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Epidemiology and Susceptibilities to Mercury Preservatives of Staphylococci Isolated from Used Eye-Drops Preserved with Thiomersal

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Abstract

Minimum inhibitory concentrations (MICs) of seven independent isolates of *Staphylococcus hominis* isolated in the same week from used eye-drops, preserved with thiomersal and collected from wards and clinics in the same hospital, ranged between 1 and 0·03 mg L^{-1} for thiomersal, 1 and 0·01 mg L^{-1} for phenyl mercuric nitrate and 10 and 3 mg L^{-1} for mercuric chloride.

Although MIC values determined on solid nutrient medium indicated a 100-fold variation in susceptibility to the bacteriostatic effect of phenyl mercuric nitrate, after 5 h in an aqueous solution containing the bactericidal concentration of 10 mg L^{-1} phenyl mercuric nitrate, the survival levels of the six *S. hominis* isolates were similar, with a mean of 13·4% (s.d. 11·0), compared with 100 and 0·8%, respectively, for the most resistant and most sensitive control staphylococcal strains tested. Antibiotic susceptibilities and plasmid profiles of the *S. hominis* isolates indicated they were the same strain.

It is concluded that laboratory indicators of preservative efficacy, such as MIC determination or susceptibility to bactericidal concentrations of preservatives, do not necessarily correlate with the epidemiology of contaminating bacterial strains or their survival in preserved pharmaceuticals.

In a study that examined a total of 556 partly-used eye-drops collected from hospital wards, clinics and out-patients, a total of 31 (5·6%) were shown to be contaminated with viable bacteria (Du Bois et al 1989). The drops had been preserved with chlorhexidine acetate 0·01%, benzalkonium chloride 0·01% or thiomersal 0·005%. Out of the seven containers (1·3%) that exhibited counts of 10^3 or greater per mL, all were thiomersal-preserved, contaminated with a Gram-positive staphylococcus, and had been collected from wards and clinics in the same hospital in the same week.

The efficacy test for preservatives in pharmaceutical systems (British Pharmacopoeia (BP) 1993) uses standard laboratory strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. To comply with the more rigorous Criterion A of acceptability for ophthalmic preparations, an inoculum of 10^6 organisms mL^{-1} should be reduced by log 2 within 6 h of challenge, and by log 3 after 24 h, with no recovery of viable organisms after 28 days. The eye-drops studied by Du Bois et al (1989) had been stored for up to 5 days before sampling, and survival of significant numbers of organisms implies a degree of resistance to the preservative higher than that of the standard strains used in the BP test. We have therefore investigated the staphylococci isolated by Du Bois et al (1989) to determine if they are indeed more resistant to mercurials than are control strains.

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Materials and Methods

Antibacterial agents

Stock solutions of phenyl mercuric nitrate, thiomersal and mercuric chloride (all from BDH Ltd, Poole) were prepared in sterile distilled water.

Bacterial strains

Strains designated D13, D18, D25, D26 and D34 were isolated from ward-used 0·1% dexamethasone eye-drops preserved with 0·005% thiomersal. Strains designated S1 and S2 came from clinic-used isotonic saline eye-drops preserved with 0·005% thiomersal. Control organisms were *Staphylococcus aureus* strain E3T, *Staphylococcus epidermidis* strain SK360, a clinically-isolated strain of *Staphylococcus saprophyticus* from Professor J. T. Smith's collection, *Staphylococcus aureus* T56 from Dr A. L. Davison, which was isolated from an eye-drop preserved with 0·01% benzalkonium chloride, *Staphylococcus hominis* NCTC 11320, and *Staphylococcus aureus* NCTC 10788, the latter being the strain used in the BP test for preservative efficacy. Eighteen-hour cultures of isolates and control strains were grown in Oxoid Nutrient Broth No. 2 (Oxoid/Unipath, Basingstoke, UK), and stored in liquid nitrogen for subsequent testing. They were revived by streaking on nutrient agar, prepared by adding lab M agar (Amersham, Bury, UK) at a concentration of 1·5% to nutrient broth.

Minimum inhibitory concentrations

These were performed on 18-h cultures grown in nutrient broth, using standard techniques (Smith 1984). One-microlitre samples of a culture and of 10^{-2} and 10^{-4} dilutions in

Table 1. Minimum inhibitory concentrations of the staphylococci strains.

Concn of antibacterial agent (mg L^{-1})	Phenyl mercuric nitrate	Thiomersal	Mercuric chloride
10			D26, S1, *S. epidermidis*, *S. aureus* T56
3			D13, D18, D25, D34, S2, *S. aureus* E3T, *S. aureus* NCTC 10788 *S. hominis* NCTC 11320, *S. saprophyticus*
1	D26, *S. aureus* T56	D26	
0·3	S1, *S. epidermidis*	S1, *S. aureus* T56	
0·1	*S. aureus* NCTC 10788	S2, *S. aureus* E3T, *S. epidermidis*, *S. hominis* NCTC 11320 *S. saprophyticus* *S. aureus* NCTC 10788	
0·03	*S. aureus* E3T *S. hominis* NCTC 11320 *S. saprophyticus*	D13, D18, D25, D34	
0·01	D13, D18, D25, D34, S2		

nutrient broth were inoculated onto nutrient agar plates, containing a range of concentrations of the compound under test, using a Denley Multipoint Inoculator (Denley Products, Billinghurst, UK). The minimum inhibitory concentration (MIC) was determined as the lowest concentration of drug that completely inhibited colony formation in the sample from the 10^{-4} dilution after 18 h incubation at 37°C. Samples of 10^{-4} dilutions applied to control plates, without antibacterial, gave between 10 and 50 colonies depending on the cell density of the 18-h cultures.

Bactericidal activities

Viable counts were performed on suspensions of organisms in solutions of antibacterial agent in Davis and Mingioli's salts solution (DM) (Davis & Mingioli 1950) at 25°C. Eighteen-hour cultures of cells grown in nutrient broth were washed in DM before use as inocula. Samples from reaction mixtures were diluted in nutrient broth containing 0·1% thioglycollic acid to inactivate mercurials before plating on nutrient agar. Viability was determined after 18-h incubation at 37°C.

Antibiotic sensitivity testing

Eighteen-hour nutrient broth-grown cultures were diluted 10^{-1} in nutrient broth, and the dilutions used to flood the surface of overdried nutrient agar plates. Once the inoculum had soaked into the medium, 6-mm antibiotic susceptibility test discs (Oxoid/Unipath, Basingstoke, UK) were applied to the surface of the agar. Diameters of zones of growth inhibition were measured after 18-h incubation at 37°C.

Isolation of plasmid DNA

Plasmid DNA was isolated according to the method of O'Sullivan & Klaenhammer (1993) and run on 0·8% agarose gels in Tris-borate buffer (pH 8·3) at 30 V for 17 h, as described by Ambler et al (1993).

Results

Minimum inhibitory concentrations

MICs of phenyl mercuric nitrate against the strains isolated from used eye-drops varied between 1 and 0·01 mg L^{-1} with strain D26 being the most resistant and strains D13, D18, D25, D34 and S2 the most sensitive (Table 1). MIC values for thiomersal varied between 1 and 0·03 mg L^{-1}, with strains exhibiting the same rank order of susceptibilities found with phenyl mercuric nitrate. MIC values of mercuric chloride were greater at 10 or 3 mg L^{-1}, but once again, strains D26 and S1 exhibited higher levels of resistance than strains D13, D8, D25, D34 or S2 (Table 1).

The eye-drop isolates, therefore, gave the widest range of MIC values against phenyl mercuric nitrate. This was also found to be the case for the control strains of staphylococci

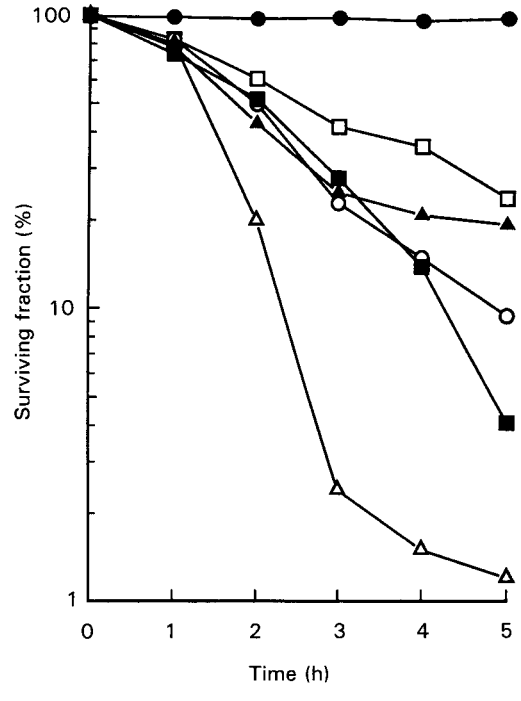


FIG. 1. Bactericidal activity of 10 mg L^{-1} phenyl mercuric nitrate solution against control strains of staphylococci. Strains used: *S. aureus* T56, ●; *S. hominis* NCTC 11320, △; *S. epidermidis* SK360, □; *S. saprophyticus*, ▲; *S. aureus* E3T, ○; *S. aureus* NCTC 10788, ■.

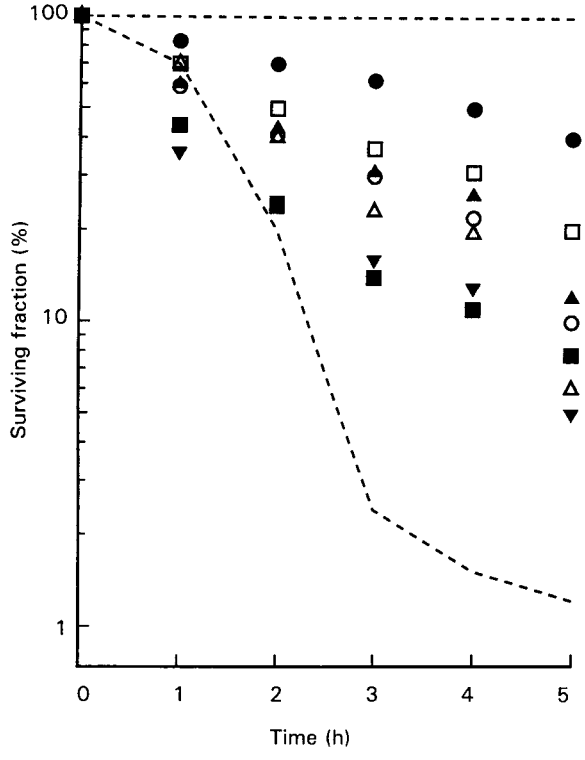


FIG. 2. Bactericidal activity of 10 mg L^{-1} phenyl mercuric nitrate solution against strains of *S. hominis* isolated from used eye-drops. Strains used: D13, ○; D18, ●; D25, ■; D26, □; D34, ▲; S1, △; S2, ▼. Dashed lines indicate survival of control *S. aureus* strain T56 (top) and *S. hominis* NCTC 11320 (bottom) from Fig. 1.

(Table 1). Mercuric chloride gave the narrowest range, but highest MIC values for both the eye-drop isolates and the control organisms (Table 1).

Susceptibilities of strains to bactericidal concentrations of phenyl mercuric nitrate

Because of the wide range of MIC values obtained with phenyl mercuric nitrate, all strains were tested for their resistance to a bactericidal concentration of this preservative. Fig. 1 shows the wide variation in susceptibility of the control strains to 10 mg L^{-1} phenyl mercuric nitrate. There is reasonable correlation of these susceptibilities with the MIC values determined on nutrient agar (Table 1). *S. aureus* T56 was the most resistant strain and had the highest MIC value, whereas *S. aureus* strain E3T and *S. hominis* strain NCTC 11320 gave the lowest MIC values and died rapidly in 10 mg L^{-1} phenyl mercuric nitrate. Fig. 1 also shows how relatively sensitive the BP test strain *S. aureus* NCTC 10788 is to phenyl mercuric nitrate solution.

The strains isolated from eye-drops had intermediate sensitivities to 10 mg L^{-1} phenyl mercuric nitrate solution (Fig. 2). The mean percentage survival of the six strains after a 5-h exposure was 13·4% with a standard deviation of 11·0%, compared with virtually 100% survival after the same time by the control *S. aureus* strain T56 and 0·8% survival of the sensitive control strain *S. hominis* NCTC 11320.

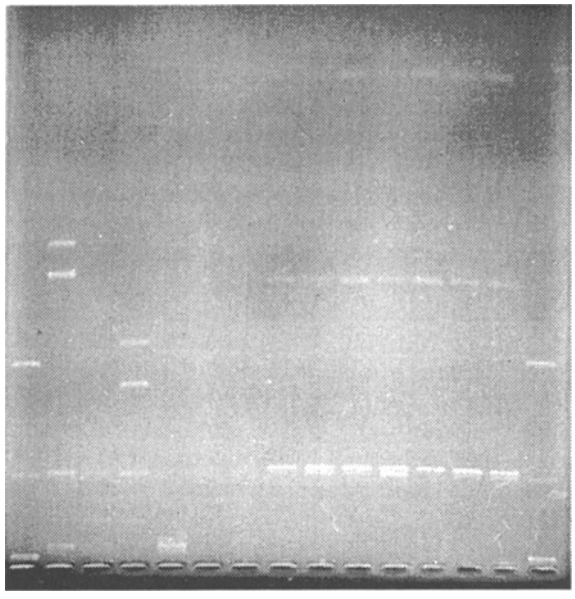
Identification and epidemiology of isolates

Although five of the strains had been isolated from hospital wards and two from hospital clinics, they were all isolated during the same week from the same hospital environment, and were identified as *Staphylococcus hominis* (Davison et al 1991). In the absence of a phage-typing system for *S. hominis*, Kloos & Lambe (1991) recommend that epidemiological investigation of the species should be carried out by comparing antibiotic sensitivities and plasmid content. Using these criteria, it was found that the seven *S. hominis* isolates gave almost identical sensitivities to a wide range of antibacterials (Table 2). The greater sensitivities to benzylpenicillin of isolates D18 and S1, which gave wider zones of inhibition than the other five strains, correlated with their similar relative sensitivities to ampicillin (Table 2). Plasmid profiles of the strains showed that, although the control staphylococcal strains gave a wide variety of plasmid bands upon gel electrophoresis of cell lysates, the seven *S. hominis* isolates all contained identical plasmid bands (Fig. 3). Note the two, approximately 40 mDa plasmids present in each isolate, together with the two other plasmids of lower molecular weights.

Table 2. Antibiograms of isolates and of control organisms. Figures are diameters of zones of inhibition (mm) around a filter paper disc (diam. 6 mm), impregnated with the antibacterial under test.

	Pen_{10}	Ap_{10}	Met_5	Cm_{30}	Km_5	Gen_{10}	Sm_{10}	Tp_{10}	Em_{10}	Tc_{10}	Su_{300}	Nov_5
S. aureus E3T	31	30	17	21	16	17	R	18	22	20	R	21
S. aureus NCTC 10788	30	28	19	22	15	16	16	17	20	19	22	21
S. aureus T56	11	11	24	23	16	15	15	15	21	20	R	21
S. epidermidis SK360	12	13	20	24	R	12	19	R	21	18	R	21
S. saprophyticus	27	26	12	25	20	21	19	R	21	22	R	R
S. hominis NCTC 11320	32	30	23	24	20	23	20	11	21	R	19	25
D13	12	13	19	24	12	18	14	24	21	23	23	22
D18	21	22	19	25	17	20	14	25	21	21	24	22
D25	12	13	19	24	13	19	15	25	25	25	23	23
D26	12	12	19	25	15	20	13	24	25	24	25	22
D34	14	15	18	25	13	20	14	25	21	22	22	23
S1	32	31	20	23	12	18	14	22	20	21	20	24
S2	11	12	19	23	12	18	14	23	25	25	24	23

Key to antibacterials: Pen = benzylpenicillin, Gen = gentamicin, Tc = tetracycline, Ap = ampicillin, Sm = streptomycin, Su = sulphonamide, Met = methicillin, Tp = trimethoprim, Nov = novobiocin, Cm = chloramphenicol, Em = erythromycin, Km = kanamycin, R = resistant: no zone of inhibition. Figures in subscript are weights of antibacterial (μg) contained in each disc.



A B C D E F G H I J K L M N O

FIG. 3. Gel electrophoresis of plasmid DNA extracted from control strains and the seven *S. hominis* eye-drop isolates. Lanes A and O: extracts of control *Escherichia coli* strain 39R861 (NCTC 50192) showing plasmids of molecular weights 98·0, 42·0 and 23·9 mDa. Lane B: *S. hominis* NCTC 11320. Lane C: *S. aureus* strain T56. Lane D: *S. epidermidis* strain SK360. Lane E: *S. saprophyticus*. Lane F: *S. aureus* E3T. Lane G: *S. aureus* NCTC 10788. Lane H: *S. hominis* isolate D26. Lane I: *S. hominis* isolate S1. Lane J: *S. hominis* isolate S2. Lane K: *S. hominis* isolate D13. Lane L: *S. hominis* isolate D18. Lane M: *S. hominis* isolate D25. Lane N: *S. hominis* isolate D34.

Discussion

The wide variation of MIC values found for the staphylococci strains isolated from thiomersal-preserved eye-drops (Table 1) illustrate how such values, determined in complex laboratory media, may not necessarily be useful predictors of bacterial survival in preserved formulations during use. Similarly, although the MIC values of phenyl mercuric nitrate for the isolates varied 100-fold, when the same strains were exposed to a lethal concentration of the preservative in a buffered salts solution, only narrow differences in strain sensitivities were recorded (Fig. 2). The isolation of what appears by antibiograms and plasmid profiles to be the same, or very closely related, strains of *S. hominis* from six independent thiomersal-preserved eye-drops during the same week from wards and clinics in the same hospital, suggest carriage of the strain by a member of the hospital personnel.

Dental amalgam is the major source of human exposure to mercury (World Health Organization 1991), and its influence on the selection of mercury-resistant oral and enteric organisms, which are also multiply-resistant to antibiotics due to plasmid-linked genes, is currently in dispute (Shearer 1993; Summers et al 1993). The possibility that mercurial preservatives may exert similar positive selection for antibiotic resistance should not be overlooked, although the data in the present paper indicates that persistence does not necessarily correlate with resistance.

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