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In vitro Activity of Sagamicin against Ocular Bacterial Isolates

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Key Words
Sagamicin · Ocular infections · Bacterial adhesion · Slime-producing staphylococci · Intraocular lenses and infections

Abstract
The in vitro antibacterial activity of sagamicin, gentamicin, tobramycin and norfloxacin was evaluated against 180 recent clinical isolates obtained from patients with ocular infections. Good activity was demonstrated for the 3 aminoglycosides against methicillin-sensitive *Staphylococcus aureus* and *Staphylococcus epidermidis.* All 4 compounds showed a lower activity against methicillin-resistant staphylococci. Sagamicin was highly effective against enterobacteriaceae with a MIC_{90} of 2 mg/l and presented good antipseudomonal activity similar to that of gentamicin. Intraocular lenses impregnated with a sagamicin solution showed a good antistaphylococcal activity immediately after preparation. In our strain collection, the ability to produce slime was more frequent among methicillin-resistant *S. epidermidis* strains than methicillin-sensitive *S. epidermidis* (85 versus 70%). The attachment of 2 *S. epidermidis* strains to plastic surfaces was partially prevented by sagamicin subinhibitory concentrations. On the contrary, sub-MIC levels of norfloxacin increased the adhesion of *S. epidermidis.*

Introduction

Bacterial eye infections may be caused by a wide variety of gram-positive and gram-negative microorganisms. Common pathogens associated with bacterial conjunctivitis include *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Haemophilus influenzae* and *Neisseria gonorrhoeae* [1]. Bacterial keratitis is most often caused by *S. aureus, Pseudomonas aeruginosa,* coagulase-negative staphylococci and sparingly enterobacteriaceae. *P. aeruginosa* is commonly associated with soft contact lens infections or contaminated solutions [2]. Sagamicin is widely used as a topical agent for the treatment of external ocular infections [3, 4]. As this compound has now been available for over 10 years, it seems appropriate to reassess its activity against pathogens, especially those which were isolated from ocular sources. Foreign bodies are often implied in the pathogenesis of ocular infections, following either trauma or surgical procedures such as implantation of an intraocular lens (IOL) [5]. Pathogens with adhesive properties, such as staphylococci and *P. aeruginosa,* particularly slime-producing strains, are the most common organisms associated with keratitis [6]. We evaluated the occurrence of slime production in 40 *Staphylococcus epidermidis* strains isolated from patients with ocular infections. As a second step of our study, the effects of subinhibitory concentrations of

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sagamicin and norfloxacin on bacterial adhesion are determined in slime-producing staphylococci, and the antibacterial activity of IOLs impregnated with sagamicin and norfloxacin was studied.

Materials and Methods

Susceptibility Test

Bacterial Strains. Organisms used in this study were isolated mainly from specimens sent by the Ophthalmology Department of IRCCS S. Matteo and were subdivided as follows: (1) methicillin-sensitive (MS) *S. aureus* (MSSA), 20 strains; (2) methicillin-resistant (MR) *S. aureus* (MRSA), 20 strains; (3) MS *S. epidermidis* (MSSE), 20 strains; (4) MR *S. epidermidis* (MRSE), 20 strains; (5) *Klebsiella pneumoniae,* 20 strains; (6) *Enterobacter* spp., 20 strains; (7) *Serratia* spp., 20 strains; (8) *Escherichia coli,* 20 strains, and (9) *P. aeruginosa,* 20 strains.

Antimicrobial Agents. Three aminoglycosides (sagamicin, gentamicin, tobramycin) and a quinolone (norfloxacin) were tested. Gentamicin, tobramycin and norfloxacin were obtained from Sigma S.p.A. (Italy) and sagamicin from Tubilux S.p.A. (Italy).

Methods. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined using the broth dilution method in cation-adjusted Mueller-Hinton broth according to guidelines of the National Committee for Clinical Laboratory Standards [7].

Evaluation of S. epidermidis *Slime Production*

A semiquantitative method was used to study the slime production of 20 MSSE and 20 MRSE strains [8]. Single colonies were picked and grown for 24 h in plastic tubes containing 10 ml of trypticase soy broth (TSB; Oxoid, UK) supplemented with 0.25% glucose and 0.25% casamino acid (Difco, USA). The tubes were emptied, rinsed twice with phosphate-buffered saline (PBS; Biomérieux, France) and stained with safranin. The internal walls of the tubes were washed twice with PBS and, through visual estimation the bacterial biofilm color intensity was evaluated and quantified as: – = no slime production; + = weak slime production; ++ = strong slime production, and +++ = very strong slime production.

Effect of Sagamicin and Norfloxacin Subinhibitory Concentrations on the Adhesion of Slime-Producing S. epidermidis

We applied a quantitative spectrophotometric technique to study the adhesion of 6 slime-producing *S. epidermidis* strains to polystyrene tissue culture plates (Flow S.p.A., Italy) [9]. The MICs of sagamicin and norfloxacin for the strains tested were determined by the broth dilution method and resulted in 0.25 mg/l. Overnight cultures of the 6 strains in TSB were diluted 1/1,000 in fresh TSB supplemented with 0.25% glucose and 0.25% casamino acid. These dilutions were further diluted by adding an equal volume of sagamicin or norfloxacin solutions to obtain final concentrations equal to the $^1/_2$, $^1/_4$, $^1/_8$ and $^1/_{16}$ MIC of the strain. Aliquots of 0.2 ml of each one of these solutions were placed into sterile tissue culture plate wells in quadruplicate. After 24-hour incubation at 37°C the content of the wells was removed by aspiration and the wells were rinsed twice with 0.2 ml of PBS at pH 7.2. The adherent microorganisms on the surface of each well were fixed by Bouin's fixative and stained with Hucker's crystal violet. The wells were again rinsed twice with PBS and, after drying, the optical density (OD) at a wavelength of 570 nm was measured with a micro ELISA autoreader (Bio-Rad Laboratories, Italy) in order to quantify the adherent bacterial film.

Antibacterial Activity of IOLs Impregnated with Sagamicin and Norfloxacin

The antibacterial activity of foldable and non-foldable IOLs impregnated with sagamicin and norfloxacin was evaluated by a modification of the standard agar diffusion test. Foldable lenses (Acrsof® IOL) were obtained from Alcon Laboratories (USA); non-foldable lenses (PC57-Kratz) were obtained from Allergan Medical Optics (USA). IOLs were incubated at 37°C for 30 min in distilled water containing sagamicin at 500 mg/l or norfloxacin at 500 mg/l. The test microorganism was a recent clinical isolate of *S. epidermidis* sensitive to the agents tested (sagamicin and norfloxacin MICs $\leq$0.12 mg/l). Mueller-Hinton agar plates were inoculated with a suspension containing about 10^5 bacteria/ml. After rinsing in distilled water, the IOLs impregnated with sagamicin or norfloxacin were placed on the surface of the plates. After 24-hour incubation at 37°C the diameter of the zone of inhibition was measured and the IOLs were transferred to fresh plates which were previously prepared by the same procedure. This step was repeated until no further inhibition zone could be observed.

Statistical Analysis

The significance of the difference between the mean values of OD obtained for the assays of each concentration and the control was evaluated by Student's t test. The levels of significance were $p < 0.05$ and $p < 0.01$.

Results

The 4 antibacterial agents were tested against 80 staphylococcal strains (40 MS and 40 MR). The antistaphylococcal activity of the 3 aminoglycosides (sagamicin, gentamicin and tobramycin) appeared to be similar, since good antibacterial activity against MS staphylococci was matched by a lower efficacy against MR strains. The MIC_{50} and MIC_{90} of norfloxacin were significantly higher (table 1) than those of the aminoglycosides. Good activity was demonstrated for sagamicin, with MIC_{50} of 0.25 mg/l versus MSSA and MIC_{90} of 0.25 mg/l versus MSSE. Norfloxacin appeared to be slightly less active with MIC_{90} values against MSSA of 32 mg/l and MIC_{90} values of 2 mg/l against MSSE. On the other hand, all 4 compounds showed a low activity against MR staphylococci. When we proceeded to *E. coli* (table 2), norfloxacin appeared to be the most active drug (MIC_{90} $\leq$0.12 mg/l), followed by gentamicin, sagamicin and tobramycin (MIC_{90} 2 mg/l). The bactericidal effect of the aminoglycosides (MBC_{90} 0.5 mg/l) was higher than that of norfloxacin (MBC_{90} 16 mg/l) against *Klebsiella* strains. On the contrary, norfloxacin was found to be more active against *Enterobacter*

Table 1. Antibacterial activity of sagamicin, gentamicin, tobramycin and norfloxacin against methicillin-sensitive (MS) *S. aureus*, MS *S. epidermidis*, methicillin-resistant (MR) *S. aureus* and MR *S. epidermidis* (mg/l)

Microorganisms (number of strains)	Antibiotics	MIC_{50}	MIC_{90}	MBC_{50}	MBC_{90}	MIC range	MBC range
S. aureus MS (20)	Sagamicin	0.25	8	0.5	16	≤0.12–>0.128	0.25–>128
	Gentamicin	0.25	4	0.25	16	≤0.12–64	≤0.12–128
	Tobramycin	0.25	2	0.25	4	≤0.12–64	≤0.12–64
	Norfloxacin	1	32	2	128	0.5–32	1–>128
S. aureus MR (20)	Sagamicin	128	>128	>128	>128	0.25–>128	0.25–>128
	Gentamicin	64	>128	64	>128	0.12–>128	≤0.12–>128
	Tobramycin	32	128	32	>128	0.25–>128	0.25–>128
	Norfloxacin	64	>128	>128	>128	32–>128	128–>128
S. epidermidis MS (20)	Sagamicin	≤0.12	0.25	≤0.12	0.25	≤0.12–>128	≤0.12–>128
	Gentamicin	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12–8	≤0.12–16
	Tobramycin	≤0.12	0.25	≤0.12	0.25	≤0.12–16	≤0.12–32
	Norfloxacin	0.25	2	0.5	4	≤0.12–>128	≤0.12–>128
S. epidermidis MR (20)	Sagamicin	32	>128	128	>128	≤0.12–>128	≤0.12–>128
	Gentamicin	16	64	16	128	≤0.12–128	≤0.12–128
	Tobramycin	16	128	32	>128	≤0.12–>128	≤0.12–>128
	Norfloxacin	2	>128	2	>128	0.25–>128	0.5–>128

Table 2. Antibacterial activity of sagamicin, gentamicin, tobramycin and norfloxacin against enterobacteriaceae and *P. aeruginosa* (mg/l)

Microorganisms (number of strains)	Antibiotics	MIC_{50}	MIC_{90}	MBC_{50}	MBC_{90}	MIC range	MBC range
E. coli (20)	Sagamicin	1	2	2	4	0.5–2	0.5–4
	Gentamicin	1	2	1	2	0.5–2	0.5–4
	Tobramycin	1	2	2	4	0.5–4	0.5–4
	Norfloxacin	≤0.12	≤0.12	≤0.12	0.5	≤0.12–0.5	≤0.12–0.5
Klebsiella spp. (20)	Sagamicin	0.25	0.5	0.5	0.5	0.25–>128	0.25–>128
	Gentamicin	0.25	0.5	0.25	0.5	≤0.12–64	≤0.12–64
	Tobramycin	0.25	0.5	0.25	0.5	0.25–64	0.25–64
	Norfloxacin	≤0.12	16	≤0.12	16	≤0.12–64	≤0.12–64
Enterobacter spp. (20)	Sagamicin	0.5	1	0.5	1	0.25–1	0.25–1
	Gentamicin	0.5	1	0.5	1	0.25–1	0.25–1
	Tobramycin	0.5	1	0.5	1	0.25–1	0.25–1
	Norfloxacin	≤0.12	0.25	≤0.12	0.25	≤0.12–4	≤0.12–4
Serratia spp. (20)	Sagamicin	0.5	1	1	2	0.25–1	0.25–2
	Gentamicin	0.5	0.5	0.5	1	≤0.12–1	≤0.12–1
	Tobramycin	2	4	4	4	0.25–4	0.25–8
	Norfloxacin	≤0.12	1	≤0.12	1	≤0.12–>128	≤0.12–>128
P: aeruginosa (20)	Sagamicin	2	8	4	8	0.5–8	1–16
	Gentamicin	2	4	2	8	0.5–4	1–8
	Tobramycin	0.5	1	1	2	0.25–1	0.25–2
	Norfloxacin	0.5	4	1	8	≤0.12–32	0.5–32

Table 3. Evaluation of the slime production of 20 *S. epidermidis* MS and 20 *S. epidermidis* MR strains using the safranin test

Microorganism (number of strains)	Number (%) of strains			
	–	+	++	+++
S. epidermidis MS (20)	6 (30)	4 (20)	6 (30)	4 (20)
S. epidermidis MR (20)	3 (15)	1 (5)	5 (25)	11 (55)

– = Absence of slime production; + = weak production; ++ = strong production; +++ = very strong production.

strains ($MIC_{90} \leq 0.12$ mg/l). All the *Serratia* strains tested were inhibited by sagamicin at a concentration of 2 mg/l (MIC_{90} 1 mg/l). Tobramycin, on the other hand, had a lower inhibitory effect against *Serratia* (MIC_{90} 4 mg/l). Sagamicin was generally as active as gentamicin against *P. aeruginosa* strains (MIC_{50} 2; MIC_{90} 8 mg/l). The antipseudomonal activity of tobramycin (MIC_{90} 1; MBC_{90} 2 mg/l) was highly superior to that of the other agents.

In our strain collection of *S. epidermidis,* the ability to produce slime was more frequent among MRSE than MSSE (table 3). On the whole, a surprisingly high percentage of staphylococcal strains were found to produce slime (70% of the MSSE strains and 85% of the MRSE strains).

The effects of sagamicin and norfloxacin subinhibitory concentrations on the adhesive behavior of 6 *S. epidermidis* strains are shown in tables 4 and 5. From the observed results, one can conclude that 4 of 6 strains of *S. epidermidis* exhibited reduced adhesion in the presence of a sagamicin concentration of $^1/_2$ MIC, with a significance corresponding to $p < 0.01$ with respect to the control, and at $^1/_4$ MIC 4 of 6 strains showed reduced adhesion with a significance of $p < 0.05$. The data obtained in this work clearly demonstrate that $^1/_2$ and $^1/_4$ MIC concentrations of sagamicin can reduce the adhesion and therefore the production of a bacterial biofilm during colonization of polymeric materials for most of the *S. epidermidis* strains tested. In contrast to the behavior induced by sagamicin, norfloxacin showed the opposite effect. The majority of the strains of *S. epidermidis* increased their adhesion in the presence of different norfloxacin concentrations. Table 5 illustrates that $^1/_2$ MIC of norfloxacin enhanced adhesion ($p < 0.01$) for 4 of 6 strains, $^1/_4$ MIC enhanced adhesion ($p < 0.01$) for 3 of 6 strains, and finally, $^1/_8$ and $^1/_{16}$ MIC had the identical effect on 3 of 6 strains. At $^1/_2$ and $^1/_4$ MIC of norfloxacin, strain 4 increased the biofilm production with a slightly lower significance corresponding to $p < 0.05$, whereas the strain 5 did not exhibit significant modifications for any of the norfloxacin concentrations tested.

IOL properties (transparency, elasticity, consistence) were maintained after immersion for 30 min in tubes containing 500 mg/l of sagamicin and norfloxacin. The acrylic foldable IOLs and the non-foldable lenses pretreated with sagamicin solution (500 mg/l in distilled water) showed good antibacterial activity immediately after the preparation. As shown in table 6, by the first transfer, no zone of inhibition was observed around the sagamicin-treated lens. On the other hand, the lenses pretreated with norfloxacin did not show any anti-staphylococcal activity. No zone of inhibition was detectable after 24 h.

Discussion

Information on the susceptibility of bacterial pathogens and the efficacy of the available agents against these pathogens is important for the ophthalmologist treating ocular infection. Mild conjunctivitis is usually self-limited and is not routinely investigated. In nonsevere conjunctivitis a calcium alginate swab may be used to obtain material from the conjunctival sacs, but in severe cases scrapings of the superior and inferior tarsal conjunctive should be taken for diagnostic smears and microbial cultures. Because of the rapid evolution to perforation in keratitis, until a definite diagnosis is substantiated, diagnostic laboratory evaluation of corneal scrapings is imperative before initiating broad-spectrum antimicrobial therapy [6].

In this study, the activity of sagamicin and other antibacterial agents was tested against staphylococci and gram-negative microorganisms isolated mainly from patients with ocular infections. Our results confirm that sagamicin has a broad spectrum of antimicrobial activity at much lower concentrations than those commonly used in topical commercial preparations. The overall activity of sagamicin was good against all the strains tested, with the exception of MR staphylococci. All the aminoglycosides tested were bactericidal at or close to its MICs.

Because the most common offending microorganisms in keratitis and in postoperative ocular infections are gram-positive cocci, such as coagulase-negative staphylococci, *S. aureus* and streptococci, and gram-negative ones, such as enterobacteriaceae and *Pseudomonas* species, in our study we tested the antibacterial activity of foldable and non-foldable lenses impregnated with 2 agents, sagamicin and norfloxacin, active against gram-positive and

Table 4. Adhesion of *S. epidermidis* strains to polystyrene in the presence of sagamicin subinhibitory concentrations (data are expressed as mean of OD)

Sagamicin	*Staphylococcus epidermidis*					
	strain 1	strain 2	strain 3	strain 4	strain 5	strain 6
MIC	0.001	0.008	0.011	0.006	0.005	0.003
$^1/_2$ MIC	0.049	0.085*	0.226*	0.31*	0.139*	0.395
$^1/_4$ MIC	0.095	0.15**	0.412**	0.445	0.285**	0.312*
$^1/_8$ MIC	0.074	0.297	0.492	0.458	0.339	0.422
$^1/_{16}$ MIC	0.11	0.303	0.527	0.502	0.369	0.419
Control	0.112	0.265	0.555	0.505	0.373	0.451

Levels of significance: * $p < 0.01$; ** $p < 0.05$.

Table 5. Adhesion of *S. epidermidis* strains to polystyrene in the presence of norfloxacin subinhibitory concentrations (data are expressed as mean of OD)

Norfloxacin	*Staphylococcus epidermidis*					
	strain 1	strain 2	strain 3	strain 4	strain 5	strain 6
MIC	0.005	0.004	0.005	0.001	0.002	0.001
$^1/_2$ MIC	0.436*	0.615*	0.951*	0.375**	0.391	2.307*
$^1/_4$ MIC	0.439	0.522*	0.588*	0.39**	0.351	1.865*
$^1/_8$ MIC	0.288	0.578*	0.439**	0.321	0.322	0.97*
$^1/_{16}$ MIC	0.237	0.588*	0.514*	0.202	0.319	0.608*
Control	0.25	0.285	0.337	0.254	0.377	0.403

Levels of significance: * $p < 0.01$; ** $p < 0.05$.

Table 6. Antibacterial activity of IOLs impregnated with sagamicin and norfloxacin against *S. epidermidis* susceptible strain

IOLs	Diameter of the zone of inhibition, mm					
	sagamicin		norfloxacin		control	
	after 24 h	after 48 h	after 24 h	after 48 h	after 24 h	after 48 h
Foldable	17	0	0	–	0	–
Nonfoldable	20	0	0	–	0	–

gram-negative microorganisms. Our data showed that the antistaphylococcal activity of sagamicin-impregnated lenses persisted for 24 h, whereas norfloxacin-impregnated lenses showed no inhibitory effect. The addition of sagamicin to the IOLs may be of interest in order to provide an optimal protracted antimicrobial activity against the most common pathogens involved in postoperative and traumatic ocular infections.

Adherence to host tissue is the initial critical step in the pathogenic process of most bacterial infections [10]. Bacteria with adhesive properties, such as coagulase-negative staphylococci, *S. aureus* and *P. aeruginosa,* are commonly associated with microbial keratitis and endophthalmitis associated with IOLs [6–11]. A highly adhesive extracellular material (slime or biofilm) produced by certain strains is associated with bacterial adherence to and growth on biomaterials [12, 13]. Our data indicated a probable important role of slime production as a virulence marker for clinically significant *S. epidermidis* strains isolated from ocular infections (about 80% slime-producing strains). In this study, the in vitro adherence of bacteria was assayed to determine the effect of sublethal concentrations of sagamicin and norfloxacin on the attachment to polystyrene of 6 strains of *S. epidermidis* with well-

characterized adherence profiles. Although there was some strain-to-strain variability, treatment with sagamicin resulted in a significant decrease in biofilm elaboration. These data suggest that bacterial attachment of *S. epidermidis,* the initiating event associated with prosthetic device infection, could be partially prevented by subtherapeutic levels of sagamicin. On the contrary, according to data we had already obtained using subMICs of pefloxacin and ofloxacin, subMIC levels of norfloxacin increased the adhesion of slime-producing staphylococci [14, 15]. Other authors have indeed shown the same increase when testing subinhibitory amounts of rifampin and vancomycin [16]. A likely explanation may be found in the cell wall changes induced by subMICs of several antimicrobial drugs. This may lead to the exposure or overexpression of molecules acting as receptors which promote adhesion on biomaterials or increase the biofilm formation and growth on prosthetic surfaces. Rupp and Hamer [17] also found that bacterial attachment by *S. epidermidis* cannot be prevented by subtherapeutic levels of fluoroquinolone, glycopeptide or β-lactam antibiotics.

In conclusion, our data indicate that: (1) sagamicin has a good antibacterial activity against gram-positive and gram-negative microorganisms; (2) sagamicin could reduce the attachment of *S. epidermidis* to biomaterials, and (3) the addition of this antibiotic to ocular lenses might provide protracted antimicrobial activity.

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