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ORIGINAL ARTICLE

A requirement for flow to enable the development of *Ureaplasma parvum* biofilms *in vitro*

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Abstract

Aims: To use a flow-based method to establish, quantify and visualize biofilms of *Ureaplasma parvum*.
Methods and Results: Absorbance readings of a *U. parvum* HPA5 culture were taken at 550 nm every 3 h for 30 h in order to establish a growth curve, with viability determined by the number of colour changing units (CCUs). Biofilms were established using the DTU flow-cell with a flow rate of 0·01 ml min^{-1} and compared to the static control. Titres of bacteria were determined by CCU and biofilm biomass was quantified by Syto9 staining and COMSTAT analysis. High-resolution images were obtained by scanning electron microscopy (SEM). Flow resulted in significantly more biofilm and higher cell titre (0·599 $\mu m^3/\mu m^2 \pm 0{\cdot}152$ and 4×10^8 CCU per ml, respectively) compared with static conditions (0·008 $\mu m^3/\mu m^2 \pm 0{\cdot}010$ and no recoverable cells, respectively). SEM revealed pleomorphic cells, with signs of budding and possible membrane vesicle formation.
Conclusions: Flow is an essential requirement for the establishment of *U. parvum* biofilms.
Significance and Impact of the Study: This is the first quantification of biofilm biomass formed by *U. parvum*. It is now possible to establish viable biofilms of *U. parvum* which will allow for future testing of antimicrobial agents and understanding of virulence-associated with adhesion.

Introduction

Mollicutes include several human and veterinary relevant pathogens. These organisms differ from many bacteria by lacking a cell wall, being pleomorphic in shape, harbouring a genome with reduced coding capacity and an essential requirement for sterols. The bacteria within the genus *Ureaplasma* are unique among Mollicutes due to their sole utilization of urea in the acquisition of energy (Robertson *et al.* 2002). The conversion of urea to ammonia and carbon dioxide results in an increase in the surrounding pH, which comes at the cost of viability when grown in vitro and therefore giving a limited window in which it is possible to work with viable organisms (Masover *et al.* 1977).

Two human associated Ureaplasma species exist, *Ureaplasma parvum* and *Ureaplasma urealyticum*. Among adults, ureaplasmas colonize mucosal membranes within the genital tract of around 40–80% of women and 30% of men (Waites *et al.* 2005). Although colonization among asymptomatic individuals is common, several disease states have been associated. Ureaplasmas can ascend the female genital tract during pregnancy, leading to *in utero* infection, chorioamnionitis and subsequent

premature birth (Sweeney *et al.* 2017; Pavlidis *et al.* 2020). Infections of the genital tract among men that result in non-gonococcal urethritis (NGU) is still under debate, with evidence of species of ureaplasma present, host adaptive immune response and bacterial load being key factors in disease outcome (Beeton *et al.* 2019). Infections with *Ureaplasma* sp. of the genital tract can be persistent even in the presence of antimicrobial therapy suggesting a potential means to evade antimicrobials and the immune clearance (Khosropour *et al.* 2015).

Capacity to form a biofilm, quantification of biomass and visualization of these three-dimensional structures have been described for a number of Mollicutes such as *Mycoplasma pneumoniae*, *Mycoplasma pulmonis* and *Mycoplasma genitalium* (Simmons *et al.* 2007, 2013; Simmons *et al.* 2007, 2013; Daubenspeck *et al.* 2020; Daubenspeck *et al.* 2020). To date, there is very little information regarding biofilm formation by the urea utilizing mycoplasmas in the genus *Ureaplasma* (García-Castillo *et al.* 2008; Pandelidis *et al.* 2013) with all published methods focusing on static models with a lack in quantification or visualization of the biofilms formed.

Here, we discuss the essential requirement for flow for *U. parvum* to develop a biofilm. Using this system, it was possible to give the first quantification of biofilm biomass produced by *U. parvum* utilizing a flow-based system. Furthermore, we present confocal scanning laser microscopy and scanning electron micrograph images of *U. parvum* which have been grown using this method.

Materials and methods

Strains and media used

All experiments were conducted with *U. parvum* strain HPA5. Cultures were grown in Ureaplasma Selective Media (Mycoplasma Experience, Surrey, UK) within microtitre plates covered with an adhesive sealing film to prevent leaching of ammonia to adjacent wells. To ensure inoculation with viable, actively growing *U. parvum*, 10-fold dilutions were grown overnight. The next morning the lowest dilution in which a colour change had occurred was used for inoculation into the flow cell.

Growth curve experiment

A 10-ml culture of *U. parvum* HPA5 was established with viable cells from an overnight dilution. Cultures were incubated under static conditions at 37°C for 30 h. Every three hours the titre of *U. parvum* was quantified by performing serial 10-fold titration in USM within microtitre plates which were sealed and incubated for 48 h until colour change had ceased (Beeton *et al.* 2012). The lowest dilution in which a colour change occurred was designated as 1 CCU. Therefore, by calculating back through the dilution series, it was possible to extrapolate the number of CCU in the original culture. In addition to CCU quantification, absorbance readings at 550 nm, as surrogate marker for growth due to media alkalisation and detectable equivalence point of phenol red indicator present in the growth media, were determined by spectroscopy (Tecan Infinite M200) (Heath *et al.* 2020).

Set up and running of the DTU flow cell

The DTU flow cell was prepared as described elsewhere (Crusz *et al.* 2012; Tolker-Nielsen and Sternberg 2014). In brief, 24 × 55-mm borosilicate glass D 263^{TM} coverslips (VWR, Lutterworth, UK) were adhered to the DTU flow cell with transparent silicone. The flow cell was connected to a Masterflex LS peristaltic pump and the system was flooded with 1% v/v hypochlorite for 4 h. The system was purged three times with air and then flushed with sterile water for 16 h, prior to displacing the sterile water with USM. Inlet tubes were surface sterilized with an alcohol wipe, and a 27G insulin needle (VWR) was used to inoculate each chamber with 150 µl of viable overnight culture (resulting puncture holes were sealed with silicone). For direct comparison of static and flow conditions, two channels were inoculated with the same overnight preparation. Both flow cells were left static for four hours to allow for attachment of *U. parvum*. Following the attachment phase, only one channel was subjected to flow rate of 0·01 ml min^{-1}, while the media in the second channel remained static. Duration of the experiment allowed to progress for a further 24 h, all media and equipment being contained incubated at 37°C.

Confocal scanning-laser microscopy imaging of biofilms and quantification of bound cells

At 28-h post-inoculation, 200 µl of sterile phosphate-buffered saline (PBS) was gently flushed through the channels to remove any non-adherent cells. Two hundred microliter of 5 mmol l^{-1} Syto9 in PBS (ThermoFisher, UK) was then injected into each channel. Samples were incubated in the dark at 37°C for 30 min followed by rinsing with a further 200 µl of PBS to remove any background Syto9. Biofilms were the visualized on a Nikkon Eclipse 80i microscope using 485-nm excitation and 498-nm emission filters. Five random sections were selected for each chamber, and Z-stack images were taken.

Following imaging, channels were triturated vigorously with 200 µl of USM to dislodge bound cells. Bacterial viability was quantified by determining the CCUs by titration as outlined above.

Preparation of biofilms for visualization by scanning electron microscopy

Flow cell experiments were repeated exactly as outlined above, except that post-removal of non-adherent cells at 28-h post-inoculation, coverslips were removed from the chamber and fixed with 2·5% glutaraldehyde in 0·1-mol l^{-1} sodium cacodylate buffer. Samples were post-fixed with 1% osmium tetroxide, dehydrated in an acetone series (50–100%) and chemically dried in hexamethyldisilazane. Samples were sputter coated with a thin layer of chromium and imaged using a field emission scanning electron microscope (JEOL JSM6301F).

Statistical analysis

Biomass was determined by COMSTAT for analysis (Heydorn *et al.* 2000). Statistical analysis by *t* test was undertaken in GraphPad Prism ver. 5.01.

Results

U. parvum rapidly lose viability following log phase under static conditions

Ureaplasma parvum was grown under static conditions for 30 h with measurements of viability and media colour change as a marker for media alkalisation taken every 3 h (Fig. 1a). Culture media remained orange for 9 h with a mean absorbance value of 1·7; however, evidence of alkalization was apparent by increased absorbance by the phenol red at 12 h (light red in colour) and the media appearing transparent and cerise red in colour after 15 h as indicated by an absorbance reading of 2·8 (Fig. 1b). In parallel, viability assessment (quantified by titration in USM) showed a lag phase between 0 and 3 h followed by an exponential increase in number between 3 and 12 h. No stationary phase was observed, showing the susceptibility of *U. parvum* to toxic by-products as viability rapidly declined between 15 and 24 h.

Evaluation of *U. parvum* biofilms under flow and static conditions

Using the DTU flow cell system *U. parvum* HPA5 was inoculated into two channels. Following adhesion, the first channel was subject to flow of 0·01 ml min^{-1}, while the second channel was left under sterile static conditions. Sporadic clusters of biofilm were observed throughout the channel subjected to flow conditions (Fig. 2a,b). Comet-like tail structures were observed from the colonies, usually observed tapering with the direction of flow. No biofilm was observed within the channel under static conditions. Figure 2c shows the evaluation of Z-stack images with COMSTAT that confirmed a statistically significant level of biomass under flow conditions (0·599 $\mu m^3/\mu m^2 \pm 0{\cdot}152$) compared to growth under no flow (0·008 $\mu m^3/\mu m^2 \pm 0{\cdot}010$). The number of viable cells attached within the channel after 28-h post-inoculation was also determined by CCU quantification (Fig. 2d). Under flow conditions 4×10^8 CCU per ml were recovered as compared to no detectable viable growth when subjected to static conditions.

Scanning electron microscopy of *U. parvum* biofilms grown under flow conditions

Scanning electron microscopy was used to obtain detailed high-magnification images of *U. parvum* HPA5 cells grown under flow conditions for 28 h (Fig. 3). Cells

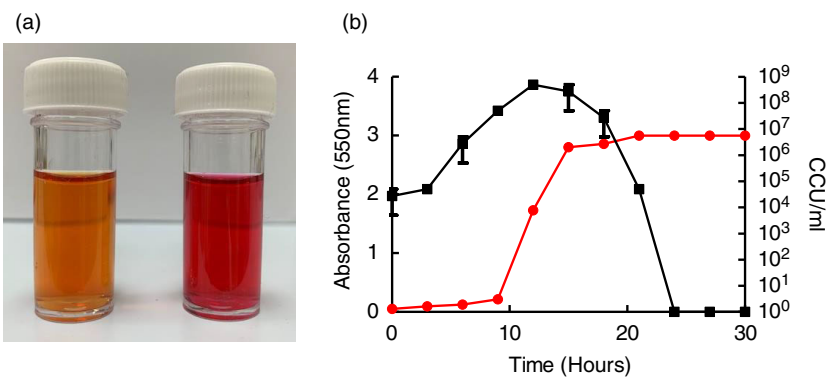

Figure 1 The relationship between *Ureaplasmaparvum* HPA5 viability and change in the colour of culture media over 30 h under static conditions. (a) *Ureaplasma parvum* culture at inoculation represented by orange media (left) compared with positive culture (red) with no visible sign of turbidity and unknown cell titre or viability (right). (b) The change in media colour over time quantified by absorbance readings at 550 nm (red line) and viability of cells quantified by determining the CCU (black line) every 3 h. CCU = colour changing units.

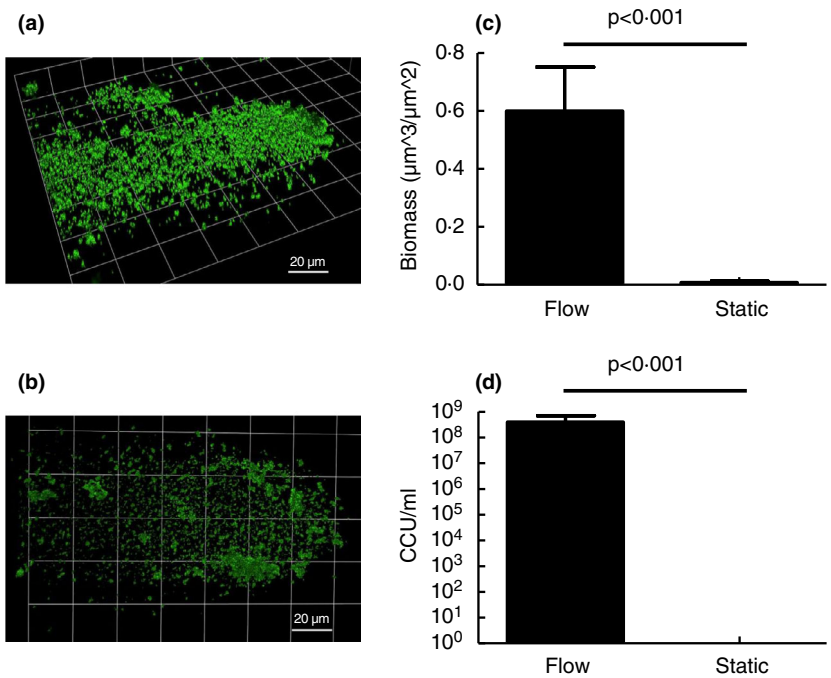

Figure 2 Presence of *Ureaplasma parvum* HPA5 biofilms formed under flow conditions. *Ureaplasma parvum* biofilms were grown under flow (0·01 ml min^{-1}) and no flow (0 ml min^{-1}) conditions for 28 h. (a & b) Representative Syto9 stained biofilms grown under flow conditions with direction of flow from right to left. (c) Z-stacks were taken and compared with no flow conditions with biomass quantified with COMSTAT2 image analysis software. (d) The number of viable cells associated with the surface was quantified by flushing PBS through the chamber following visualisation and subsequently determining the number of colour changing units (CCU).

attached to the glass cover slip were sporadic after preparation for imaging (Fig. 3a). Cells which remained were associated with an extracellular matrix linking cells together (Fig. 3b). At a magnification of 15 000× (Fig. 3c) cells were pleomorphic in shape and variable in size. On closer inspection, some cells also displayed signs of budding with the possibility of membrane vesicles formation and conjugative apparatus-like appearing protrusions (Fig. 3d; indicated with * and ^, respectively).

Discussion

Ureaplasma sp. are unique in their essential requirement for urea in the production of ATP (Robertson *et al.* 2002). Although human-associated ureaplasmas are recognized causes in a number of persistent infections such as those associated with preterm birth, neonatal lung disease and suggested links with NGU among men, very little is known regarding the ability to form biofilms.

The first indications to suggest *Ureaplasma* sp. have the capacity to form a biofilm was presented by García-Castillo *et al.* (2008) and then subsequently by Pandelidis *et al.* (2013). Both studies utilized a static peg-lid-based approach to grow biofilms of clinical isolates of *Ureaplasma* sp. whereby lids were transferred to fresh media following the change of culture media from orange to red. Using this methodology, the studies found 82 and 95% of clinical isolates had the capacity to form biofilms, respectively. Although most isolates were found to form biofilms using this method, in some instances, some isolates were unable to. It is not clear if this was either true absence of biofilm-forming capacity, or an artefact of overincubation and viability loss between transfer to fresh media resulting in biofilm failure (Masover *et al.* 1977). Inferring from our viability data with *U. parvum* HPA5, it suggests that the viability window from the point of detectable colour change is very narrow: bacterial viability peaks before full visible colour change to red has occurred (Fig. 2b). Therefore, media change 6–9 h after this point would see significant loss of viable cells and strains with shorter generation time would fair even worse.

Due to the limitations of a static system resulting from toxic metabolite accumulation and subsequent drop in cell viability, we reasoned that a flow-based system would sustain the development of *Ureaplasma* biofilms. By using the DTU flow cell method, it was possible to run parallel culture, with the same inoculum, under static and flow conditions. Confocal scanning laser microscopy was used to obtain the first images of biofilms formed by

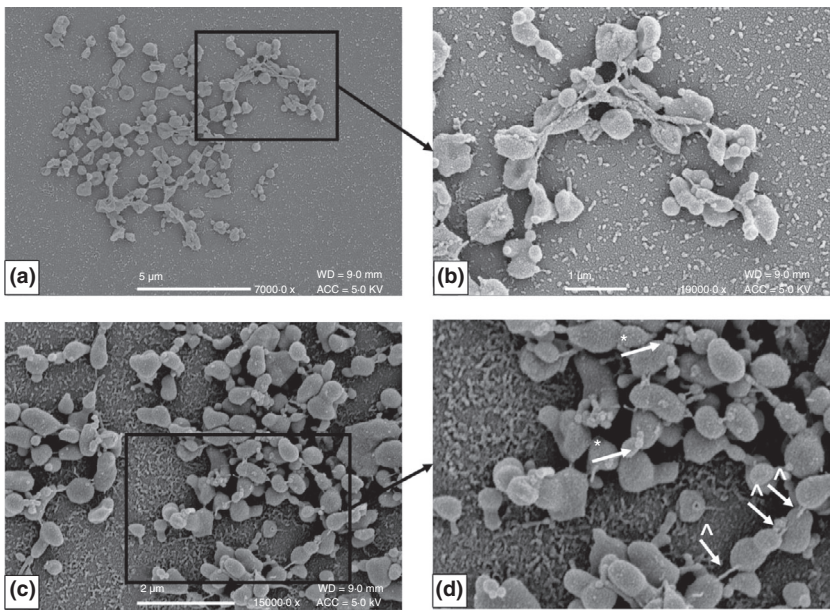

Figure 3 Scanning electron micrographs of *Ureaplasma parvum* HPA5 biofilms formed under flow conditions. *Ureaplasma parvum* biofilms were grown under flow conditions for 28 h and prepared for SEM (a) with indication of an extracellular matrix (b). Cells were highly pleomorphic exhibiting budding (c). White arrows indicated by * suggest the presence of membrane vesicles and white arrows indicated by a ^ indicate the possibility of conjugation pilus (d).

U. parvum in our axenic culture. Biofilms took on a sporadic microcolony appearance and did not form large areas of biomass unlike that seen with other bacteria (Crusz *et al.* 2012). It is not known if the development of these microcolonies are the result of cell aggregates settling and continuing to expand through cell division as observed with *M. pneumoniae* tower formation (Feng *et al.* 2020). This socially distanced phenotype of sporadic microcolonies could be an adaptive trait in order to prevent the accumulation of localized toxic metabolites by large areas of biomass upon a surface. As ureaplasmas exist as obligate parasites (requiring nucleotides, sterols and other key nutrients they cannot synthesize), this would also prevent damage to the cells on which they are bound. In addition to this sporadic microcolony phenotype, a comet-like tail appearance was apparent which has described in other bacteria under flow, such as *Hemophilus influenzae* (Cho *et al.* 2015). This finding was in stark contrast to the channel which was subjected to static conditions which lacked any detectable biofilm. This absence of biofilm was not surprising based on the growth curve viability data.

A common method for quantifying biofilm utilizes crystal violet staining of biomass, which has been successfully used with other mycoplasmas (Simmons and Dybvig 2015). Our previous attempts to utilize this method suggested that it was not sensitive enough due to the low levels of biomass (data not shown). We therefore utilized image data Z-stacks obtained from CLSM using COMSTAT analysis, to give the first quantifiable measurement of biomass formed by *U. parvum*. A second level of quantification was the recovery of high titre of viable organisms when subjected to flow, past the point at which viability was lost within the static channel.

Using SEM, it was possible to get the first high-resolution images of ureaplasma biofilms when grown in axenic culture. Previously Torres-Morquecho *et al.* (2010), used SEM to visulise ureaplasmas attached to A549 lung epithelial cells. The void in SEM images of ureaplasma attached to surfaces in axenic culture may be related to the difficulties of balancing high cell titres to localized toxic concentrations of ammonia and CO_2 production unless flow or chelation can remove these by-products. A further observation from the SEM images was the presence of budding and potentially the formation of membrane vesicles. Budding has previously been observed among ureaplasmas, but little is known with regards to the formation of membrane vesicles (Whitescarver and Furness 1975). Finally, from the SEM images, structures which resembled suspected conjugation pili were apparent. Literature on horizontal gene transfer among ureaplasmas is limited; however, attempts to use qPCR target panels to serotype clinical ureaplasmas established that extensive horizontal gene transfer rendered this impossible (Xiao *et al.* 2011), which was then confirmed by detailed whole genome analysis of 19 strains of

ureaplasma subsequently (Paralanov *et al.* 2012). Identification of a mobile antibiotic resistance gene, *tet*(M) element, which is present on a transposable element has also been identified in both *U. parvum* and *U. urealyticum* (Dégrange *et al.* 2008; Beeton and Spiller 2017). Although HPA5 is *tet*(M)-negative, the presence of these pili may suggest the presence of other uncharacterized transposable elements as suggested by the presence of the UU372 DDE-type transposase (NP_078206.1), the integrase-recombinase protein UU404 (NP_078239.1) and the tyrosine recombinase XerC (UU222; NP_078055.1) found within the whole genome sequence of HPA5 (unpublished data). Although data suggest the presence of transposable elements within the HPA5 genome, it should be emphasized that the presence of a conjugation pili is speculative and based on observations from the electron micrograph. Further work is underway to examine if this is a true finding or an artefact from the preparation of the samples.

In this study, we present a flow-based method for growing biofilms of *U. parvum* superior to static methods, given the removal of toxic-metabolite mediated cell death. Using this flow-based approach, it was possible to use fluorescent and electron microscopy to gain the high-resolution images of the resulting biofilm. Confocal laser scanning microscopy images were then used to give the first quantification of biomass formed by *U. parvum*. With this platform, it is now feasible to interrogate the role of individual surface associated adhesins, such as the multiple banded antigens, for their role in biofilm formation, as well as the capacity to investigate biofilm-mediated resistance to antimicrobials.

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Conflict of interest

No authors have any conflict of interest to declare.

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Author contributions

M.L.B, R.S.R, S.S, S.E.M, A.B and O.B.S conceived and designed the study. R.S.R, S.S and M.L.B undertook experimental procedures. M.L.B and K.K undertook analysis. MLB and R.S.R drafted the manuscript. All authors approved the final manuscript.

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