Microbial biofilms on the surface of intravaginal rings worn in non-human primates

Manjula Gunawardana,^{1,2} John A. Moss,¹ Thomas J. Smith,^{1,2,3} Sean Kennedy,¹ Etana Kopin,² Cali Nguyen,^{1,2} Amanda M. Malone,² Lorna Rabe,⁴ Christoph Schaudinn,⁵ Paul Webster,⁵ Priya Srinivasan,⁶ Elizabeth D. Sweeney,⁶ James M. Smith⁶ and Marc M. Baum¹

Correspondence

Marc M. Baum

m.baum@oak-crest.org

¹Department of Chemistry, Oak Crest Institute of Science, 2275 E. Foothill Boulevard, Pasadena, CA, USA

²Auritec Pharmaceuticals Inc., Suite 3, 1434 6th Street, Santa Monica, CA, USA

³Department of Ophthalmology, University of Kentucky, Lexington, KY, USA

⁴Magee-Womens Research Institute, Pittsburgh, PA, USA

⁵Ahmanson Advanced EM & Imaging Center, House Ear Institute, 2100 W. 3rd Street, Los Angeles, CA, USA

⁶Laboratory Branch, Division of HIV/AIDS Prevention, National Center for HIV, STD, TB Prevention, Coordinating Center for Infectious Diseases (CCID), Centers for Disease Control and Prevention, Atlanta, GA, USA

Millions of intravaginal rings (IVRs) are used by women worldwide for contraception and for the treatment of vaginal atrophy. These devices also are suitable for local and systemic sustained release drug delivery, notably for antiviral agents in human immunodeficiency virus pre-exposure prophylaxis. Despite the widespread use of IVRs, no studies have examined whether surface-attached bacterial biofilms develop *in vivo*, an important consideration when determining the safety of these devices. The present study used scanning electron microscopy, fluorescence *in situ* hybridization and confocal laser scanning microscopy to study biofilms that formed on the surface of IVRs worn for 28 days by six female pig-tailed macaques, an excellent model organism for the human vaginal microbiome. Four of the IVRs released the nucleotide analogue reverse transcriptase inhibitor tenofovir at a controlled rate and the remaining two were unmedicated. Large areas of the ring surfaces were covered with monolayers of epithelial cells. Two bacterial biofilm phenotypes were found to develop on these monolayers and both had a broad diversity of bacterial cells closely associated with the extracellular material. Phenotype I, the more common of the two, consisted of tightly packed bacterial mats approximately 5 µm in thickness. Phenotype II was much thicker, typically 40 µm, and had an open architecture containing interwoven networks of uniform fibres. There was no significant difference in biofilm thickness and appearance between medicated and unmedicated IVRs. These preliminary results suggest that bacterial biofilms could be common on intravaginal devices worn for extended periods of time.

Received 16 November 2010

Accepted 4 March 2011

INTRODUCTION

Bacteria are known to colonize surfaces and establish a sessile mode of growth in a chemically and morphologically heterogeneous matrix of extracellular polymeric substances

Abbreviations: BV, bacterial vaginosis; CLSM, confocal laser scanning microscopy; CVL, cervicovaginal lavage; EPS, extracellular polymeric substances; FISH, fluorescence *in situ* hybridization; HIV, human immunodeficiency virus; IL, interleukin; IVR, intravaginal ring; SEM, scanning electron microscopy; TFV, tenofovir; WGA, wheatgerm agglutinin.

(EPS) known as a biofilm. Microbial biofilms are widely recognized as being implicated in chronic infections (Costerton *et al.*, 1999), and these communities have been found to be more resistant to antimicrobial agents and the immune system than their planktonic counterparts (Davies, 2003; Stewart, 2002). Biofilm bacteria also are difficult to cultivate in the laboratory, frequently resulting in misdiagnosis of the associated infections (Costerton *et al.*, 2003).

The formulation of pharmaceutical agents into intravaginal rings (IVRs) represents an attractive approach to achieving sustained release of compounds to the vaginal cavity for

local or systemic delivery (Valenta, 2005), thereby increasing efficacy and adherence to therapy while potentially decreasing toxic side effects when compared to daily oral administration. This strategy has become popular in contraception and in oestrogen replacement therapy (Yoo & Lee, 2006), and several products are available commercially. Sustained vaginal delivery of antiviral agents from IVRs constitutes a potential route for human immunodeficiency virus (HIV) pre-exposure prophylaxis in women, particularly in the developing world (Nel *et al.*, 2009; Saxena *et al.*, 2009). Given the widespread use of IVRs, it is surprising that few studies have examined how these abiotic surfaces are colonized by micro-organisms *in vivo*.

Bacterial vaginosis (BV) has been found to be associated with characteristic biofilms adherent to the vaginal epithelium (Swidsinski *et al.*, 2005), which were composed of confluent *Gardnerella vaginalis* and other bacterial groups. These biofilms persisted on the vaginal epithelium after standard therapy with oral metronidazole (Swidsinski *et al.*, 2008). *Staphylococcus aureus* infections have been associated with mortality resulting from tampon-related toxic shock syndrome in menstruating women (Begley & Barnes, 2007; Tang *et al.*, 2010). Veeh *et al.* (2003) used fluorescence *in situ* hybridization (FISH) to examine 44 paired – tampon and vaginal – wash specimens from 18 pre-screened women and detected *S. aureus* biofilms in 37 of these specimens. These results suggest that *S. aureus* colonization of IVRs in the vaginal canal is possible, especially since the devices remain in the vagina for a longer time than tampons.

Scanning electron microscopy (SEM) was used in *ex vivo* experiments to show that yeast isolates from vaginal exudates of patients with vulvovaginal candidiasis were able to adhere to a combined contraceptive vaginal ring (NuvaRing; Organon Pharmaceuticals) (Camacho *et al.*, 2007) and to contraceptive intrauterine devices (Chassot *et al.*, 2008), with concomitant biofilm formation. Infected intrauterine devices recovered from patients suffering from reproductive tract infections were found to be tainted with *Candida* biofilm (Lal *et al.*, 2008). Miller *et al.* (2005) used SEM to examine a NuvaRing worn for 28 days by a healthy female volunteer and did not observe embedded bacteria, erosion or structural changes compared to an unused ring. This is, to the best of our knowledge, the only report on bacterial biofilms and IVRs in an *in vivo* setting.

We have developed IVRs that deliver the nucleotide analogue reverse transcriptase inhibitor tenofovir (TFV) at controlled rates for up to 3 months (Moss *et al.*, 2010), with the goal of preventing HIV infection in women. In the present study six IVRs worn for 28 days by female pig-tailed macaques were examined by SEM, FISH and confocal laser scanning microscopy (CLSM). Four of the IVRs contained and released TFV, while the remaining two rings were non-medicated. The aim of the study was to determine if bacterial biofilms formed on the IVRs *in vivo* and, if so, whether there would be a difference between the medicated and control rings. This is believed to be the first

report of microbial biofilms forming on the surface of IVRs in a model of relevance to humans.

METHODS

Manufacture of silicone IVRs. Silicone IVRs were prepared in a multi-step process from Nusil MED-4840 liquid silicone elastomer (Nusil Silicone Technology) using an injection moulding system developed in house. The ring dimensions (outer diameter 25 mm; inner diameter 15 mm; cross-sectional diameter 5 mm) recommended by Promadej-Lanier *et al.* (2009) were used. The ring manufacture was accomplished in two separate injection moulding steps. The delivery window (1 mm diameter) was created in a half ring, and four pods consisting of 3 mg each of ({[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy)methylphosphonic acid (TFV; Sinoway International) coated with poly-DL-lactide ($M_r \sim 15\,000$, Resomer R 202 S; Boehringer Ingelheim) were placed in pre-moulded, evenly spaced cavities. The second half of the ring then was injection moulded onto the first. Control rings contained poly-DL-lactide-coated silicone plugs of the same dimensions as the drug pods.

Macaques. Six sexually mature female pig-tailed macaques (*Macaca nemestrina*) were used for this study. All macaques were housed under an approved Centers for Disease Control and Prevention animal care and use protocol and standard guidelines [Department of Health, Education and Welfare (DHEW) no. NIH 86-23] (NIH, 1996). The animal study was approved by the Centers for Disease Control and Prevention IACUC (Institutional Animal Care and Use Committee) (2003DOBMONC-A3). Four macaques (PHD2, PMD2, PID2 and PRB2) received IVRs containing four pods of TFV. IVRs containing blank control pods were placed in two macaques (PZA2 and PVC2). The IVRs were inserted on day 0 into the vaginal vault and retained for a period of 28 days. Vaginal colposcopy was used to confirm placement and retention of the vaginal rings. The animals were placed in ventral recumbency while under anaesthesia. A paediatric speculum was used to open the vaginal vault to visualize the IVR placement. A Rebel T1i /EOS 500D digital camera (15 megapixels; Canon) was used along with a model LR66238 colposcope (Carl Zeiss). Photographs were taken at $\times 0.6$ and $\times 1.0$ magnification. Plasma samples were analysed for progesterone levels to monitor the menstrual cycle by the Wisconsin National Primate Research Center.

Cytokine analysis. Cervicovaginal lavages (CVLs) and blood were obtained up to 3 weeks prior to the insertion of the ring in order to establish baseline cytokine profiles. PBS solution (4–5 ml) was gently infused into the vaginal vault via a sterile 10 ml syringe attached to a sterile gastric feeding tube (size 5 or 8 French) of adjusted length and CVL was drawn out with the same device. Blood and CVLs were obtained on days –21, –14, –5, 0, 3, 7, 14, 21, 28 and 35. The CVL was observed for the presence of blood or any other discoloration. Amicon Ultra-4 10 kDa concentrators (Millipore) were used to concentrate the CVLs. Induction of mucosal inflammation was monitored by measuring vaginal and systemic cytokines as previously described using fluorescent multiplexed bead-based assays (Invitrogen and Bio-Rad) in accordance with the manufacturer's instructions (Promadej-Lanier *et al.*, 2009). Interleukin-8 (IL-8), granulocyte colony-stimulating factor, Rantes, IL-1 receptor antagonist (IL-1Ra), macrophage inflammatory protein 1 β (MIP-1 β), IL-6, eotaxin, IL-15 and IL-12p40 were analysed by the above methods.

Vaginal microbiological assessment. Sterile swabs were collected from all six macaques to complete a microbiological characterization on day –14, 0, 7, 21, 28 and 35. Each swab was placed individually in a Port-A-Cul transport tube (Becton Dickinson) and transported on ice packs to Magee-Womens Research Institute within 24 h of collection for quantitative culture analysis. This transport system has

been shown to maintain the viability and quantity of organisms for up to 48 h (Stoner *et al.*, 2008). Quantitative cultures and bacterial identification were performed as previously described (Patton *et al.*, 2006). *Lactobacillus* and viridans streptococci were tested for hydrogen peroxide production in a qualitative assay on tetramethylbenzidine agar plates (Rabe & Hillier, 2003).

IVR processing for biofilm characterization. TFV and blank pod IVRs were removed on day 28 from the macaques. The rings were cut into sections and segments without pods were placed in either 2.5 % glutaraldehyde in phosphate buffer, pH 7.2, or 50 % ethanol in water and transported on ice to the Oak Crest Institute of Science for biofilm characterization.

SEM. Glutaraldehyde-fixed samples were prepared for SEM as described previously (Webster *et al.*, 2004). Dehydration was carried out in an ethanol line followed by critical point drying. The dried ring segments were cut lengthwise, mounted on metal specimen stubs, coated with a 16 nm thick platinum film, and imaged by an XL-30 S FEG 6 SEM (FEI Company) operating at 5 kV.

Biofilm thickness. Horizontal cross-sections (1 mm) of glutaraldehyde-fixed ring segments were cut at random points using a razor blade under an inspection scope. These samples were cut into four equal-sized quadrants and processed for SEM analysis as described above. Examination of the quadrants under low magnification ($\times 500$) by SEM allowed the height of the biofilm from the ring surface to be determined. At least two images were captured at random points for each quadrant representing the inner and outer surface of the ring. A minimum of four images were obtained for each sample. Random measurements of biofilm thickness were made for each image using ImageJ (<http://rsbweb.nih.gov/ij/>), affording means and SDs.

FISH. Samples for FISH were preserved in 50 % ethanol in water, as described above, and stored at 4 °C prior to processing. Sample preparation was carried out as described by Macalady *et al.* (2006), except that the paraformaldehyde fixing step was omitted. We have found this approach to afford strong, selective FISH signals with minimal biofilm disruption (Romero *et al.*, 2008). The oligonucleotide probe EUB338 was used to target most bacterial groups and was synthesized and labelled at the 5' end with the fluorescent dye Cy5 (Integrated DNA Technologies). SEM and FISH/CLSM samples were fixed as described above (SEM, glutaraldehyde; FISH/CLSM, ethanol).

CLSM. Thin (approximately 2 mm) vertical cross-sections of ring segments were incubated in the presence of fluorescent dyes, rinsed, placed in sterile water in a slide chamber (Lab-Tek; Electron Microscopy Sciences) and imaged by confocal microscopy (LSM710; Carl Zeiss MicroImaging). The following fluorescent dyes were used either alone or in conjunction with FISH experiments: 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; MP Biomedicals); concanavalin, FITC (Integrated DNA Technologies); wheatgerm agglutinin (WGA), FITC (Integrated DNA Technologies); Syto60 (Invitrogen).

Image processing and manipulation. All images in this study were digitally captured and manipulated to adjust image size, contrast and brightness. Linear adjustment of size, contrast or brightness was always applied equally to the entire image.

RESULTS

Cytokine analysis

Mucosal and plasma levels of the proinflammatory cytokines IL-6 and IL-8 remained stable throughout the

study period in all animals with no significant increases observed as a result of the IVRs. This is typical and has been observed previously in pigtailed macaques in the context of IVRs (Promadej-Lanier *et al.*, 2009). Changes in local mucosal cytokine levels therefore were not a contributing factor to differences observed in the biofilms.

Microflora

The facultative and anaerobic micro-organisms recovered from vaginal swabs from the six pig-tailed macaques are listed in Table 1. The following organisms were not detected in any of the samples: group B, C, G, and non-group β -haemolytic *Streptococcus*, *Micrococcus* spp. and *Proteus* spp. Fig. 1 compares the mean cell counts for hydrogen peroxide (H_2O_2) producers and anaerobes recovered from vaginal samples. The microbial production of lactic acid and H_2O_2 is important to maintain a balanced vaginal microflora ecosystem and to prevent colonization by other, pathogenic micro-organisms (Martín & Suárez, 2010). These results indicate a drop in H_2O_2 -producing populations on insertion of the IVRs, but no significant impact on anaerobe populations. One of the control animals (PZA2) developed BV-like flora following insertion of the IVR, evidenced by a drop in H_2O_2 -producing bacterial populations and a concomitant infection by *G. vaginalis* (Table 1 and Fig. 1), which subsided after the IVR was removed. *Escherichia coli* and *Enterococcus* spp., micro-organisms typically found in faeces, were not detected in most samples with the exception of PID2, which had elevated levels of both organisms (*E. coli* $8.5 \times 10^5 \pm 1.1 \times 10^6$ c.f.u. g^{-1} ; *Enterococcus* spp. $1.1 \times 10^5 \pm 2.0 \times 10^5$ c.f.u. g^{-1}). These results agree with those from other reports that macaque vaginas only contain minor contamination from faecal matter (Patton *et al.*, 1996).

Visual examination of IVRs

Fig. 2 shows colposcopic images taken *in vivo* with the IVRs (indicated by arrows) in place. The white cylinders, labelled 'P' in Fig. 2, are the TFV pods. Visual inspection of the IVRs following the animal study revealed no discoloration of devices recovered from PHD2 (medicated), PMD2 (medicated), PRB2 (medicated) and PVC2 (control). The rings worn by PID2 (medicated) and PZA2 (control) were covered with low and copious amounts of brown residue, respectively. When examined at low magnification ($\times 20$) under the stereo light microscope, a colourless deposit was seen to have accumulated on all rings, mostly located on the inner surface, on the ridge formed by the two ring halves (the IVRs were injection-moulded as two separate halves), and where sprues were cut during the production process.

SEM examination of biofilms

SEM examination of the biological material covering the IVR surface revealed a high degree of heterogeneity that

Table 1. Vaginal facultative and anaerobic micro-organisms recovered from six *M. nemestrina* during the course of the study

Data are shown as numbers (%).

Organism(s)	Day -14		Day 0		Day 7		Day 21		Day 28		Day 35	
	TFV*	Control†	TFV*	Control†	TFV*	Control†	TFV*	Control†	TFV*	Control†	TFV*	Control†
<i>Lactobacillus</i> H ₂ O ₂ +	4 (100)	1 (50)	4 (100)	2 (100)	3 (75)	1 (50)	3 (75)	1 (50)	4 (100)	2 (100)	4 (100)	2 (100)
<i>Lactobacillus</i> H ₂ O ₂ -	4 (100)	0 (0)	2 (50)	1 (50)	3 (75)	1 (50)	2 (50)	2 (100)	2 (50)	0 (0)	3 (75)	0 (0)
<i>G. vaginalis</i>	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)
Diphtheroids	4 (100)	1 (50)	4 (100)	2 (100)	4 (100)	2 (100)	4 (100)	2 (100)	4 (100)	2 (100)	3 (75)	2 (100)
<i>Enterococcus</i> spp.	0 (0)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)
<i>E. coli</i>	1 (25)	1 (50)	1 (25)	0 (0)	1 (25)	1 (50)	1 (25)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)
<i>Viridans</i> <i>streptococci</i> H ₂ O ₂ +	3 (75)	2 (100)	4 (100)	1 (50)	4 (100)	1 (50)	4 (100)	1 (50)	3 (75)	1 (50)	1 (25)	1 (50)
<i>Viridans</i> <i>streptococci</i> H ₂ O ₂ -	4 (100)	2 (50)	3 (75)	2 (100)	4 (100)	2 (100)	2 (50)	1 (50)	4 (100)	1 (50)	4 (100)	2 (100)
Group F <i>Streptococcus</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)
<i>S. aureus</i>	1 (25)	1 (50)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)
Coagulase- negative staphylococci	4 (100)	2 (100)	4 (100)	2 (100)	3 (75)	2 (100)	4 (100)	2 (100)	4 (100)	2 (100)	3 (75)	2 (100)
<i>Bacillus</i> spp.	3 (75)	1 (50)	0 (0)	0 (0)	1 (25)	1 (50)	0 (0)	0 (0)	0 (0)	1 (50)	1 (25)	0 (0)
Aerobic Gram- positive rods	4 (100)	1 (50)	3 (75)	2 (100)	3 (75)	2 (50)	4 (100)	1 (50)	4 (100)	1 (50)	2 (50)	1 (50)
Aerobic Gram- positive cocci	1 (25)	0 (0)	1 (25)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)
Other Gram- negative rods	2 (50)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)	2 (50)	0 (0)	0 (0)	0 (0)
Anaerobic GNR non- pigmented	4 (100)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)
Black anaerobic GNR	4 (100)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)	3 (75)	2 (100)

GNR, Gram-negative rods.

*IVRs containing and releasing TFV (*n*=4).†Control IVRs that did not contain TFV (*n*=2).

could be classified in terms of consistently recurring structural features as illustrated by Figs 3 and 4. There was no observable difference between the IVRs containing TFV and the controls, with the exception of the device recovered from experimental animal PZA2 as discussed below. Large areas (>4 mm²) of the IVRs were covered in monolayers of epithelial cells (Figs 3a and 4a). In some cases, these monolayers were closely associated with bacterial cells and biofilm EPS (Fig. 3c and Fig. 4b, c). Some regions of the biofilms embedded reticulated structures (Figs 3b and 4b), which were concluded to be the epithelial cell surface. While bacterial biofilms formed on the epithelial cells that covered the silicone IVR surfaces, there were no observed instances of epithelial cells growing on a bacterial mat.

Two biofilm phenotypes were observed in this study and were classified as phenotype I and II. Phenotype I consisted of 2–5 µm thick (Fig. 3e) mats of tightly packed bacteria embedded in an amorphous matrix. The bacterial communities in these structures were highly diverse both in terms of the shape and size of the cells. Biofilm phenotype II was made up of an open, fibrous matrix (Fig. 3f and Fig. 4d, f). These architectures were populated by a high diversity – in terms of cell shape and size – of exposed bacteria and many appeared to be connected by thin fibres of uniform diameter (Fig. 4d). Bacteria within both biofilm phenotypes were a mixture of cocci, rods and spirochaetes (Fig. 3d, e and Fig. 4d, e). Overall, phenotype I was observed more frequently than phenotype II.

Fig. 1. Box and whisker plots of vaginal microflora in the absence and presence of IVR. (a) H_2O_2 + producers (*Lactobacillus* spp. and viridans streptococci). (b) Anaerobic Gram-negatives (black and non-pigmented). The IVR consisted of either control devices without TFV (blank pod) or devices with four TFV pods per ring (TVF pod). The horizontal line represents the median c.f.u. (g vaginal fluid)⁻¹. The data are pooled by group.

Biofilm samples on the IVR recovered from experimental animal PZA2 (control, not exposed to sustained release TFV) were unusual as they contained spherical cells (diameter 4 μ m), which were embedded in biofilm EPS and were surrounded by bacterial cells. This macaque was the only animal in the group to develop BV-like microflora while wearing the IVR (positive for *G. vaginalis*, Table 1). Large areas of the IVR also were covered in brown residue as a result of recent menstruation. The spherical cells

appeared to be eukaryotic in origin and could have been yeast cells, B-cells or red blood cells; the cell in the centre of Fig. 3(b) appears to have the characteristic dimple of red blood cells. None of these interpretations are fully satisfactory, however, as no budding or other surface features were observed on most of these cells. *Candida albicans* cells isolated from vaginal exudates of patients with vulvovaginal candidiasis were 1–2 μ m in diameter by SEM analysis (Camacho *et al.*, 2007), significantly smaller than the 4 μ m spherical cells seen here, while B-cells and red blood cells are larger than 4 μ m. No yeast cells could be cultured from any of the samples suggesting the absence of *Candida* spp.

Biofilm thickness

Biofilm measurements of the inner and outer IVR surfaces were carried out using SEM and the results are presented in Table 2. Most of the biofilm mass was located on the inner surface of the rings, presumably due to mechanical removal from the outer surface when the IVRs were worn. This is not surprising given the images shown in Fig. 2. The thickness of biofilm phenotype I was typically ten times less than that of phenotype II, with mean values ranging between 0.5–7.5 μ m and 16.3–59.2 μ m for phenotypes I and II, respectively.

FISH–CLSM analysis

Fig. 5 shows IVR samples hybridized with EUB338 (red) and labelled with WGA (green). Yellow areas represent overlap between signals from both probes. EUB338 is a universal FISH oligonucleotide probe designed to exclusively target members of the domain Bacteria (Amann *et al.*, 1995). The fluorescently labelled lectins WGA and concanavalin have been shown to efficiently bind biofilm EPS (Johnsen *et al.*, 2000; Strathmann *et al.*, 2002), and provide useful probes for CLSM examination. Both were evaluated in the present work, and WGA appeared to be superior for the visualization of the studied EPS (data not

Fig. 2. Colposcopic images taken *in vivo* with the IVRs (indicated by arrows) in place. The white cylinders are the TFV pods (P).

Fig. 3. Scanning electron micrographs of the surface features on IVRs containing blank (control) pods after 28 days implantation. The corresponding macaque ID and scale bar dimensions for each frame are given in parentheses below. (a) Portions of the ring surface were covered with a monolayer of epithelial cells along with EPS (PZA2; bar, 1 mm). (b) Amorphous biofilms contained numerous bacterial cells and thin fibres (right side). Other regions (left side) of the biofilm had a reticulated appearance. Spherical cells (diameter 4 μm) were associated with the biofilms (PZA2; bar, 10 μm). (c) The monolayer of epithelial cells was covered with bacteria and biofilm EPS. Bacterial cells with different sizes and shapes (see inset) were associated with the epithelial cell surface (PVC2; bar, 20 μm). (d) Some regions of the biofilms contained dense clusters of bacteria with variable size and shape. Long (>2 μm) rod-shaped bacteria were the most abundant and were interconnected by thin fibres (PVC2; bar, 2 μm). (e) Other regions of the biofilms appeared to have more associated extracellular material (PVC2; bar 2 μm). (f) Side view of bacterial biofilm cross-section used in thickness measurements. The clear material at the bottom of the image corresponds to the ring surface (PZA2; bar, 50 μm).

shown). Fig. 5 supports the SEM results suggesting that large areas of the IVRs (Fig. 5b) are covered with bacterial biofilms and that these structures are comprised of high densities of bacterial cells attached to one another in a matrix of extracellular material. In some regions, the bacteria were clustered together (red zones) in the absence of material labelled by the WGA (see Fig. 5e). Examination of these systems at higher magnification (Fig. 5a) indicated considerable heterogeneity in the clustering of the bacterial cells and their distribution within the EPS.

DISCUSSION

Several commercial IVR products are available for contraception and for the treatment of vaginal atrophy (Dezarnaulds & Fraser, 2003). Millions of these devices are used by women worldwide. Additionally, the CAPRISA 004 trial represents a recent demonstration of prophylaxis against HIV and herpes simplex virus using a topical microbicide (Abdool Karim *et al.*, 2010). Protection was based on the antiviral drug TFV and was greater with

Fig. 4. Scanning electron micrographs of surface features on IVRs containing TFV after 28 days implantation. The corresponding macaque ID and scale bar dimensions for each frame are given in parentheses below. (a) Portions of the ring surface were covered with a monolayer of epithelial cells and EPS (PRB2; bar, 1 mm). (b) Biofilms, consisting of bacterial cells linked by thin fibres and amorphous extracellular material, were observed on the surface of epithelial cells (PID2; bar, 10 μ m). (c) In other regions, bacterial mats (see inset) were associated with the epithelial cell monolayer (PMD2; bar, 20 μ m). (d) Rod-shaped bacteria were interconnected by thin fibres on an amorphous extracellular structure (PID2; bar, 2 μ m). (e) At higher magnification, the bacterial mat consisted of bacteria with a variety of shapes. In this image, which shows a disruption at the edge of the biofilm, the bacteria can be clearly observed embedded in the extracellular matrix (PMD2; bar, 2 μ m). (f) Side view of bacterial biofilm cross-section used in thickness measurements. The dark stripe at the bottom of the image corresponds to the ring surface (PRB2; bar, 50 μ m).

increased adherence. Since IVRs are believed to increase adherence, the development of ring formulations of antiviral drugs is an urgent global priority and likely will lead to increased use of IVR products in the future (Geonnotti & Katz, 2010; Promadej-Lanier *et al.*, 2009; Romano *et al.*, 2009; Saxena *et al.*, 2009; Smith *et al.*, 2008). The potential for microbial biofilms to develop on the surfaces of these devices *in vivo* therefore needs to be better understood.

In this study we have conducted a safety study of silicone IVRs developed in house using a pig-tailed macaque model

(Promadej-Lanier *et al.*, 2009). Patton *et al.* (1996) have demonstrated that the vaginal microflora of the pig-tailed macaque is a useful model in the evaluation of intravaginal devices prior to widespread intravaginal use in women. Like humans, *M. nemestrina* typically has a 28 day menstrual cycle and no significant changes in microflora have been observed over that period (Patton *et al.*, 1996). More recent studies using culture-independent techniques confirmed the usefulness of the macaque model in studies involving the vaginal microbiota (Spear *et al.*, 2010; Yu *et al.*, 2009). In the present study, the vaginal microflora of

Table 2. Biofilm thickness measurements on the inner and outer surfaces of the IVRs

Measurements were made on cross-sections of glutaraldehyde-fixed ring segments using SEM.

Test animal ID and analysed ring surface	Structure thickness [mean $\pm$ SD (μ m)]		
	Biofilm phenotype I*	Biofilm phenotype II†	Other‡
PHD2 inner§	6.48 $\pm$ 4.11		
PHD2 outer			0.74 $\pm$ 0.30
PID2 inner§	7.50 $\pm$ 5.17		
PID2 outer	2.37 $\pm$ 0.78		0.56 $\pm$ 0.27
PMD2 inner§	4.72 $\pm$ 1.98	16.28 $\pm$ 5.06	1.31 $\pm$ 0.62
PMD2 outer			
PRB2 inner§		59.17 $\pm$ 13.55	
PRB2 outer	0.47 $\pm$ 0.11		
PVC2 inner	2.18 $\pm$ 0.59		
PVC2 outer			
PZA2 inner	5.17 $\pm$ 2.05	38.28 $\pm$ 16.76	
PZA2 outer	4.77 $\pm$ 1.77		

*Dense bacterial mat, usually on the surface of epithelial cells.

†Fibrous and defined by highly porous, open architecture.

‡Resembles monolayer of epithelial cells, not a biofilm.

§IVR containing and releasing TFV.

||Control IVR that did not contain TFV.

Fig. 5. FISH micrographs of IVR biofilm samples (test animal PZA2) hybridized with EUB338 (red, labels bacteria) and labelled with WGA (green, labels biofilm). Yellow areas represent overlap between signals from both probes. (a) Inner surface of IVR showing the distribution of biofilm and bacteria. Bar, 100 μ m. (b) Low magnification view of biofilm and bacteria on an IVR segment, visible as the grey rectangular object. Bar, 1 mm. (c) Imaged ring surface showing dense biofilm. (d) Imaged ring surface showing the location of bacteria. (e) Superimposed image of (c) and (d). Bar, 100 μ m.

five of the six macaques was normal and was not significantly affected by retaining the IVR for 28 days. One of the control animals developed BV-like flora after insertion of the ring that resolved spontaneously without treatment when the ring was removed.

Bacterial biofilms formed on all six rings at 28 days, with most of the material accumulating on the inner surfaces. Epithelial cells also were observed covering large areas of the IVRs. In an SEM study on a contraceptive IVR worn for 28 days by a human volunteer, Miller *et al.* (2005) observed 'visible mucus and vaginal discharge' on the ring surface, but did not recognize that this material likely was a monolayer of epithelial cells. Most of the bacterial biofilms observed here formed on the surface of the epithelial cell monolayer, an unexpected result. It appears that epithelial cells colonize the surface of the ring followed by bacterial attachment and subsequent adherent biofilm formation. No significant difference in biofilm architecture and thickness was observed between the animal positive for *G. vaginalis* and the remaining five healthy animals. These results suggest that multispecies biofilms form on IVRs coated with epithelial cells, and likely also on the vaginal epithelium, independent of the presence of *G. vaginalis*. No other studies have examined the characteristics of bacterial biofilms in healthy vaginal ecosystems (Witkin *et al.*, 2007).

Two biofilm phenotypes were observed in most of the IVR samples and a broad diversity of bacterial cells were closely associated with the extracellular material. Phenotype I, the more common of the two, consisted of tightly packed bacterial mats approximately 5 µm in thickness. Phenotype II was much thicker, typically 40 µm, and had an open architecture containing interwoven networks of uniform fibres reminiscent of structures observed in monospecies *Pseudomonas* spp. laboratory cultures (Baum *et al.*, 2009). These so-called nanowires have been observed as a consistent feature of bacterial biofilms (Baum *et al.*, 2009; Gorby *et al.*, 2006; Schaudinn *et al.*, 2007). There was no significant difference in biofilm thickness and appearance between medicated and unmedicated IVRs.

More research is required to better understand the microbial ecology of biofilms developing on the surface of IVRs and the implications to the user. These preliminary results suggest that bacterial biofilms could be common on intravaginal devices worn for extended periods of time.

ACKNOWLEDGEMENTS

The authors thank the National Institutes of Health (grant number 5R21AI079791 and 5R21AI076136), CONRAD (service contract number PSA-08-10 and PPC-09-017), the International Partnership for Microbicides, the US Agency for International Development (cooperative agreement number GPO-A-00-05-00041-00) and the National Science Foundation (award number 0722354) for funding support. The authors also thank the Ahmanson Foundation for continued financial support of advanced imaging at the House Ear Institute. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for

Disease Control and Prevention. The authors have no commercial or other associations that might pose a conflict of interest. The use of trade names is for identification only and does not constitute endorsement by the US Department of Health and Human Services, the Public Health Service or the Centers for Disease Control and Prevention.

REFERENCES

- Abdool Karim, Q., Abdool Karim, S. S., Frohlich, J. A., Grobler, A. C., Baxter, C., Mansoor, L. E., Kharsany, A. B. M., Sibeko, S., Mlisana, K. P. & other authors (2010). Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* **329**, 1168–1174.
- Amann, R. I., Ludwig, W. & Schleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**, 143–169.
- Baum, M. M., Kainović, A., O'Keefe, T., Pandita, R., McDonald, K., Wu, S. & Webster, P. (2009). Characterization of structures in biofilms formed by a *Pseudomonas fluorescens* isolated from soil. *BMC Microbiol* **9**, 103.
- Begley, J. S. & Barnes, R. C. (2007). Group B streptococcus toxic shock-like syndrome in a healthy woman: a case report. *J Reprod Med* **52**, 323–325.
- Camacho, D. P., Consolaro, M. E. L., Patussi, E. V., Donatti, L., Gasparetto, A. & Svidzinski, T. I. E. (2007). Vaginal yeast adherence to the combined contraceptive vaginal ring (CCVR). *Contraception* **76**, 439–443.
- Chassot, F., Negri, M. F. N., Svidzinski, A. E., Donatti, L., Peralta, R. M., Svidzinski, T. I. E. & Consolaro, M. E. L. (2008). Can intrauterine contraceptive devices be a *Candida albicans* reservoir? *Contraception* **77**, 355–359.
- Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.
- Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C. & Ehrlich, G. (2003). The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest* **112**, 1466–1477.
- Davies, D. (2003). Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov* **2**, 114–122.
- Dezarnaulds, G. & Fraser, I. S. (2003). Vaginal ring delivery of hormone replacement therapy – a review. *Expert Opin Pharmacother* **4**, 201–212.
- Geonnotti, A. R. & Katz, D. F. (2010). Compartmental transport model of microbicide delivery by an intravaginal ring. *J Pharm Sci* **99**, 3514–3521.
- Gorby, Y. A., Yanina, S., McLean, J. S., Rosso, K. M., Moyles, D., Dohnalkova, A., Beveridge, T. J., Chang, I. S., Kim, B. H. & other authors (2006). Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proc Natl Acad Sci U S A* **103**, 11358–11363.
- Johnsen, A. R., Hausner, M., Schnell, A. & Wuerz, S. (2000). Evaluation of fluorescently labeled lectins for noninvasive localization of extracellular polymeric substances in *Sphingomonas* biofilms. *Appl Environ Microbiol* **66**, 3487–3491.
- Lal, P., Agarwal, V., Pruthi, P., Pereira, B. M. J., Kural, M. R. & Pruthi, V. (2008). Biofilm formation by *Candida albicans* isolated from intrauterine devices. *Indian J Microbiol* **48**, 438–444.
- Macalady, J. L., Lyon, E. H., Koffman, B., Albertson, L. K., Meyer, K., Galdenzi, S. & Mariani, S. (2006). Dominant microbial populations in

- limestone-corroding stream biofilms, Frasassi cave system, Italy. *Appl Environ Microbiol* 72, 5596–5609.
- Martin, R. & Suárez, J. E. (2010). Biosynthesis and degradation of H₂O₂ by vaginal lactobacilli. *Appl Environ Microbiol* 76, 400–405.
- Miller, L., MacFarlane, S. A. & Materi, H. L. (2005). A scanning electron microscopic study of the contraceptive vaginal ring. *Contraception* 71, 65–67.
- Moss, J. A., Baum, M. M., Malone, A. M., Friend, D. R., Smith, J., Kopin, E., Kennedy, S., Nguyen, C., Cortez, C. & other authors (2010). *In vitro* release of tenofovir and acyclovir from a silicone vaginal ring platform. In *Abstracts of the International Microbicides Conference 2010, Pittsburgh, PA, USA*, abstract 259, p. 153. Pittsburgh, PA: Microbicide Trials Network.
- Nel, A., Smythe, S., Young, K., Malcolm, K., McCoy, C., Rosenberg, Z. & Romano, J. (2009). Safety and pharmacokinetics of dapivirine delivery from matrix and reservoir intravaginal rings to HIV-negative women. *J Acquir Immune Defic Syndr* 51, 416–423.
- NIH (1996). *Guide for the Care and Use of Laboratory Animals*, NIH publication no. 85-23. Washington, DC: National Institutes of Health.
- Patton, D. L., Sweeney, Y. C., Rabe, L. K. & Hillier, S. L. (1996). The vaginal microflora of pig-tailed macaques and the effects of chlorhexidine and benzalkonium on this ecosystem. *Sex Transm Dis* 23, 489–493.
- Patton, D. L., Sweeney, Y. T. C., Agnew, K. J., Balkus, J. E., Rabe, L. K. & Hillier, S. L. (2006). Development of a nonhuman primate model for *Trichomonas vaginalis* infection. *Sex Transm Dis* 33, 743–746.
- Promadej-Lanier, N., Smith, J. M., Srinivasan, P., McCoy, C. F., Butera, S., Woolfson, A. D., Malcolm, R. K. & Otten, R. A. (2009). Development and evaluation of a vaginal ring device for sustained delivery of HIV microbicides to non-human primates. *J Med Primatol* 38, 263–271.
- Rabe, L. K. & Hillier, S. L. (2003). Optimization of media for detection of hydrogen peroxide production by *Lactobacillus* species. *J Clin Microbiol* 41, 3260–3264.
- Romano, J., Variano, B., Coplan, P., Van Roey, J., Douville, K., Rosenberg, Z., Temmerman, M., Verstraelen, H., Van Bortel, L. & other authors (2009). Safety and availability of dapivirine (TMC120) delivered from an intravaginal ring. *AIDS Res Hum Retroviruses* 25, 483–488.
- Romero, R., Schaudinn, C., Kusanovic, J. P., Gorur, A., Gotsch, F., Webster, P., Nhan-Chang, C. L., Erez, O., Kim, C. J. & other authors (2008). Detection of a microbial biofilm in intraamniotic infection. *Am J Obstet Gynecol* 198, 135.e1–135.e5.
- Saxena, B. B., Han, Y. A., Fu, D. Y., Rathnam, P., Singh, M., Laurence, J. & Lerner, S. (2009). Sustained release of microbicides by newly engineered vaginal rings. *AIDS* 23, 917–922.
- Schaudinn, C., Stoodley, P., Kainović, A., O’Keeffe, T., Costerton, J. W., Robinson, D. H., Baum, M. M., Ehrlich, G. & Webster, P. S. (2007). Bacterial biofilms, other structures seen as mainstream concepts. *Microbe* 2, 231–237.
- Smith, D. J., Wakasiaka, S., Hoang, T. D. M., Bwayo, J. J., Del Rio, C. & Priddy, F. H. (2008). An evaluation of intravaginal rings as a potential HIV prevention device in urban Kenya: behaviors and attitudes that might influence uptake within a high-risk population. *J Womens Health (Larchmt)* 17, 1025–1034.
- Spear, G. T., Gilbert, D., Sikaroodi, M., Doyle, L., Green, L., Gillevet, P. M., Landay, A. L. & Veazey, R. S. (2010). Identification of rhesus macaque genital microbiota by 16S pyrosequencing shows similarities to human bacterial vaginosis: implications for use as an animal model for HIV vaginal infection. *AIDS Res Hum Retroviruses* 26, 193–200.
- Stewart, P. S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* 292, 107–113.
- Stoner, K. A., Rabe, L. K., Austin, M. N., Meyn, L. A. & Hillier, S. L. (2008). Quantitative survival of aerobic and anaerobic microorganisms in Port-A-Cul and Copan transport systems. *J Clin Microbiol* 46, 2739–2744.
- Strathmann, M., Wingender, J. & Flemming, H. C. (2002). Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of *Pseudomonas aeruginosa*. *J Microbiol Methods* 50, 237–248.
- Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L. P. & Lochs, H. (2005). Adherent biofilms in bacterial vaginosis. *Obstet Gynecol* 106, 1013–1023.
- Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., Lochs, H. & Verstraelen, H. (2008). An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* 198, 97.e1–97.e6.
- Tang, Y. W., Himmelfarb, E., Wills, M. & Stratton, C. W. (2010). Characterization of three *Staphylococcus aureus* isolates from a 17-year-old female who died of tampon-related toxic shock syndrome. *J Clin Microbiol* 48, 1974–1977.
- Valenta, C. (2005). The use of mucoadhesive polymers in vaginal delivery. *Adv Drug Deliv Rev* 57, 1692–1712.
- Veeh, R. H., Shirliff, M. E., Petik, J. R., Flood, J. A., Davis, C. C., Seymour, J. L., Hansmann, M. A., Kerr, K. M., Pasmore, M. E. & Costerton, J. W. (2003). Detection of *Staphylococcus aureus* biofilm on tampons and menses components. *J Infect Dis* 188, 519–530.
- Webster, P., Wu, S., Webster, S., Rich, K. A. & McDonald, K. (2004). Ultrastructural preservation of biofilms formed by non-typeable *Hemophilus influenzae*. *Biofilms* 1, 165–182.
- Witkin, S. S., Linhares, I. M. & Giraldo, P. (2007). Bacterial flora of the female genital tract: function and immune regulation. *Best Pract Res Clin Obstet Gynaecol* 21, 347–354.
- Yoo, J. W. & Lee, C. H. (2006). Drug delivery systems for hormone therapy. *J Control Release* 112, 1–14.
- Yu, R. R., Cheng, A. T., Lagenaur, L. A., Huang, W. J., Weiss, D. E., Treece, J., Sanders-Beer, B. E., Hamer, D. H., Lee, P. P. & other authors (2009). A Chinese rhesus macaque (*Macaca mulatta*) model for vaginal *Lactobacillus* colonization and live microbicide development. *J Med Primatol* 38, 125–136.