CLINICAL MICROBIOLOGY

An examination of the clonal variants of *Serratia marcescens* that infect the eye during contact lens wear

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Serratia marcescens colonises contact lenses during wear, although the frequency of isolation is generally low (0.6% contamination rate). A method for typing the *S. marcescens* colonising the eye or contact lens was developed, based upon ribotyping, serotyping and biotyping. Twelve different types of *S. marcescens* were isolated from the eyes, contact lenses, contact lens cases and fingers of contact lens wearers in the Sydney area over a 2-year period. There was no evidence of a specific type being more readily able to colonise the contact lenses than other types. Indeed, eight *S. marcescens* strains were isolated from the lenses and these belonged to seven types. The diversity of types isolated from the eye indicates that there is probably not a subset of *S. marcescens* that can colonise the eye, although the results suggest that the types of strains isolated from contact lenses are different from those isolated from nosocomial infections.

Introduction

Serratia marcescens is an opportunist pathogen that has been implicated in a variety of nosocomial infections including septicaemia, urinary tract infections and meningitis [1]. *S. marcescens* has also been implicated in ocular infections such as keratoconjunctivitis [2], endophthalmitis [3] and keratitis [4] – all of which may be associated with loss of vision if not treated properly – and an inflammation reaction called ‘contact lens-induced acute red eye’ (CLARE) in which *S. marcescens* has been cultured in large numbers from 24% of lenses at the time of an event [5]. CLARE occurs in 7% of contact lens wearers per annum, is one of the major reasons for the discontinuation of wearing lenses and occurs specifically during the overnight extended wear of contact lenses. The symptoms of CLARE are acute pain, corneal infiltration, lachrymation, photophobia and reddening of the conjunctiva [6].

Various methods have been used to type nosocomial strains of *S. marcescens*, including biotyping [7, 8], serotyping, antibiograms [9], phage typing [10] and bacteriocin production and susceptibility [9]. These methods lack sensitivity, can be costly and may require lengthy periods of time to perform [11]. The Grimont biotyping system has been modified by

Sifuentes-Osornio *et al.* [12] and although this method is sensitive, molecular methods are needed to subdivide strains further.

There are various molecular typing methods available, including protein fingerprinting [13], zymotyping [14], plasmid analysis [15, 16], restriction endonuclease analysis [13] and ribotyping. In previous reports, protein fingerprinting appeared to lack sufficient sensitivity to distinguish between isolates [13]. Gargallo-Viola [14] found that zymotyping (the analysis of different enzymes) was sensitive enough to subdivide isolates, but the zymotypes did not correlate with the pathogenicity of strains. McGeer *et al.* [13] used restriction endonuclease analysis (REA) to type nosocomial isolates of *S. marcescens*. This method was sensitive, but it was difficult to distinguish strains due to the large number of fragments generated. Ribotyping has been used to type hospital strains [10, 17, 18]. This method was shown to be effective in discriminating between isolates; the results were easier to interpret than REA and enabled the investigators to track the spread of infection and sites of colonisation of *S. marcescens*. The types of *S. marcescens* that colonise contact lenses have not yet been determined. In nosocomial infections specific types of *S. marcescens* have been implicated in disease. The majority of isolates are non-pigmented [14], at least 24% of non-pigmented strains contain plasmids [14, 15] and the majority of isolates are of serotype O14 [8, 18].

The aim of this study was to develop an effective

method to discriminate between *S. marcescens* strains and to use this method to determine the range of clonal variants of *S. marcescens* that colonise the eye during contact lens wear.

Materials and methods

Patient details

Seventy subjects participated in the study, which was conducted at the Cornea and Contact Lens Research Unit, School of Optometry, University of New South Wales, Sydney, Australia. Twenty-six subjects used soft hydrogel contact lenses on a daily wear basis and 44 on an extended wear basis. All subjects were free of ocular and systemic disease, had had no previous ocular surgery, and required visual correction for low refractive errors only. Informed consent was obtained before lenses were fitted.

The base materials of the lenses used were etafilcon A (Vistakon, Johnson and Johnson, Jacksonville, FL, USA), a 58% water content ionic hydrogel material, and polymacon (Bausch and Lomb, Rochester, NY, USA), a 38% water content non-ionic hydrogel material. A different lens type was worn in each eye and lenses were allocated randomly. Extended wear lenses were worn on a schedule of 6 nights per week continuously, with lenses replaced weekly. Daily wear users replaced lenses fortnightly.

Lenses from outside practitioners ($n = 4$) were also sent to this laboratory for culturing, these patients wore their lenses on either a daily wear or extended wear basis.

Determination of carriage of *S. marcescens* on the ocular surface, contact lenses, contact lens cases and fingers of subjects

The microbial flora of the lids and conjunctiva was sampled before lens fitting as a baseline and subsequent sampling together with the contact lenses was performed after 1, 3, 6, 9 and 12 months of wear. Ocular sites were sampled with calcium alginate swabs moistened with sterile saline. Samples were taken from the upper bulbar conjunctiva, with avoidance of contact with the lids, lashes and tarsal conjunctiva. A second swab was passed along the lower lid margin, with avoidance of contact with the bulbar conjunctiva and lashes. Swabs were placed immediately into 2 ml of sterile phosphate-buffered saline (PBS; NaCl 8 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.15 g/L, KH₂PO₄ 0.2 g/L) and vortex mixed for 30 s. After removal of the swab, a 0.4-ml sample was used to inoculate a chocolate agar plate (Becton Dickinson, North Ryde, Sydney, Australia). The plate was incubated aerobically at 35°C and read after incubation for 48 h.

For enumeration of *S. marcescens* on contact lenses

(after 1, 3, 6, 9 and 12 months of wear), the contact lens was removed aseptically with sterile gloves and placed in a bottle containing 2 ml of sterile PBS. The lens was then removed, placed concave side down on a chocolate agar plate, covered in 10 ml of sterile nutrient agar and incubated in air + CO₂ 5% v/v for 24 h at 35°C. The PBS was also examined for bacterial contamination by plating 200 µl on a chocolate agar plate and incubating for 24 h at 35°C.

Contamination of lens cases was measured by vigorously swabbing lens cases with a dry calcium alginate swab. The swab was then processed according to the protocol for ocular swabs (see above). The swab was dry prior to sampling as the cases contained up to 500 µl of liquid. Contamination of subjects' fingers was assessed by asking the subjects to press gently all four fingers and thumb of each hand on to a chocolate agar plate. The plate was then incubated for 48 h at 35°C.

After incubation of all agar plates, colonies were streaked for purity. Gram-negative, oxidase-negative rods were counted and identified with API 20E test kits (Vitek, Baulkham Hills, Australia).

Biotyping

Production of pigment was examined after growth aerobically at 35°C for 18 h on peptone (Difco, Michagen, USA) 0.5% w/v, glycerol (BDH, Kilsyth, VIC) 1% v/v agar by examining colonies for pink colouration. Strains were biotyped, three times for each isolate, with API 20E strips (Vitek) according to the manufacturer's instructions. API 20E comprises 23 biochemical tests to characterise bacteria from the family Enterobacteriaceae (Table 1).

Serotyping

The strains were serotyped with 'Seiken' Serratia-O-grouping immune sera kit (Accurate Chemical and Scientific Corp, Westbury, NY, USA) according to the manufacturer's instructions. Briefly, bacterial antigen was generated by heating a bacterial suspension, which was prepared by emulsifying all bacterial colonies

Table 1. Differentiation of biotypes based on biochemical tests

Biotype no.	β			
	Galactosidase production	Urease production	Indole production	Arabinose fermentation
1	+	—	—	+
2	+	+	—	+
3	+	—	—	—
4	—	+	—	—
5	+	—	+	+

grown on a chocolate agar slant (incubated at 35°C for 18 h) in 10 ml of saline, at 120°C for 20 min. The suspension was then cooled and centrifuged. The pellet was weighed, resuspended to 10 mg of protein/ml, then mixed with the O antisera and incubated at 50°C for 2 h. After incubation overnight at 5°C, the tubes were observed and the presence of agglutination was considered positive.

DNA extraction and separation of fragments

All products for ribotyping were obtained from Boehringer (Mannheim, Germany) unless otherwise stated. Growth from overnight broth cultures in trypticase soy broth (TSB) was harvested by centrifugation, washed and resuspended in 10 ml of 50 mM Tris-HCl, pH 8.0; 400 µl of 0.5 M EDTA was then added and incubated for 10 min at 37°C. Cells were lysed by the addition of 400 µl of lysozyme (Sigma) 20 mg/ml and incubated at 37°C for 10 min. After incubation, 400 µl of SDS 10% was added to further lyse the bacteria and 10 µl of RNAase A 10 mg/ml and 25 µl of proteinase K 20 mg/ml were added. This mixture was incubated at 37°C for 1 h. DNA was purified by three phenol:chloroform:isoamyl alcohol (25:24:1) extractions and precipitated with a 1 in 10 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of ice-cold ethanol 100%. DNA was spooled on to a hooked-end pasteur pipette, washed in ethanol 70%, dried and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA yield and purity was determined by measuring the optical density at 260 nm and 280 nm [19]. Before digestion with restriction endonucleases, DNA was subjected to agarose gel electrophoresis (see below) and stained with ethidium bromide to examine for the presence of plasmids.

Purified DNA (20 µg) was digested with *Eco*RI and *Bam*HI according to the manufacturer's instructions. The digests were analysed by gel electrophoresis at 30 mA for 18 h through agarose 0.7% gels containing ethidium bromide 0.5 µg/ml in TBE buffer (0.089 M Tris-base, 0.089 M boric acid and 2 mM EDTA, pH 8.0). After electrophoresis, Southern transfers [19] of the gels were performed by blotting to nitrocellulose membranes (Hybond-N⁺, Amersham, North Ryde, NSW, Australia).

Preparation of probe and hybridisation

A cDNA probe was made from *Escherichia coli* 16S and 23S RNA with AMV reverse transcriptase and a mixture of hexanucleotides in the presence of digoxigenin-11-dUTP (DIG-11-UTP) according to the manufacturer's instructions (Boehringer). The membranes were hybridised overnight at 68°C in 5 × SSC buffer (750 mM NaCl, 75 mM sodium citrate, pH 7.0), blocking reagent 1%, N-lauroylsarcosine 0.1%, SDS 0.02% and DIG-labelled cDNA to a final concentration of 5–25 ng/ml. Chemiluminescence was detected with

an anti-DIG-alkaline phosphatase-conjugated antibody and the substrate LumigenTM PPD according to the manufacturer's instructions. The membranes were autoradiographed by exposure to photographic film (Amersham) overnight at –70°C.

Results

Microbial contamination of contact lenses, ocular sites, lens cases and subjects fingers

Contamination of ocular surfaces and devices with *S. marcescens* was rare. No *S. marcescens* strains were isolated from the lid swabs. However, conjunctival swabs showed a 0.16% contamination rate (625 sampling occasions). Contact lenses showed a 0.6% contamination rate (1427 sampling occasions). Contact lens cases showed the highest contamination rate of 1% (211 sampling occasions) and fingers showed a contamination rate of 0.5% (203 sampling occasions). There were no significant differences between the contamination rates of any of the surfaces sampled.

Contact lenses gave the greatest number of cfu (median >300; range 1–>300), the lens case gave the next highest level of contamination (median 36 cfu; range 8–65), the conjunctiva gave the next highest level of contamination (median 4 cfu; range 3–10) and the fingers gave the lowest level of contamination (3 cfu). For only one patient was *S. marcescens* isolated from more than one site; this strain was isolated from both the conjunctiva and the lens.

There was a tendency for a greater rate of isolation from patients on a daily wear lens schedule (23%) than from those on extended wear (9%). However, by the proportions test this was not significantly different. There was no difference in isolation rate from Etafilcon or Polymacon lens types.

Strains

Sixteen *S. marcescens* isolates were examined; 11 of these were isolated from the lenses or conjunctiva of asymptomatic patients, two from lens cases, one from fingers and one from a contact lens at the time of a CLARE event. Type strain ATCC 274 was used for comparative purposes. Isolates were divided into 13 types based on differences in their biotypes, serotypes and ribotypes (Table 2).

Biochemical characterisation

The only strain that was pigmented was ATCC strain 274; all clinical isolates were non-pigmented. API 20E biotypes were defined by the characteristic number assigned by the manufacturer to describe a bacterial strain's ability to ferment carbohydrates and produce several other enzymic reactions. The differences in reactions between biotypes are shown in Table 1. Five

Table 2. Source, serotype, biotype and ribotype of various *S. marcescens* strains

Isolate no.	Isolation site	Date of isolation	Serotype*	Biotype†	Ribotype‡	Type§
Smar 5	CLARE, lens	June 1991	O:13	1	1	1
Smar7	Conjunctiva	June 1991	O:13	1	1	1
Smar9	Lens	May 1992	O:13	1	1	1
Smar12	Lens	March 1993	O:13	1	2	2
Smar12b	Conjunctiva	March 1993	O:13	1	2	2
ATCC 274	---	Type strain	O:6	5	2	3
Smar1	Lens	Nov. 1992	O:5	UT	3	4
Smar4	Lens	May 1992	UT	3	3	5
Smar11	Conjunctiva	Nov. 1992	UT	1	3	6
Smar13	Case	June 1993	O:13	2	4	7
Smar14	Conjunctiva	June 1993	O:14	2	4	8
Smar2	Lens	Aug. 1992	O:6	3	5	9
Smar3	Lens	Feb. 1992	UT	4	6	10
Smar6	Lens	Sep. 1991	UT	1	7	11
Smar15	Fingers	June 1993	O:5	UT	8	12
Smar17	Case	Oct. 1993	O:16	2	9	13

UT, untypable.

*Serology was performed on whole cells with commercially available antisera.

†Biotyping differentiated strains on five key biochemical tests (Table 1).

‡Ribotyping was performed by digesting chromosomal DNA with restriction enzyme *Eco*RI (Fig. 1).

§The final 'type' for each strain was based on similarities in biotype, serotype and ribotype.

biotypes were generated with API 20E (Table 2). The biotypes were determined by testing strains on three different occasions. Sometimes the biotype varied and so was assigned a type when a minimum of two of three tests gave consistent results. The exceptions to this rule were isolates Smar1 and Smar15 which gave variable results and, therefore, were classified as untypable.

Serotyping

The isolates fell into five serotypes (13, 14, 5, 6 and 16), the most common of these being serotype 13 (Table 2). There was generally a good correlation between serotype O13 and biotype 1 (Table 2). However, strains of serotype O6 were divided between biotypes 5 and 3. With our antisera 25% of the isolates showed no agglutination reaction with the antisera and so were classed as untypable.

DNA analysis and ribotyping

No strain was shown to possess plasmid DNA. After digestion with the restriction endonuclease *Eco*RI probing with labelled 16S and 23S rRNA, nine distinct ribotypes were found (Fig. 1, Table 2). A ribotype was defined as a distinct pattern of banding. The most common ribotypes were ribotypes 1 and 2, representing 22% of isolates each. Serotype 13 and ribotype 1 was the most common combination; the isolate from a CLARE episode fell into this group. Serotype 13 and ribotype 2 was also a common combination and this was an internal control as two strains (Smar12 and 12b) from the same patient isolated on the same day from different sites fell into this group. With *Bam*HI-digested chromosomal DNA, no differences between isolates could be discerned.

Discussion

This study has developed a rapid typing scheme for *S. marcescens* based on the genetic and physiological diversity of these strains. *S. marcescens* has been found to cause corneal infections related to the wearing of contact lenses which may be due to colonisation of the lenses by bacteria. This study demonstrated that many different types of *S. marcescens* are able to colonise the eye during contact lens wear.

The diversity of types isolated from the eye indicates that there is probably not a subset of *S. marcescens* that can colonise the eye. Similarly, to the majority of nosocomial isolates [20, 21] all ocular isolates proved to be non-pigmented, although the significance of this is not known. Six of the 15 isolates were of serotype O13. In comparison, none of 209 strains was O13 in a study conducted by Alonso *et al.* [18] and only one of 117 strains in the study by Rubin *et al.* [8]. These results are very different from nosocomial studies where O14 is the major serotype isolated [8, 18], whereas only one of 15 strains belonged to this serotype in the present study. Simor *et al.* [15] and John and McNeill [16] used plasmid analysis to type nosocomial strains of *S. marcescens*. However, this method was not suitable for the present study, as none of the strains so far isolated from contact lenses has contained plasmids. John and McNeill [16] found that 92% of nosocomial strains contained plasmids. Our strains may not contain plasmids due to the lack of selection pressure. The serotyping results combined with the lack of plasmids suggest that *S. marcescens* strains that colonise the contact lens are community acquired and distinct from nosocomial strains.

The preponderance of serotype O13 in this study is of

Fig. 1. Ribotyping of *S. marcescens*. Chromosomal DNA from each *S. marcescens* isolate was digested with *Eco* RI and separated by agarose gel electrophoresis. Lane 1, ATCC 274; 2, Smar 1; 3, Smar 2; 4, Smar 3; 5, Smar 4; 6, Smar 6; 7, Smar 7; 8, Smar 5; 9, Smar 9; 10, Smar 11; 11, Smar 12; 12, Smar 12b; 13, Smar 13; 14, Smar 14; 15, Smar 15; 16, Smar 17.

interest. There is some evidence that the O13 serotype antigen is not lipopolysaccharide but an acidic polymer [22]. The O13 serotype has been reported to be a rough phenotype [22]. However, the colony morphology of strains in the present study does not suggest a correlation between O13 serotype and roughness.

Grimont and Grimont [7] have developed a sensitive biotyping system. However, this method is time consuming (requiring up to 14 days for measuring utilisation of carbon sources) and labour intensive. The systems used in the present study were a commercially available biochemical test kit, a commercially available serotyping kit and a rapid analysis for restriction endonuclease fragment patterns of chromosomal DNA with *Eco* RI. *Eco* RI gave an adequate number of bands to distinguish between strains, as was found also by Alonso *et al.* [18]. To determine the type of each *S. marcescens* isolate only 4 days were required from first isolating the strain from the eye in the present typing scheme. Thus the present system was quicker than the system of Grimont and Grimont [7]. In view of the large number of fragments generated by restriction endonuclease analysis, the development of a ribotyping system allowed a more reliable comparison of a large number of strains.

S. marcescens grows well in moist environments, and solutions and damp surfaces have been implicated as reservoirs in several nosocomial outbreaks [23–25]. However, more recent evidence [13] has shown that *S. marcescens* strains colonising children in hospital are not commonly acquired from the environment, but colonise the pharynx secondarily to rectal colonisation. Furthermore, hand wash cultures of hospital staff were negative [13]. In the present investigation, the single isolate from the fingers of a lens wearer possessed a unique ribotype (type 6) that was not represented in the isolates from contact lenses or conjunctiva. This, albeit preliminary evidence from a small number of isolates, suggests that the route of contamination of eye or contact lenses is not *via* hands.

The source of colonisation of contact lenses by *Pseudomonas aeruginosa* strains that have caused corneal ulceration has been traced by Mayo *et al.* [26] with various methods including serotyping and plasmid profiling. They demonstrated that an isolate from the cornea was identical to that found in the saline solution from the same patient. In the present investigation, there were two isolates from contact lens cases but these were of unique types.

In the future, typing of isolates from environmental sources such as water and from symptomatic patients

by this method would enable the source of contamination of contact lenses to be traced. This method may define specific pathogenic types of *S. marcescens* and enable development of strategies to prevent contact lens contamination.

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