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Full Length Research paper

Biofilm formation by *Mycoplasma fermentans* on intrauterine devices

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Microbial biofilm are communities of sessile microorganisms formed by cells that are attached irreversibly to a substratum or interface or to each other and embedded in hydrated matrix of extracellular polymeric substances. Bacterial biofilm formation on intrauterine devices is a very important event in the pathogenesis and evolution of infections associated with the use of these medical devices. Mycoplasmas are typical surface parasites colonizing the mucous membranes of animals and man. Efficient adherence mechanisms are therefore a prerequisite for survival and, in some species, for pathogenicity. The purpose of this study was to examine biofilm formation by *Mycoplasma fermentans* on intrauterine devices. Intrauterine devices were placed in *M. fermentans* cultures during 72 h. Mycoplasmas were analyzed for biofilm formation and cell counts compared for both biofilm and planktonic cells. The examination was carried with stereoscopic and scanning electron microscope. Crystal violet staining and scanning electron microscopic analysis of intrauterine devices indicated that *M. fermentans* formed a biofilm. This study might allow for clearer understanding of the rate of biofilm formation and the importance of different materials, contaminating organisms, and treatments which could control the process.

Key words: Adherence, biofilm, *Mycoplasma fermentans*, intrauterine devices.

INTRODUCTION

Mycoplasmas are a heterogeneous group of the smallest organisms capable of self-replication. Some mycopla-smas cause respiratory or urogenital diseases in human, but others chronically colonize our respiratory and uro-genital tracts without apparent clinical significance. In this respect, wall- free mycoplasmas are among the few pro-karyotes that can grow in close interaction with mamma-lian cells, often silently for a long period of time. How-ever, prolonged interactions with mycoplasmas with see-mingly low virulence could, through a gradual and pro-gressive course, significantly affect many biological pro-perties of mammalian cells (Loo et al., 1991; Taylor-Ro-binson, 1989).

Because mycoplasmas have an extremely small geno-me (0.58-2.20 Mb compared with the 4.64 Mb of *Escheri-chia coli*), these organisms have limited metabolic op-tions for replication and survival. These micro-organisms have evolved molecular mechanisms needed to deal with the host immune response and the transfer and coloniza-tion in a new host (Rottem, 2003). Many mycoplasmas depended on adhesion to host tissues for colonization and infection. In the mycoplasmas the adherence is the major virulence factor, and adherence- deficient mutants are avirulent. These organisms exhibit the typical poly-morphism of mycoplasmas, with the most common flask and filamentous shaped. Cytadherence of these orga-nisms to cells in the respiratory or urogenital epithelium is an initial essential step in tissue colonization and subseq-uent disease pathogenesis (Baseman and Tully, 1997; Razin and Jacobs, 1992) . Because these surfaces are sometimes providing quite inhospitable environments, the mycoplasmas had to develop very efficient mechanisms to adherence to the various cells and substrates.

Mycoplasma fermentans is an infectious agent of the human urogenital and pulmonary systems (Nicol and Ed-ward, 1953; Taylor-Robinson, 1995). Because it has been shown to be associated with lesions in the kidneys of human immunodeficiency virus-positive patients (Bau-er et al, 1991) and causes fulminating disease in monkey

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(Lo et al., 1993), this agent is under investigation as a potential human pathogen. Whether this organism is a cofactor in the progression of AIDS, as has been postulated (Lo, 1992), or represents an opportunistic infection in immunocompromised patients, investigation of the surface proteins of this organism is needed to understand its role in infection, pathogenicity, virulence, and host-cells interaction.

A considerable number of attachment mechanisms are known for bacteria and some of them are under intensive study (Bredt et al., 1981).However, adherent biofilm cells are resistant to antibiotic, antibodies and phagocytes. In addition, biofilms can cause host damage as phagocyte are attracted but phagocytosis is frusted, and phagocytic enzymes are released which damage surrounding tissue and exacerbate infection. As well as enabling chronic infection of hosts, biofilms may cause bouts of acute infection when planktonic cells are periodically released from the biofilm. Despite their discovery in many other bacterial species studied to date, biofilms have not been studied in importance medical mycoplasmas (McAuliffe et al., 2006).

Intrauterine devices are highly effective, long-term methods of contraception; however intrauterine devices use is limited to some regions of the world due to concerns about an increased risk of pelvic inflammatory disease and subsequent complications, such as infertility and ectopic pregnancy. The major complication associated with the use of medical implants such as intrauterine devices, intravascular catheters and tubes is infection. Microorganisms originating from the normal flora can colonize these devices and form biofilms consisting of layer of host cells and bacteria/fungi embedded within a matrix material. Foreign materials of this type are the most probable sites of biofilm formation (Gristina, 1994). The main component of the biofilm produced by the bacteria and/or fungi is an exopolisaccharide layer, which is the pivotal factor responsible for the behaviour of biomaterial-centred infection. The biofilm bacteria are usually resistant to attack by antimicrobial agents and host phagocytes. The objective of this study was to examine biofilms formation by *M. fermentans* on intra-uterine devices.

MATERIAL AND METHODS

Organisms and growth conditions

M. fermentans previously isolated from an asthmatic patient was used throughout the study; the mycoplasma was grown for 24 to 72 h at 37^{O}C in Eaton´s medium and 5 ml aliquots were frozen at -80^{O}C. The frozen inoculum contained 1^{6} CFU per ml of broth. The number of viable cells was determined by the plating method and is presented as CFU.

Analysis of biofilm grown by crystal violet staining in intrauterine devices

Intrauterine copper devices (T 380 A) were placed in *M. fermentans* cultures to a concentration of 1^{6} CFU/ml during 72 h. Cells adherents on intrauterine devices were rinsed briefly in PBS to remove non-adherent cells and stained with 0.5% crystal violet solution for 30 min. Cells adherents were then washed profusely in distilled water before being left to dry at room temperature for at least 30 min. *M. fermentans* was analyzed for biofilm formation and the stationnary-phase cell counts compared for both biofilm and planktonic cells.

Scanning electron microscopy

Intrauterine devices were cut into 1 cm stripes with sterile scissors, and then subjected to chemical dehydratation (in 30, 50, 70, 90, 100% ethanol for 1 h each, then in 30:70, 50:50, 70:30 ethanol: acetone mixtures for 20 min each). The samples were placed in the critical -point drier in 100% acetone, and rinsed three times in liquid CO_2, then the critical point was identified, after which the samples were secured onto racks and coated in gold in a sputter coater. The examination was carried with a JEOL JSM 5410-LV scanning electron microscope and the pictures were digitally recorder.

RESULTS

M. fermentans was assessed for their ability to form a biofilm. Crystal violet staining on intrauterine devices revealed the ability to form a biofilm. Intrauterine devices were only placed in Eaton´s medium did not show adhesion, discarding with this unspecific adhesion of medium components (Figure 1).

Microscopic analysis of crystal violet *M. fermentans* biofilms on intrauterine devices indicated that individual cells were aggregating together to form microcolonies. Biofilm growth by *M. fermentans* was followed over a time-course of 72 h. By 24 h post- inoculation, cells had begun to adhere to the intrauterine devices and thin covering of individual cells could clearly be seen; by 48 h the cells had began to form small group, and by 72 h larger groups of microcolonies were evident (Figure 2). Scanning electron microscopic analysis of intrauterine devices also indicated that *M. fermentans* formed a biofilm (Figure 3). Counts for planktonic cells showed a correlation with result obtained using crystal violet staining (Table 1).

DISCUSSION

The formation of biofilms by most microorganisms involves the regulation of genes that are essential for attachment to surface and the production of extracellular matrices (Danese et al., 2001). Several mycoplasma species have recently been shown to form biofilm (McAuliffe et al., 2006), but the macromolecules and the mechanisms that contribute to biofilm structure are unknown.

M. fermentans can form aggregates on intrauterine devices, it follows that if such aggregates constitute a biofilm. Consistent with what has been described for the extracellular matrices formed by other bacteria, the matrix formed by the mycoplasmas contained protein (VsaA epitopes), lipid, DNA, and saccharide (Beveridge et al., 1997; Whitchurch et al., 2002). Vsa is anchored to the

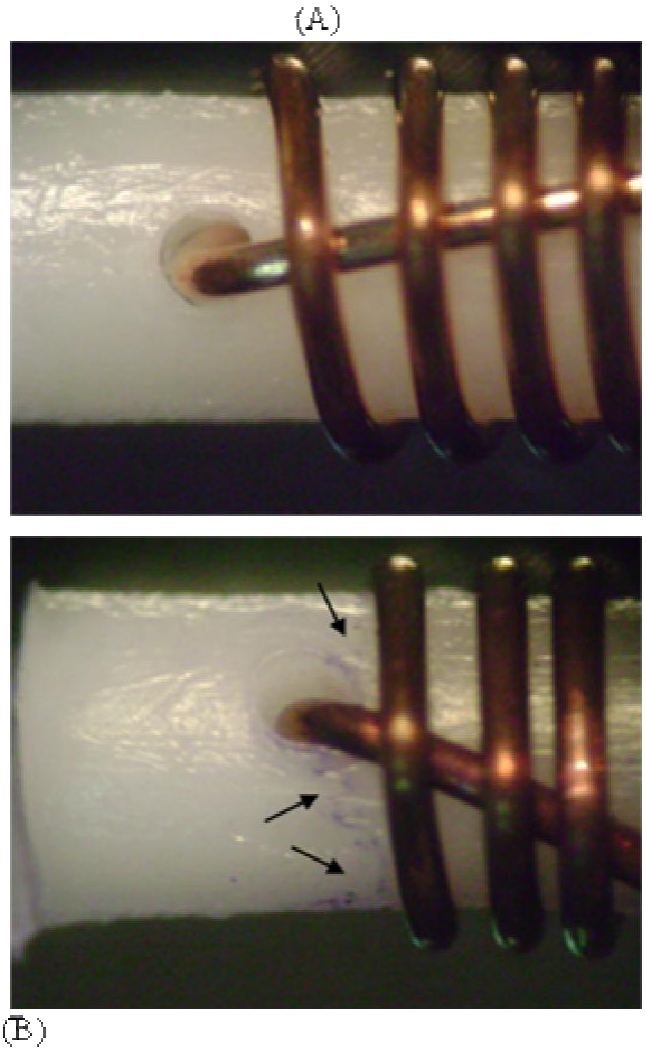

Figure 1. A) Intrauterine devices that were only placed in Eaton´s medium do not present unspecific adhesion, and B) intrauterine devices in presence of *M. fermentans* culture adherence developed 72 hours postinoculation using crystal violet staining (4X), (arrows) adherence zone.

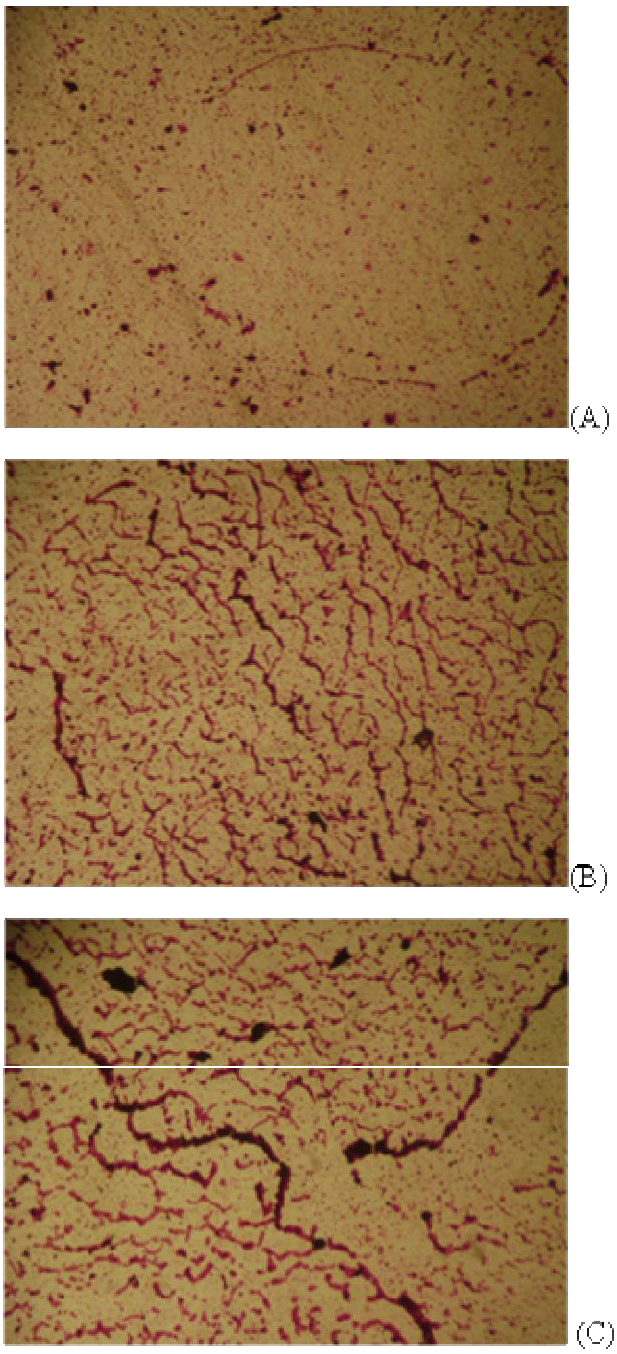

Figure 2. Microscopic analysis of a biofilm formation on intrauterine device by *Mycoplasma fermentans* at (A) 24 hours, (B) 48 h and (C) 72 h postinoculation using crystal violet staining (40X).

cell surface via the acyl moiety of this lipoprotein. The VsaA epitopes could have been released into the extracellular matrix by blebbing of the mycoplasma cell membrane and/or cell death, as have been extensively described for other prokaryotes (Simmons et al., 1996; Kuehn and Kesty, 2005) . The ability of *M. fermentans* to form a biofilm may be a virulence factor and biofilm formation may be an alternative mechanism to resist the host´s immune system of the same form with *Mycoplasma pulmonis* (Simmons and Dybvig, 2007).

Mycoplasma cells form aggregates and microcolonies suggesting that components of the biofilm, perhaps the towers, could form in the intrauterine devices. Recently, several distinct surface lipoproteins in *M. fermentans* were identified by using specific antibodies. Screening of nitrocellulose lifts of colonies with these antibodies revealed a system of high-frequency antigenic variation involving most of these lipoproteins (Theiss et al., 1993). The ability to differentially mask cell surface structure may contribute to the survival of *M. fermentans* in different environments, particularly those in which host recognition of specific regions of surface proteins could mediate damaging immune reactions (Theiss et al., 1996).

Table 1. Ability of *Mycoplasma fermentans* to form a biofilm on intrauterine devices.

	Quantitative crystal violet assay of biofilm formation	Planktonic cell counts (CFU/ml)		
		24 h	48 h	72 h
M. fermentans	+	9^5	7^5	4^5

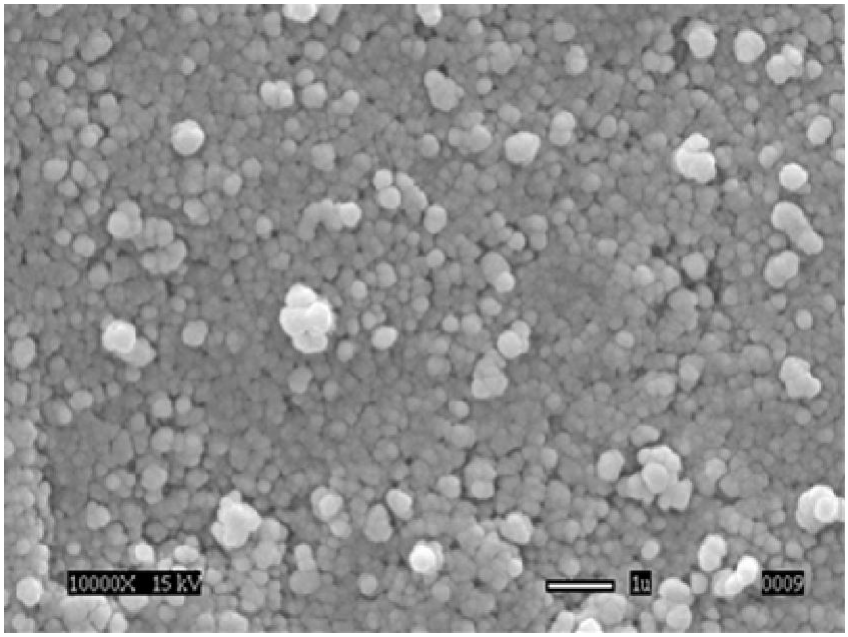

Figure 3. Scanning electronic microscopic analysis of biofilm formation by *Mycoplasma fermentans* on intrauterine devices at 72 hours postinoculation.

Mycoplasmas that produced a short form of the VsaA, VsaG, or VsaH protein grow adhered to inert surfaces and formed a biofilm. After 3 days of growth, as the biofilms became increasingly dense, the prominence of the cavities was reduced and the diameter of the towers increased (Simmons et al., 2007).

The Vaa antigen, which is an abundant surface lipoprotein adhesin that apparently mediates interactions of *Mycoplasma hominis* with its human host, is subjected to high-frequency phase variation in expression, which correlates precisely with the ability of *M. hominis* to adhere to cultures human cells. It was shown that an oscillating mutation involving a single nucleotide deletion or insertion in a short homopolymeric tract of adenine residues correlates with the Vaa expression state (Zhang and Wise, 1997). The poly (A) tract was localized near the 5´ end of the sequence encoding mature Vaa sequence, creating a translational frameshift UAG stop codon immediately downstream of the poly (A) tract. A similar translation control of variable surface lipoprotein was demonstrated for the human pathogen, *M. fermentans* (Razin et al., 1998).

Recent reviews suggest that the overall risk of pelvic inflammatory disease with modern intrauterine devices is lower than previously thought, at least in regions where medical advice is followed by the patients, and where there is a low prevalence of sexually transmitted infections. The risk of pelvic inflammatory disease may be higher, however, in places where screening for sexually transmitted infections is limited and where it is difficult to ensure aseptic conditions for insertion (Steen and Shapiro, 2004).

Biofilms of various medical devices have been studied extensively over the last 20 years, though much of the published research used very basic tools, such as viable culture techniques and scanning electron microscopy, to characterize the microbial diversity and visualize the biofilms. For certain devices, such as urinary catheters and contact lenses, research has also elucidated the susceptibility of biofilm formation. Development of a reproducible nonanimal model system for growing and evaluating intrauterine devices biofilms might allow a clearer understanding of the rate of biofilm formation and the importance of different materials, contaminating organisms, and treatments which could control the process (Donland and Costerton, 2002).

Conclusion

The biofilm mode of growth offers a selective advan-

tage in mycoplasmas, as it contributes to the persistence of many mycoplasmas species. As mycoplasmas possess only a small genome, it seems likely that biofilm growth is beneficial, if not essential, for many mycoplasmas, other wise it is likely that the genes necessary for this mode of growth would have been lost during degenerative evolution. It is important to remember that this study has examined biofilm formation under laboratory conditions. The adherence process consists of several consecutive stages: first the contact with some surface material like mucus or a host cell appendage using energy requiring processes, then an unspecific contact with the cellular surface and finally, a specific interaction of binding sites.

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