molecules of the outer membrane. Lipopolysaccharide consists of 3 main regions: lipid A (endotoxin), core oligosaccharide, and O-specific antigen.[12,13] It is a major virulence factor that causes inflammatory responses in tissues and gives protection against the bactericidal effect of serum. Of specific relevance to corneal disease, LPS is thought to be involved in bacterial invasion of the corneal epithelium.[23]

The strain of *K. pneumoniae* isolated from our patient showed the mucoid phenotype. The capsular polysaccharide and the mucoid phenotype are important virulence factors contributing to *K. pneumoniae* pathogenicity. The capsular K antigen protects the organism from complement-mediated serum killing and phagocytosis.[16] Some encapsulated *Klebsiella* strains form glistening mucoid colonies of viscid consistence. The degree of mucoidy has been shown to correlate positively with the establishment of infection.[16,24] The way in which the mucoid phenotype increases virulence is unclear. This phenotype may facilitate bacterial colonization of mucosal surfaces and may reduce uptake of the organism by phagocytes.[16]

In addition, many clinical *Klebsiella* isolates possess fimbrial adhesins (type 1 and 3 fimbriae), which mediate specific attachment to the host cell surface and facilitate biofilm formation.[12,14,17]

Bacterial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to the surface of tissues and biomaterials.[25,26] According to a basic model of biofilm structure, bacteria form microcolonies bound together by a copious amount of exopolysaccharide and surrounded by water-filled channels that deliver nutrients and remove waste products.[26] In vitro experiments have demonstrated that bacterial biofilms are considerably less susceptible to antibiotics than their planktonic counterparts.[27,28] In particular, the biofilm formed by *K. pneumoniae* was shown to survive prolonged antibiotic treatment with ampicillin and ciprofloxacin at concentrations that would soon kill the corresponding freely suspended bacteria.[27,28] Two principal hypotheses have been formulated to explain the reduced susceptibility of biofilms to antibiotics: the first is that antibiotics fail to penetrate biofilms fully, the second supposes that at least some of the bacteria in a biofilm enter a slow-growing or nongrowing state in which they are less likely to be killed. We are unaware of reports of biofilm producing strains of *K. pneumoniae* isolated from the eye. Although the ability to form biofilm contributes to the pathogenicity of this organism, its clinical importance in ocular *Klebsiella* infection is unknown.

The mucoid phenotype and the ability to form biofilm may have played a role in the development of *K. pneumoniae* keratitis in our patient. Indeed, these virulence determinants may mediate bacterial attachment to corneal epithelial cells, followed by microcolony formation within matrix-enclosed biofilm. Furthermore, the gram-negative LPS may be responsible for bacterial invasion of the corneal epithelium, thus allowing proliferation of the organism within the corneal tissue.

N-acetylcysteine is a mucolytic agent with antibacterial (bacteriostatic) properties.[18] The molecule is a thiol-containing antioxidant that disrupts disulfide bonds in mucus. The effect of *N*-acetylcysteine on bacteria and bacterial biofilms is still relatively unknown. Pérez-Giraldo et al[29] showed that *N*-acetylcysteine reduced the formation of biofilm by clinical isolates of *Staphylococcus epidermidis*. Furthermore, Olofsson et al[18] demonstrated that the growth of various bacteria isolated from a paper mill, including *K. pneumoniae*, was inhibited at different concentrations of *N*-acetylcysteine. In our study, *N*-acetylcysteine had a dose-dependent inhibitory effect not only on biofilm formation but also on preformed biofilm produced by a corneal isolate of *K. pneumoniae*. Altogether, these data suggest that *N*-acetylcysteine may be an interesting candidate for use as an inhibitor of *K. pneumoniae* biofilm formation. The presence of *N*-acetylcysteine may disrupt the texture of *Klebsiella* biofilm and make the organism more susceptible to antibiotics. Further in vivo studies are necessary to establish what dose and frequency of dosing would be necessary for treatment of a *K. pneumoniae* corneal ulcer.

The choice of antibiotics to treat ocular *Klebsiella* infection must take into consideration the increasing prevalence of isolates resistant to penicillins, first-generation cephalo-

sporins, and gentamicin. Third-generation cephalosporins, fluoroquinolones, and aminoglycosides, such as tobramycin and netilmicin, are appropriate first-line drugs.[1] In the case reported here, early aggressive treatment with systemic ciprofloxacin and piperacillin, topical tobramycin and ciprofloxacin, and subconjunctival gentamicin resulted in slow but progressive healing of the corneal ulcer. Conversely, in the case described by Aung and Chan,[7] *K. pneumoniae* keratitis progressed to corneal perforation despite the use of ciprofloxacin, to which the organism was susceptible. Likewise, previous reports of endogenous *Klebsiella* endophthalmitis have shown poor visual outcome, despite the use of intravenous and intravitreal antibiotics to which the organisms isolated were susceptible.[3,30] Furthermore, endogenous endophthalmitis may still develop in patients already receiving appropriate intravenous antibiotics for *Klebsiella* sepsis.[31] In a recent study, Ang et al.[3] reviewed the literature regarding the successfully treated cases of endogenous *Klebsiella* endophthalmitis and found that only 4 of 72 patients (5.5%) achieved good vision. A common factor in these successfully treated patients was early diagnosis and treatment, which was probably the major contributing factor toward a favorable visual outcome.

The ability of *K. pneumoniae* to form antibiotic-resistant biofilm might explain the poor results seen in endogenous endophthalmitis[3] and in the *Klebsiella* keratitis described by Aung and Chan.[7] The fact that the strain of *K. pneumoniae* isolated from our patient was a strong biofilm producer and that response to appropriate antibiotic therapy was slow seems to favor this hypothesis.

It is difficult to draw conclusions about the best treatment regimen for *Klebsiella* keratitis from our case study and the small number of other reports described in the literature. Indeed, what was effective in one case may not bear out with other similar infections. Overall, topical fluoroquinolones and fortified aminoglycosides, given early, seem to be the best treatment for corneal ulcers caused by *Klebsiella* organisms. Subconjunctival aminoglycosides and systemic ciprofloxacin or third-generation cephalosporins may be considered in severe cases when there is impending perforation or frank perforation of the cornea.

Ophthalmologists should bear in mind that *K. pneumoniae* can cause severe keratitis, especially in the elderly. The mucoid phenotype and the ability to form biofilm are important virulence factors. *N*-acetylcysteine has an in vitro dose-dependent inhibitory effect on *K. pneumoniae* biofilm production. However, further studies are necessary for a better understanding of the mechanism by which this opportunistic pathogen may cause keratitis and to confirm the inhibitory effect of *N*-acetylcysteine on biofilm production in vivo.

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