Journal of Industrial Microbiology (1995) 15, 243–247

Bacterial adhesion measurements on soft contact lenses using a Modified Vortex Device and a Modified Robbins Device

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***S. marcescens* 8100 and *P. aeruginosa* 15442 were used to study bacterial adhesion to hydrogel contact lenses which had not been worn. Bacterial removal from unworn lens materials was assessed with a calibrated vortex device modified with a digital rpm readout and fitted with a test tube attachment (MVD). The MVD, which relies on a whirlpool-like force to remove the bacteria, showed that bacteria adhered to the same degree to etafilcon A, vifilcon A and polymacon lenses under standardized conditions. Tracking the isoenzyme patterns of these bacterial species over time showed instability of *S. marcescens* upon repeated passage. This instability was not evident with *P. aeruginosa*. Bacterial adhesion of *P. aeruginosa* 15442, to human worn and unworn etafilcon A materials was determined with a Modified Robbins Device. The MRD was closed off at both ends stopping medium and bacterial movement after 1 h of fluid flow over the lens surface. The results show that immediately following this 1-h period more bacteria adhere to unworn contact lenses than to worn lenses. However, bacterial counts were equivalent on worn and unworn lenses following 5 h of static incubation.**

Keywords: adhesion; contact lens; bacteria; isoenzyme; hydrogel

Introduction

Bacterial adhesion and biofilm development have been concerns in the medical device arena for many years [1,3,4,6,10,17]. More recently, bacterial adhesion has become a concern relative to extended-wear soft contact lenses and possible correlation to contact lens-related eye disease [8,25]. The methods used to evaluate bacterial adherence to contact lenses vary with the group reporting the work [1,3,5,13,14,19,20]. However, the stability of bacterial isolates used in such experiments has not been studied. Conflicting data are sometimes generated due to the variety of techniques, species of bacteria, incubation times and temperatures, and media used to evaluate both worn and unworn contact lenses.

There are several areas of interest relative to the study of bacterial resistance to a solid phase matrix in the industrial setting. An area of interest in the contact lens industry is the development of bacterial adhesion-resistant materials. As an alternative to costly animal testing of new contact lens materials, specifically for properties of bacterial adhesion, an *in vitro* method was developed to use as a screening tool. The Modified Vortex Device (MVD), relies on a whirlpool-type force to remove bacteria from a solid matrix such as a contact lens. We used a test method with standardized reagents, equipment, and culture techniques to perform evaluations on potential new contact lens materials, as well as contact lenses already on the market for their susceptibility to bacterial attachment. Vortex-type actions have been shown to recover low levels of bacteria from human contact lenses which have been worn [13]. Other groups have used vortex-type actions in their protocols to recover bacteria from lenses; however, this step in the protocols is not emphasized [5,13,14].

Another area of interest is the study of bacterial adhesion to human worn contact lenses as part of the disease process. Bacterial attachment can occur either during a sleep period or during storage in a lens case. The attachment and eventual adhesion of a biological molecule onto a contact lens may be one of the initial steps in the process of infection [15]. Biofilm formation begins with the attachment of bacteria onto a surface [15,17,18]. The Modified Robbins Device (MRD) has been used extensively in other settings for evaluation of biofilm development over longer periods of time in a free-flow fluid environment [15,17]. The MRD was used to evaluate worn and unworn etafilcon A lenses in a minimal medium for bacterial adhesion properties.

This report discusses two *in vitro* techniques used to study different aspects of bacterial adhesion by two different bacterial species as they relate to contact lenses. We performed isoenzyme analysis on cellular extracts to determine the extent of divergence from newly freeze-dried cultures. The MVD was used to quantify bacteria removed from unworn contact lens materials. The MRD was used to study adhesion of bacteria using worn and unworn hydrogel lens materials as the matrix.

Materials and methods

Microorganisms

The bacteria used in this study were *Serratia marcescens* ATCC 8100 and *Pseudomonas aeruginosa* ATCC 15442. They were cultured using tryptic soy broth (TSB) and agar (TSA), (Difco Laboratories, Detroit, MI, USA). These media were prepared and evaluated for optimal growth by standard United States Pharmacopeia methods [26]. Incubation temperatures were 22° C and 35° C for experiments conducted with *S. marcescens* and 35° C for *P. aerugi-*

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Received 22 December 1994; accepted 24 February 1995

nosa [11,23]. The number of bacteria was determined from serial dilutions in sterile filtered 0.85% NaCl, pH 7.2 (PS) plated on TSA.

Contact lenses

The contact lens materials were etafilcon A (Vistakon, Jacksonville, FL, USA), vifilcon A (Ciba Vision Corp, Atlanta, GA, USA), and polymacon (Bausch and Lomb Inc, Rochester, NY, USA). In addition, hydrogel lenses, one with a positive charge (experimental lens A) and the other set with a negative charge (experimental lens B) (compositions proprietary) were used as positive and negative controls, respectively. All lenses were washed three times in sterile physiological saline (PS) prior to *in vitro* assays.

Isoenzyme analysis

Isoenzyme extraction from bacteria and analysis were performed essentialy as previously described [21,22,24]. Enzymes were extracted with a detergent at 37° C. Observations were made following denaturing electrophoresis of bacterial extracts and staining for particular enzymes using the Authentikit System (Innovative Chemistry, Marshfield, MA, USA). Migration was measured from the middle of the inoculation origin to the bottom of the resulting electrophoresis band. A migration difference greater than 2 mm was considered significant. Isoenzymes were extracted at passages one and ten from *S. marcescens* and *P. aeruginosa*. The isoenzymes measured were adenylate kinase, EC 2.7.4.3 (AK); glutamate oxalacetate transaminase, EC 2.6.1.1 (AST); glucose-6-phosphate dehydrogenase, EC 1.1.1.49 (G6PD); isocitrate dehydrogenase, EC 1.1.1.42 (ICD); lactate dehydrogenase, EC 1.1.1.27 (LD); malate dehydrogenase, EC 1.1.1.37 (MD); malic enzyme, EC 1.1.1.40 (ME); mannose phosphate isomerase, EC 5.3.1.8 (MPI); purine nucleoside phosphorylase, EC 2.4.2.1 (NP); peptidase A, EC 3.4.11 (PEP A); phosphoglucose isomerase, EC 5.3.1.9 (PGI); and phosphoglucomutase, EC 2.7.5.1 (PGM).

Bacterial adhesion using the MVD

Bacteria were grown in TSB for 18 h at 22° C or 35° C resulting in densities of 5.0×10^8–1.0×10^9 cells per milliliter. One milliliter of this stock bacterial culture was diluted in PS, and 1.0-ml portions were added to sterile glass scintillation vials (Baxter Scientific, McGraw Park, IL, USA) for a final inoculum of 3.0–7.0×10^4 CFU ml^{-1}. Unworn washed contact lenses were then added to these vials. Incubation was continued at either 22° C or 35° C for 18-h, agitating the vials at 0.13 *g*. Bacterial counts increased over time. The lenses were then transferred to a new scintillation vial containing 5 ml of PS and washed for 1 min, agitating the vial at 0.54 *g* to remove bacteria associated with the fluid film around the lens. The lenses were then transferred to a 50-ml conical centrifuge tube containing 10 ml of PS and vortexed at 63 *g* using a modified vortex device (MVD) (Baxter Scientific, McGraw Park, IL, USA) for 3 min. Vortexing for 3 min, gave the most reproducible results. The MVD has a calibrated digital readout and requires only 1.5 ft^2 of bench space. The speed is controlled manually and can range from 0.13 *g* to 84 *g*. Rotation may occur in the clockwise or counterclockwise direction. A speed of 63 *g* was used. This type of whirlpool action removes bacterial cells from the contact lens surface [13]. One milliliter of this cell suspension was serially diluted in PS and plated in duplicate on TSA. Each data point represents results from four lenses of the same material. Experiments were conducted in triplicate.

Modified Robbins Device time course experiments

Human worn and unworn etafilcon A contact lenses were washed in PS and cut so they completely covered a 7-mm diameter rubber disc. Each lens was cut into three pieces. The worn lenses used in this study had been on human eyes for six overnight periods. Experiments conducted with the MRD were done in duplicate. The MRD has been described in detail elsewhere [15,17,18]. The lens pieces were glued onto discs with Vetmed Tissue Glue (3M, St Paul, MN, USA). The discs were then fitted into a cylinder which was placed in the flow chamber section of the Modified Robbins Device. *P. aeruginosa* 15442 was allowed to flow past the lenses for 1 h at 32° C using a peristaltic pump drawing fluid through the effluent part of the MRD. The supernatant fluid was filter-sterilized 0.85% NaCl supplemented with 500 μg ml^{-1} of bovine serum albumin (ICN, Cleveland, OH, USA) pH 7.1 [4,20]. The medium was contained in a sterile 1-L bottle equipped with a bottom side port and a stirring bar. The inoculum was mixed on a magnetic stirring plate during the 1-h flow with a rate of 50 ml min^{-1}. The device was then clamped off with forceps at both ends and incubated at 35° C for an additional 5 h with gentle agitation. Sampling was conducted at 0, 3 and 5 h by expressing the lens and its backing from the cylinder support. The lens material was transferred into 2 ml of PS in a conical centrifuge tube and stirred vigorously on a vortex mixer for 30 s. The supernatant fluid was serially diluted in PS and samples were plated on TSA. Experiments were conducted in duplicate with three lens pieces per data point.

Results

Comparison of bacterial adhesion to unworn contact lenses

Results obtained using the MVD with *P. aeruginosa* as the indicator organism are shown in Figure 1. The data show no significant difference in bacterial recovery as measured by the MVD when unworn etafilcon A, vifilcon, and polymacon lens materials were compared. However, control lens A with a positive charge yielded greater than 10^5 cells per lens, whereas less than 10^3 bacteria were recovered for control lens B (Figure 1).

In contrast to *P. aeruginosa*, *S. marcescens* 8100 showed little adhesion to any of the three commercially available lens types at 35° C; adherence to experimental lens A was relatively high (Figure 2). When experiments were conducted at 22° C however, there was an increase in the degree of adhesion for all the lens types tested except for experimental lens A. *S. marcescens* bound to a greater degree to the experimental A lens even when experiments were conducted at 35° C. This is not an optimal adhesion temperature for *S. marcescens* as the data in Figure 2 demonstrate. *Pseudomonas* or *Serratia* adhesion to etafilcon A,

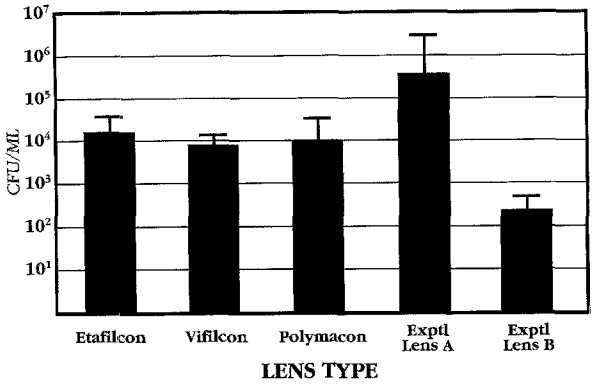

Figure 1 Viable counts of *P. aeruginosa* 15442 removed by the MVD from unworn contact lenses. Bacteria were grown and the experiments were conducted at 35° C. Reagents were prewarmed to 35° C. The final inoculum concentration was 5×10^5 CFU ml^{-1}. Error bars = one standard deviation ($n = 12$)

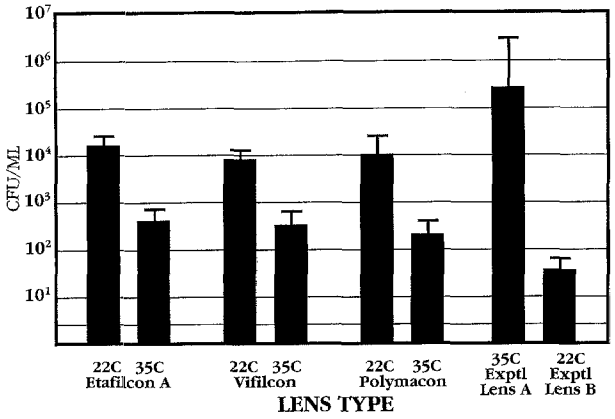

Figure 2 *S. marcescens* 8100 adhesion to unworn contact lenses. Bacteria were grown and the experiments were conducted at 22° C or 35° C as indicated. The final inoculum concentration was 5×10^5 CFU ml^{-1}. The positive control lens material was incubated at 35° C and the negative control material was incubated at 22° C

vifilcon and polymacon lenses were similar at each organism's optimal adhesion temperature.

Fewer *S. marcescens* cells were recovered from the negative control (lens B) compared to the other lens materials tested. Upon repeated passage of *S. marcescens* at 22° C, the degree of adhesion to all materials decreased to about the levels observed when experiments were conducted at 35° C (data not shown). This was observed by passage ten in TSB.

Isoenzyme profile of S. marcescens *8100 and* P. aeruginosa *15442*

There was no change in the isoenzyme profiles of *S. marcescens* isolates cultured at 22° C or 35° C for NP, MD, PEPA, AST, ICD, AK, PGM or PGI at first passage (Table 1). However, there were banding pattern differences for LD, MPI, G6PD and ME after the first passage. Additional bands occurred as the *S. marcescens* isolates were passaged regardless of temperature. There was a converging of enzyme patterns of the isolates at passage ten. This convergence of the isoenzyme patterns was accompanied by a reduction in the degree of bacterial adhesion to the etafilcon A lenses.

Table 1 Isoenzyme profile of *S. marcescens* 8100 grown at 22° C and 35° C

	P_1		P_{10}	
	22° C	35° C	22° C	35° C
Isoenzyme	Migration distance (mm)		Migration distance (mm)	
NP	31	30	29	29
MD	8	8	8	9
PEPA	21	21	21.5	23
AST	26	27	5,25	5,25
ICD	20	20	20	20
AK	13	13	13	13
PGM	21	20	23	23
PGI	18	18	4,15	5,14
LD	5	0	5	5
MPI	5,14	15	5,15	5,15
G6PD	7,20	7	8	8
ME	22,29	0	0	0

P_1 was extracted after passage one from frozen stock and passage ten (P_{10}) was extracted from a continually-passaged culture. There were two growth temperatures for both extraction points. Two numbers indicate two bands. A difference of 2 mm or more is considered significant

The isoenzyme profiles for *P. aeruginosa* 15442 extracted from cells harvested at zero and ten passages essentially were identical (Table 2). Additionally, no differences in the relative adhesion properties were observed after repeated passages in TSB.

Time course experiments with the Modified Robbins Device

The results summarized in Figure 3, show a recovery of 300–400 CFU per worn lens and approximately 800–900 CFU per unworn lens following the 1-h flow period. This is consistent with the findings of other investigators who have shown greater levels of adhesion on unworn lenses [3]. The increases in CFU per lens at 3 and 5 h for

Table 2 Isoenzyme profile for *P. aeruginosa* 15442, passage one using TSB

Isoenzyme	Migration distance (mm) P_1
LD	0
ME	27
ICD	28
MPI	0
PEPA	0
NP	−2
MD	0
G6PD	26.5
AST	19
AK	18
PGI	30
PGM	0

Migration distances were measured in millimeters. A difference of 2 mm or more is significant. Bacteria were grown at 35° C. The isoenzyme profile for passage 10 was the same as for passage 1

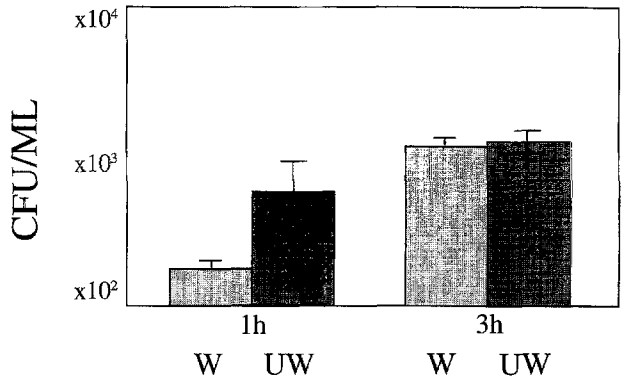

Figure 3 Time course experiments using the MRD comparing worn and unworn etafilcon A lenses with *P. aeruginosa* 15442 as the indicator organism. There were differences in counts at 1 h for worn and unworn lenses. These differences were no longer evident at the 3-h sampling time. Bacterial recovery at 5 h was similar to counts obtained at 3 h

both worn and unworn etafilcon A lenses were such that there was virtually no difference in recoverable microorganisms when comparing human-worn with unworn lenses. Final recoverable counts were in the high 10^3–10^4 range after 5 h in a static system. This was in contrast to the results after 1 h incubation.

Discussion

Since there is a lack of an industry standard for bacterial adhesion measurements to lens materials, individual investigators have used different techniques for adhesion measurements [1,3,5,8,9,19].

An important experimental variable to control is the bacteria used as the indicator of adhesion. Isoenzyme analysis was used as a monitor of strain stability. The shift in enzyme pattern which was observed with *S. marcescens* 8100 was not observed with *P. aeruginosa* 15442. Upon multiple passages the *S. marcescens* isolate was less stable with respect to enzyme profile and bacterial adhesion.

P. aeruginosa 15442 is used in the multi-item challenge assay recommended in the FDA guidelines for the evaluation of lens disinfection systems. Thus, an organism that is used in an accepted industrial test was used in this work. This isolate is stable relative to bacterial adhesion to soft contact lenses and its isoenzyme pattern. Other groups have used isoenzyme analysis to study intraspecies genetic relationships and to perform cell culture quality control [2,7,12,21,22]. In our experiments we have transferred inoculum at the same time prior to experiment initiation, performed routine culture identification and calibration of equipment, tracked the biochemical makeup of the bacteria being used, controlled the number of passages of a bacterial culture and used a medium which supports bacterial growth from a minimal starting inoculum [26]. In these experiments with *P. aeruginosa* 15442 the data were consistent with respect to variation in adherence levels over time and in multiple experiments. The variation was less than 10% when lens types were compared with one another. Of particular interest is that when passage number is controlled for both species, the densities of bacteria recovered are similar at different temperatures. This is exclusive of the controls. *S. marcescens* 8100 was less stable relative to biochemical makeup with repeated passage on artificial media. In addition, phenotypic shifts are common in genus *Serratia*, especially with respect to pigmentation [11].

The Modified Robbins Device was used in a series of experiments as a measure of adhesion to worn and unworn lenses under changing flow conditions. We wanted to determine the number of bacteria we could recover using a minimal medium following 3–5 h incubation time in a simulated closed eye environment. The numbers of bacteria recovered were in a range which can cause ocular irritation [16].

Initially, bacterial adhesion to unworn lenses was greater than to worn lenses in a static environment. This difference between worn and unworn lenses disappeared with continued incubation. We are continuing experiments with the Modified Robbins Device in order to understand the dynamics of static environments relative to the growth of bacteria under the influence of different tear constituents.

Acknowledgements

The authors thank Ms G Pelletier for secretarial assistance.

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