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In vitro interaction between human pathogenic viruses and *Candida albicans* biofilm

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Academic Year 2013-2014

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Department of Diagnostic and Clinical Medicine and of Public Health

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"It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change"

Charles Darwin

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Abstract

BACKGROUND: *Candida albicans* is known as a major cause of infections related to biofilms on medical devices such as catheters, artificial valves and prostheses. The clinical impact of such infections is very heavy: the microrganisms grown as biofilms become resistant to chemotherapy and the related infections are difficult to eradicate. Candida biofilm is a source of invasive candidemia with high mortality rates (30-50%). So far, only few studies investigated the interactions between human pathogenic viruses and biofilms, mainly focusing on water biofilms. To our knowledge, there are no studies on the interplay between biofilms in humans and viruses.

AIM: In this study, we evaluated: i) whether Herpes Simplex Virus type 1 (HSV-1) and Coxsackievirus type B5 (CVB5) can be encompassed in *Candida albicans* biofilm, retaining their infectivity, and then be released; ii) whether Candida biofilm has the ability to hold non adhering HSV1-infected cells; iii) whether different materials for catheters (silicone and PVC) have a different behaviour as far as virus interactions with biofilm; iv) the localization, inside the biofilm, of entrapped viruses and cells; v) antiviral drug sensitivity of virus infected cells inside the biofilm..

MATERIALS AND METHODS: *Candida albicans* biofilms were grown in tissue culture microplates and then exposed to cell free inocula of HSV-1 or CVB5 for 48h: after deep washing and energetic scapring of the wells to detach and rescue the biofilm, the viral load in this material was end-point titrated on VERO cells. In parallel, wells with a strain of non-biofilm producer *Candida albicans* (planktonic) and negative controls with only medium were processed at the same way. Moreover, Candida biofilms were exposed to non-adhering lymphoblastoid HSV1-infected cells and then, after washing and scraping, the number of living cells attached to the biofilms and virus titer were determined. In addition, Candida biofilm were also grown on silicone and PVC medical catheters as well on silicone disks: on these samples biofilm mass and metabolic activity were measured by

Crystal Violet staining and XTT assay and then the ability to encompass free HSV-1 particles and HSV1-infected cells was ascertained. Virus antigens and infected cells were localized inside the biofilm by immunofluorescence staining with anti HSV-1 monoclonal antibody and UVitex2. In biofilms with embedded HSV1-infected cells sensitivity to antivirals drug (acyclovir and foscarnet) was asceretained by adding scalar doses of the two drugs to HSV1-infected cells embedded or not in Candida biofilm, determining the Inhibiting Dose 5 (ID50).

RESULTS AND DISCUSSION: Free virus particles of HSV-1 and CoxB5 as well as HSV1-infected cells remained embedded in the biofilm, retaining their infectivity, with a significantly higher load than in the presence of planktonic Candida or in the negative controls. This phenomenon was observed regardless of the surface type (plastic microplate, silicon, PVC) used. Viral material was observed dispersed inside the biofilm. HSV-1 infected cells embedded in biofilm displayed a reduced sensitivity to antiherpetic drugs, with significantly higher ID50. These results provide the first evidence that infectious viruses, after being entrapped in Candida biofilms, can retain their infectivity and be released. Biofilm might therefore be a source also of viral infections displaying reduced sensitivity to antivirals so posing a health risk for patients with implanted medical devices.

Key words: Herpes simplex virus 1, Coxsackievirus, viruses, *Candida albicans,* biofilm, antivirals

Riassunto

STATO DELL'ARTE: *Candida albicans* è uno dei patogeni più coinvolti nelle infezioni associate ai biofilm che si formano su impianti medicali quali cateteri, valvole artificiali, protesi. L'impatto clinico di tali infezioni è molto pesante: i microrganismi cresciuti in forma di biofilm mostrano una diminuita sensibilità ai chemioterapici e le infezioni associate sono difficili da eradicare. Il biofilm di Candida diventa infatti una sorgente di candidemie invasive associate ad un'alta mortalità (30-40%). Finora pochi studi hanno indagato le interazioni tra virus patogeni umani e biofilm, focalizzandosi sui biofilm in ambiente acquatico. Non risulta invece indagata l'interrelazione tra biofilm nell'ospite umano e virus.

SCOPO. In questo studio abbiamo valutato: i) se l'Herpes Simplex Virus tipo 1 (HSV-1) e Coxsackievirus B5 (CVB5) possono essere trattenuti nel biofilm di *Candida albicans,* mantenendo la loro infettività, e poi rilasciati; ii) se il biofilm di Candida può intrappolare cellule non aderenti infettate da HSV-1; iii) se differenti materiali per cateteri (silicone e PVC) si comportano in maniera diversa per quel che concerne le interazioni tra virus e biofilm; iv) la localizzazione, all'interno del biofilm, dei virus e delle cellule inglobate; v) la sensibilità a farmaci antivirali di cellule infettate da virus e inglobate nel biofilm.

MATERIALI E METODI. I biofilm di *Candida albicans* venivano cresciuti in micropiastre da coltura cellulare e poi esposti a inoculi acellulari di HSV-1 e CVB5 per 48h: dopo un abbondante lavaggio e raschiamento dei pozzetti per staccare e recuperare il biofilm, la carica virale in questo materiale veniva titolato con il metodo della diluizione limite. In parallelo, venivano allestiti anche pozzetti con un ceppo di *Candida albicans* non produttore di biofilm (planctonica) e controlli negativi con solo mezo di coltura processati allo stesso modo. Inoltre, i biofilm di Candida sono stati esposti anche a cellule linfoblastoidi non aderenti infettate con HSV-1 e, dopo lavaggio e raschiamento, sono stati determinati il numero di cellule vitali attaccate al biofilm e il titolo virale. Successivamente a queste prove effettuate su plastica, il biofilm è stato fatto crescere su cateteri di silicone e di PVC

e anche su dischi di silicone: in questo caso sono stati determinati la massa e l'attività metabolica del biofilm mediante colorazione con Cristal Violetto e saggio XTT e l'abilità di inglobare particelle di HSV-1 e cellule infettate con HSV-1. Per la localizzazione degli antigeni virali edelle cellule infettate all'interno del biofilm è stata utilizzata una doppia colorazione in fluorescenza con un anticorpo monoclonale contro HSV-1 e UvitexB. E' stata valutata la sensibilità ad acyclovir e foscarnet in cellule infettate da HSV-1 inglobate o no nel biofilm aggiungendo dosi scalari dei due farmaci alle colture determinando la Dose Inibente 50 (DI50).

RISULTATI E DISCUSSIONE. Particelle virali di HSV-1 e di CVB5 e anche linfociti infettati con HSV-1vengono trattenuti nel biofilm, mantenendo la loro infettività, con titoli significativamente più elevati che in presenza di Candida planctonica o nei controlli negativi. Questo fenomeno si verifica indipendentemente dal tipo di superficie (plastica delle piastre da coltura, silicone, PCV). Le prove di sensibilità ai farmaci antiherpetici hanno evidenziato un aumento di molte volte della DI50 nelle cellule inglobate nel biofilm. Il materiale virale è stato osservato disperso irregolarmente all'interno del biofilm. Questi risultati mostrano per la prima volta che il biofilm di Candida può intrappolare virus e cellule infettate da virus che mantengono la loro infettività e vengono poi successivamente rilasciati. Questi riscontri suggeriscono l'ipotesi che il biofilm che cresce su impianti biomedicali possa costituire un ulteriore rischio anche di infezioni virali meno responsive ai farmaci antivirali nei pazienti con impianti biomedicali.

Parole chiave: Candida albicans, biofilm, virus, Herpes simplex virus 1, Coxsackievirus B5, farmaci antivirali

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List of original paper based on thesis

Mazaheri-Tehrani E, Sala Ariana, Orsi CF, Blasi E, Cermelli C.
Candida albicans biofilm can retain and release human pathogenic viruses *in vitro*. Virus Research 2014; 179: 153- 160

Conference proceedings

E. Mazaheri-Tehrani, A. Sala, C. F. Orsi, R. Neglia, E. Blasi, C. Cermelli.
In vitro interactions between viruses and Candida biofilm.
The Italian Society of Virology, Sep 17-19. 2012, Orvieto. Italy

Elham Mazaheri-Tehrani, Ariana Sala, Carlotta Francesca Orsi, Elisabetta Blasi, Claudio Cermelli.
Candida albicans biofilm can retain and release human Herpes virus type-1 *in vitro*. European conference on virology, Sep 11-14, Lyon, France
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Abbreviations

Confocal laser scanning microscopy	CLSM
Exopolymeric matrix	EPS
Transmission electron microscopy	TEM
Scanning electron microscopy	SEM
Quorum sensing	QS
Human T-cell leukemia virus type 1	HTLV-1
Herpes simplex virus type 1	HSV-1
Coxsakievirus Type B5	CVB5
Unique Long	UL
Unique Short	US
Terminal repeats long	TRL
Terminal repeats Short	TRS
Internal repeat long	IRL
Internal repeat short	IRS
Latency associated transcripts	LATs
Human enterovirus	HEV
Virus protein	VP
Swine vesicular disease virus	SVDV
Polyvinyl chloride	PVC
Extracellular matrix	ECM
Human T cell line	JJHAN
Yeast Peptone Dextrose	YPD
Minimal Essential Medium	MEM
Fetal calf serum	FCS
Phosphate buffer saline	PBS
Proteinase K	PK
Crystal Violate	CV

Optical densities	OD
Plaque forming unit	PFU
Tissue culture infective dose 50%	TCID50
Fluorescein Isothiocyanate	FITC
Monoclonal antibody	MAb
Indwelling medical devices	IMD

Chapter one
Introduction

1.1- Microbial biofilm

Over the past century, microorganisms have primarily been characterized as planktonic, freely suspended cells and described on the basis of their growth properties in nutritionally rich culture media. Rediscovery of a microbiological phenomenon first described by Antonie Van Leeuwenhoek, that microorganisms attach to and grow on exposed surfaces, led to studies which revealed that surface associated microorganisms (biofilms) exhibit a distinct phenotype with respect to gene transcription and growth rate. Biofilm formation involves specific mechanisms for initial attachment to a surface, development of a community structure and ecosystem, and detachment (1). Earlier studies by Heukelekian and Heller (2) demonstrated that bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach. Also, Zobell (3) found that the number of bacteria on surfaces was dramatically higher than in the surrounding medium (seawater). Moreover, Characklis (4) studied microbial slimes in industrial water systems and showed that these microorganisms were not only very tenacious but also highly resistant to disinfectants such as chlorine. Costerton *et al.* (5), based on observations of dental plaque and sessile communities in mountain streams, formulated a biofilm hypothesis that explained the mechanisms whereby microorganisms adhere to either biotic (living materials such as tissues and cells) and abiotic (nonliving materials) and the benefits derived from this ecologic niche. Later on, Carpentier and Cerf (6) simplified the concept of a biofilm as a community of microbes embedded in an organic polymer matrix, adhering to a surface. Furthermore, Elder *et al.* (7) and Mah and O'Toole (8) described a biofilm in more co-operative terms as a functional consortium of microorganisms that is difficult to eradicate by conventional antimicrobial therapy and can cause

indolent infections. It has become clear that biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents (Fig. 1.1).

Over the last two decades several definitions of a biofilm have been proposed by researchers. One which is widely quoted (9) states that a biofilm is “a microbial derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription”. However, there has been a paradigm shift as the link between sessile (surface attached and heterogeneous cells) and microbial pathogenesis and human infection is now widely accepted (10). Underlying this definition are the three basic components of a biofilm: microbes, matrix, and surface. If one of these essentials is removed from the environment, the biofilm will not form. Clearly, this is an over simplification of a fairly complex process that does not take into account the type of microorganisms, the composition of the surface, or the influences of environmental factors (11).

For long time, biofilms were considered literally as a side issue and they experienced little awareness, although they were a common sight all the time. Their relevance for environmental processes as well as in medicine and public hygiene has gained attention only in the past few decades. Since then, sophisticated methods have been introduced into biofilm research such as fluorescence microscopy and confocal laser scanning microscopy (CLSM), micro-electrodes, advanced chemical analysis, and, most powerful, molecular biology (12). All this has allowed investigating biofilm biology in much

reater detail and, thus, taking views of the life of microorganisms in the real world (13).

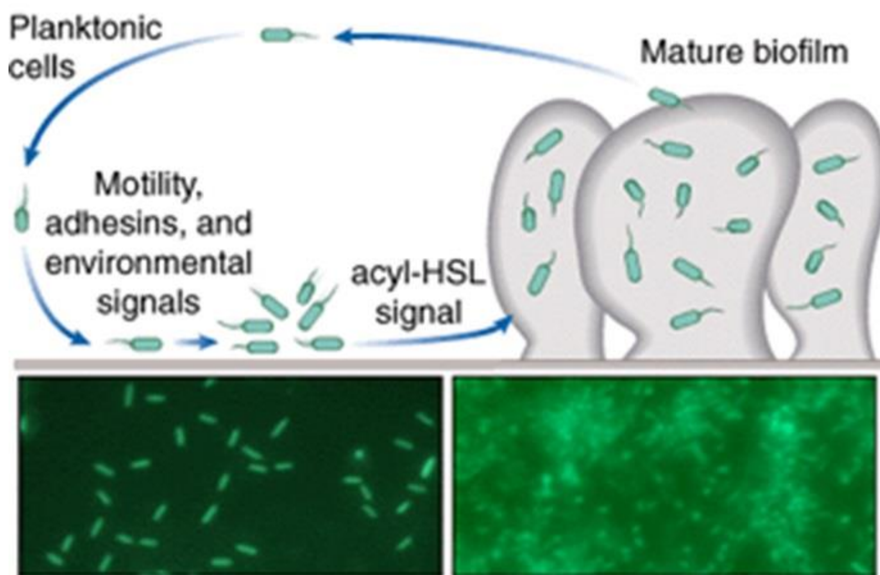


Fig. 1.1: Formation of microbial biofilm. Free (planktonic) bacteria assemble on a surface (**left**). Cell-to-cell communication then induces the formation of multicellular pillars and columns (**right**) (14)

Biofilm formation has brought huge amounts of problems to our everyday life (Fig.1.4) since they may form on a wide variety of surfaces, including living tissues such as tooth enamel, heart valves, or the lung, and middle ear, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems (15). Biofilms are problematic in water and sewage treatment facilities, causing metal corrosion, increased contamination of products, decreased quality of water, and reduced efficacy of heat exchangers (16-18). In many cases, biofilms irreversibly attach to processing equipment and can thrive in high flow systems (19). Moreover, biofilms

existing within these facilities serve as reservoirs for contamination on production lines, potentially creating a health hazard to the community (20, 21).

Microbial biofilms cost billions of dollars every year world-wide in equipment damage, product contamination, energy losses and medical infections. Conventional methods for eradicating bacteria such as antibiotics, and disinfection are often ineffective to biofilm populations due to their special physiology and physical matrix barrier (22). So novel strategies based on a better understanding of the biofilm formation mechanisms are urgently needed by many industries as well as hospitals.

It should not be overlooked that biofilms have very beneficial aspects. They are the carriers of the self-cleaning potential of soil, sediment and water by mineralizing organic matter. They are employed for biological purification of drinking water in biofilters (23), of biological waste water treatment and they are the drivers of biological waste disposal (24).

1.2- Biofilms in medical areas

Biofilms that form in the human body can have direct consequences (25). For example, acid-producing streptococci in dental plaque are responsible for cavity formation in teeth, and some plaque inhabitants are associated with periodontal disease (9, 26). Certain oral Streptococci can, if provided access to the bloodstream, colonize cardiac tissue or valves, causing potentially fatal endocarditis (27). Biofilms are especially problematic on in-dwelling medical devices such as urinary catheters, prosthetic heart valves, endotracheal tubes, surgical sutures, orthopedic devices, contact lenses, and dentures (9, 22, 28). Colonization of medical devices plays a key role in the problem of healthcare-associated infections (Table. 1.1). Cystic fibrosis

related infections, native valve endocarditis, otitis media, periodontitis, and chronic prostatitis all appear to be caused by biofilm-associated microorganisms. The exopolysaccharides (EPS) matrix shields most of the bacteria from effective opsonization and phagocytosis, making them very difficult to dislodge. Antibiotic treatment is frequently unsuccessful in resolving biofilm-based infections, so surgical removal of infected tissue or replacement of a colonized device may be necessary.

Table1.1: Microorganisms involved in producing biofilm on indwelling medical devices (9)

Organisms
Coagulase-negative staphylococci, *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans*
Viridans *Streptococcus,* coagulase-negative staphylococci, enterococci, *Staphylococcus aureus*
Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Proteus mirabilis
Coagulase-negative staphylococci, *β*-hemolytic streptococci, enterococci, *Proteus mirabilis, Bacterioides* species, *Staphylococcus aureus,* viridans *Streptococcus, Escherichia coli, Pseudomonas aeruginosa*
Candida albicans, Streptococcus mitis, Streptococcus salivarius, Rothia dentrocariosa, Candida tropicalis, Streptococcus sobrinus, Staphylococcus epidermidis, Stomatococcus mucilaginous
Staphylococcus epidermidis, Corynebacterium species, *Staphylococcus aureus, Micrococcus* species, *Lactobacillus plantarum,* group B streptococci, *Enterococcus* species, *Candida albicans*

1.3- Stages of biofilm development

Biofilm development begins when planktonic microrganisms- unattached individual cells — adhere to a surface. Adherence mechanisms vary depending on the microbe and surface. The steps for the early stages of biofilm formation were outlined by Palmer and White (29) that included cell-surface and cell-cell interactions, followed by the development of the mature

biofilm. Briefly, when microorganisms are transported to the surface by sedimentation, liquid flow, they first make weak and transient attachments. The solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms (Fig.1.2) (1). As adherent cells grow and divide, proximity to the surface induces physiological adaptations, including secretion of EPSs to create a protective matrix surrounding the cells (30). The next phase, irreversible attachment, depends on the properties of both biomaterial surface and the microbial cell surface. During irreversible attachment, a monolayer of single cells firmly adhered to a surface is produced. Next, microcolonies develop, finally forming the mature biofilm which is a surprisingly elaborate structure. Eventually, if the experimental conditions are no longer favorable, cells can detach from the biofilm and revert to a planktonic lifestyle (1, 31, 32).

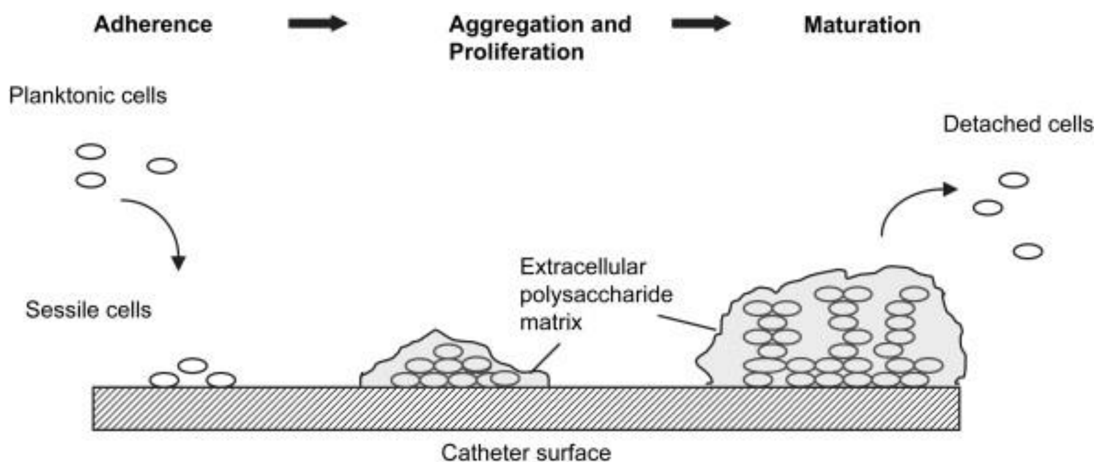


Fig. 1.2: Schematic picture of growing biofilm on a catheter surface

1.4- Biofilm structure and architecture

During the transition from initial attachment to formation of a mature biofilm, the microbial cells undergo significant changes. This process of

biofilm development results in the formation of a complex, three-dimensional architecture that usually includes mushroom-like structures, water channels, and pores (33, 34). Biofilm architecture can be visualized using a variety of microscopy methods such as transmission electron microscopy (TEM), fluorescence microscopy, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM) (9). It was generally assumed that the biofilm structures in mature biofilms are rigidly “cemented” to their place on a surface until they detach. (34). For instance, Tolker-Nielsen *et al*. (35) showed that cells of *Pseudomonas* sp. move actively by their flagella inside and between the microcolonies of a developing biofilm. In particular, CLSM which allows the visualization of fully hydrated samples, has revealed the complex three dimensional structure of biofilms (10). Moreover, Lawrence *et aI.* (36) used this method to study biofilms formed by *P. aeruginosa, P.jluorescens,* and *Vibrio parahaemolyticus* in flow chambers.

They found that while each biofilm varied in depth, structure, and ratio of cellular to noncellular material, all of the biofilms developed water channels (36). Many conditions such as surface and interface properties, the composition of the microbial community, and hydrodynamics, can affect biofilm structure and architecture, especially nutrient availability in the environment (37, 38).

1.5- Biofilm matrix and its composition

A characteristic feature of biofilms is the production of an extracellular matrix that envelops the attached cells. This is generally composed of water and microbial macromolecules and provides a complex group of micro environments surrounding the microorganisms (39). The extracellular matrix is arguably the most critical component of biofilms as it constitutes the

framework that holds the component cells together (40). In addition, the matrix contains a range of enzymic and regulatory activities. The matrix architecture is based upon a combination of intrinsic factors such as the genotype of the attached cells and a number of extrinsic factors, including fluctuations in nutrient and gaseous levels and fluid shear. Together, these intrinsic and extrinsic factors combine to produce a dynamic, heterogeneous microenvironment for the attached cells (41).

Much research during last two decades has focused on characterizing the matrix material of both Gram-negative and Gram-positive bacterial biofilms, such as *P. aeruginosa* biofilms (42) and S. *epidermidis* biofilms (43). Matrix polymers of bacterial biofilms are primarily exopolysaccharides and many of them are negatively charged. Smaller amounts of proteins, nucleic acids and various other components may also be present. However, much of the biofilm matrix - up to 97% - is water (39). The water may be bound within the capsules of the bacterial cells or can exist as a solvent whose physical properties are determined by the solutes dissolved in it (39). In Gram-negative bacteria, some exopolysaccharides are neutral, whereas others are polyanionic because of the presence of uronic acids such as D-glucuronic, D-galacturonic, and D-mannuronic acids, or phosphate or sulphate residues (44). In contrast to Gramnegative bacteria, Gram-positive bacteria often produce polycationic extracellular polymeric substances (45, 46). However, some polysaccharides carry hydrophobic groups which may be involved in hydrophobic interactions when adhering to hydrophobic interfaces (47).Additionally, many matrix materials possess backbone structures that contain sequences of 1,3- or l,4-p-linked hexose residues that are likely to be more rigid in structure, and in some cases less soluble or even insoluble (44). Moreover, in the presence of ions, the extracellular polymeric material shows

increased viscosity or gelation (48). EPS contain apolar regions, groups with hydrogen-bonding potential, anionic groups (in uronic acids and proteins) and cationic groups (for example, in amino sugars) (49). Owing to this stickiness of the matrix, particles and nanoparticles can be trapped and accumulated. Interestingly, heavy metals such as Zn2+, Cd2+, and Ni2+ bind to cell walls of bacteria in activated sludge, whereas hydrophobic compounds such as benzene, toluene and xylene are present in the matrix (50). Biofilms may be very dynamic and contain migrating bacterial subpopulations, and it appears that the biofilm matrix may serve as a framework that the bacteria can migrate on. Evidence is emerging that exopolymer material is necessary for coordinated bacterial migration during structural development in biofilms. (51).

1.6- *Candida albicans* infections

Candida species are all opportunistic pathogens capable of inflicting disease only when most defenses are in some way impaired, locally or systemically. The incidence of invasive Candida infections has risen along with the increased use of intravenous catheters, broad-spectrum antibiotics, parenteral nutrition, immunosuppressive therapy, and invasive procedures, all of which are important relevant risk factors (52-54). In the USA, candidaemia is the third and fourth most common nosocomial bloodstream infection in ICUs and non-ICU wards, respectively behind coagulase negative staphylococci, *S. aureus* and enterococci (55). In Europe, the rate of candidaemia ranges from 0.17 to 20 per 1000 hospital admissions, depending on country and patient population (8, 5, 6 56-58).

The principal pathogen of the genus, *Candida albicans,* is a commensal fungus that is frequently a benign member of the skin and mucosal flora.

Candida albicans, which can grow either as oval budding yeasts, as continuous septate hyphae or as pseudohyphae; all of these morphological forms are usually seen in infected tissue (Fig. 1.3) (7, 59). Vulvovaginal candidiasis is commonplace and may affect up to 75% of women at least once in their lifetime (8, 60). A small subset of women (5–10%) experience chronic recurrent episodes that substantially affect their quality of life. Patients with AIDS are prone to oral and oesophageal candidiasis and such infections are also commonly associated with oral cancers, the use of dentures and terminally ill patients who fail to produce sufficient saliva (7, 59). Patients suffering from burns and newborn (especially premature) babies are also subject to C. albicans skin infections. In vulnerable groups of patients and frail patients in intensive care units, *C. albicans* can cause a bloodstream infection known as candidaemia, which can develop into disseminated candidiasis when the infection spreads to internal organs (9, 61). Candidaemia and disseminated candidiasis are extremely serious medical conditions with mortality rates documented in different surveys of between 30–50%; some surveys have found them to be the second most common cause of death from nosocomial infections (10, 11, 62, 63).

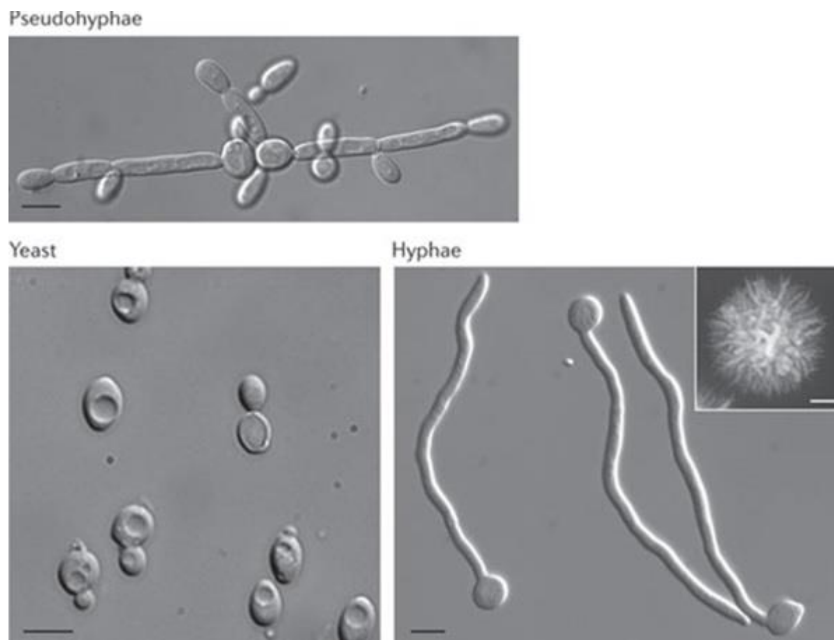


Fig. 1.3: Morphology of yeast, hyphae and pseudo-hyphal forms (63)

1.6.1-Candida albicans biofilm

Candida species can form biofilms on almost any medical device and host surfaces. Devices such as stents, shunts, prostheses, implants, endotracheal tubes, pacemakers, and various types of catheters, to name a few, have all been shown to support colonization and biofilm formation by Candida. Formation and structure of Candida biofilms is influenced by the nature of the contact surface, environmental factors, Candida morphogenesis, and the Candida species involved. (Fig1.4). (64-66).

C. albicans biofilms represent an increasing problem in the clinical setting where they form on natural surfaces, such as teeth and indwelling medical devices, such as urinary catheters, endothelial tubes, prosthetic heart valves and cardiac pacemakers (15, 64, 66). Implant-related infections in particular are a significant problem in this context. *C. albicans* cells may reach an indwelling device through occasional breach of the gastrointestinal tract barrier (67) or, perhaps, through external contamination (68).

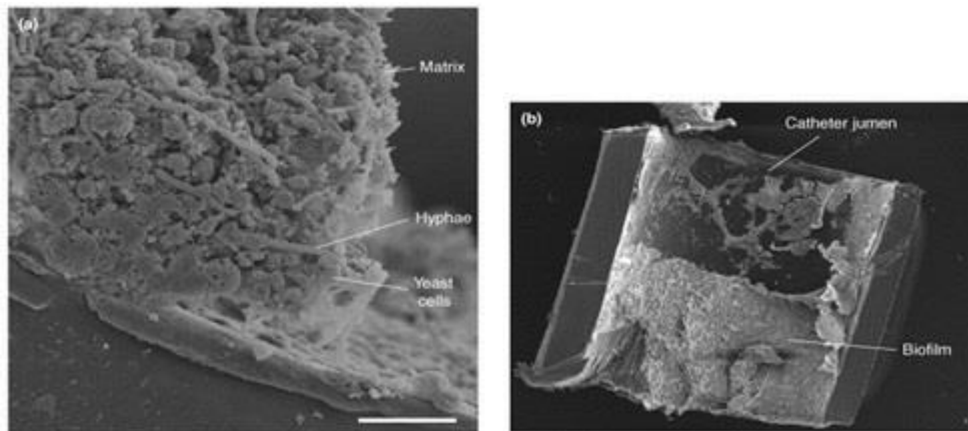


Fig. 1.4: Scanning electron micrographs of a *C. albicans* biofilm on the inside lumen of a vascular catheter from a rat central venous catheter model. (a) Cross section of a biofilm. Yeast and filamentous cells are seen encased in matrix material (b) Image of section of venous catheter (68).

1.6.2-Ultrastructure of Candida albicans biofilm

Our understanding of *C. albicans* biofilm ultrastructure began with scanning electron microscopic visualization of yeast cells, pseudohyphae and hyphae in *C. albicans* biofilms (69). Initial attachment of yeast cells to a catheter disc was followed, after 3 to 6 h, by germ tube formation. Fully mature biofilms, produced after incubation for 24 to 48 h, consisted of a dense network of yeasts, hyphae and pseudohyphae (69).

C. albicans biofilm development has been characterized in various model systems both *in vitro* and *in vivo* (70, 71). Several studies (69, 72-75) show that biofilm formation *in vitro* can be broken down into three basic stages: (i) attachment and colonization of yeast cells to a surface, (ii) growth and proliferation of yeast cells to allow formation of a basal layer of anchoring cells
and (iii) growth of pseudohyphae and extensive hyphae concomitant with the production of extracellular matrix material (Fig 1.5).

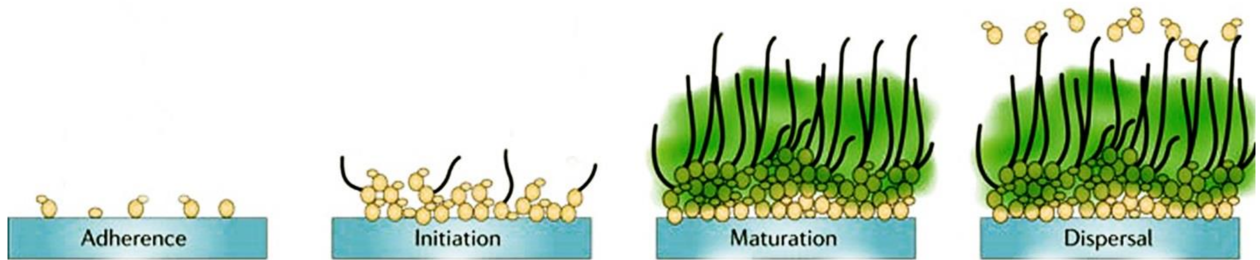


Fig. 1.5: Stages in the formation of a *Candida albicans* biofilm. Adherence and initial yeast (yellow) adhesion to the surface plus formation of the basal layers of yeast microcolonies. These anchor each microcolony to the surface. Completion of microcolony formation by addition of the upper, mainly hyphae layer and matrix material (green) that surrounds both yeasts (yellow) and hyphae (black) (Maturation). Final step is Dispersal which hyphae growth is elevated, and extracellular matrix envelops the biofilm

One study of *C. albicans* biofilms from denture stomatitis patients confirm the presence of yeast, hyphae and extracellular matrix *in vivo* (75) plus, biofilm architecture in two animal catheter models appears to include numerous yeast cells in the basal region, as well as extracellular matrix (70). Hyphae are also present in both *in vivo* biofilm models (70).

1.6.3-The role of Extracellular Matrix (ECM) in *Candida albicans* biofilm

The matrix, also known as the exopolymeric substance (EPS), is perhaps the most mysterious aspect of the biofilm state. It is made by the cells within the biofilm, and consists carbohydrate (41%), protein (5%), phosphorus and hexosamine but the matrix contained significantly less carbohydrate and protein. It also has a higher proportion of glucose (16%) than mannose, and contains galactose, suggesting that it might possess components unique to biofilms (64, 65, 73, 76). One main extracellular carbohydrate constituent is β-1,3 glucan, increased production of which is associated with biofilm cells rather than planktonic cells. (77). In *C. albicans*, more EPS is produced in biofilms grown under high-flow conditions than under low-flow or static conditions (76); thus EPS may strengthen biofilm structure under harsher

environmental conditions. The inherent drug resistance of *C. albicans* biofilms, however, does not seem to vary with EPS content (76). Therefore, the *C. albicans* matrix may not be a major determinant of biofilm drug resistance *in vitro*. Clearly, the elucidation of *C. albicans* EPS structure, function and regulation remains a major challenge.

1.6.4-Quorum sensing and Candida albicans biofilms

Quorum sensing (QS), is the “language” of molecular signaling in biofilms and was first discovered in two marine bacteria, *Vibrio fischeri* and *Vibrio harveyi* (78). It is a mechanism by which bacteria regulate specific genes as an answer to a certain concentration of a signaling molecule produced by microrganisms. Microbial behaviors or responses are governed by cell density (79). Such community behaviors are usually determined by secreted signalling molecules, the accumulation of which is a measure of cell density (80). Quorum sensing is involved in the regulation of several traits such as virulence, sporulation, mating, bioluminescence, and biofilm formation of all kinds (78, 81, 82). Several different types of QS molecules have been described for a wide variety of microbial species.

The best studied quorum-sensing molecule in *Candida albicans* is E,E-farnesol, an inhibitor of hyphal formation. Exogenous farnesol inhibits biofilm formation if provided early during adherence (83, 84). The limited biofilms that form in the presence of farnesol comprise mainly yeast and pseudohyphal cells, rather than hyphae. Farnesol also accumulates in supernatants of mature biofilms (85), where stimulation of yeast cell production might promote biofilm dispersal. Tyrosol, an alcohol derived from tyrosine, has the opposite activity to farnesol: it stimulates hyphal formation. The addition of exogenous tyrosol does not have a measurable effect on overall biofilm development but can partially overcome the

inhibition of biofilm formation by exogenous farnesol. Tyrosol also accumulates in mature biofilm supernatants (84,85), and the overall inhibition of hyphal formation by such supernatants (83-85) seems to reflect the dominant activity of farnesol. Several other small molecules are detectable in biofilm supernatants, including phenyl ethyl alcohol, dodecanol and nerolidol (85). Each of these compounds can inhibit hyphal formation, and thus all might aid in biofilm dispersal by promoting yeast cell formation.

1.6.5-*Candida albicans* biofilm and interaction with other microorganisms

Poly-microbial biofilm communities may be defined as a varied collection of organisms (fungi, bacteria, and viruses) that exist at a phase or density interface and are coated in a self- and/or host-derived hydrated matrix, often consisting of polysaccharide (86). Figure 1.6 shows the sites and types of poly-microbial diseases commonly located throughout the human body. The composition of early colonizers determines which microbes colonize at later time points. This process of sequential attachment is commonly referred to as co-aggregation (87).

Co-aggregation is believed to be mediated in two distinct ways (fig.1.7): either a secondary colonizer in suspension binds to specific molecules on the surface of a biofilm and begins the co-aggregation cascade, or several bacteria form an aggregate that results in phenotypic changes promoting further co-aggregation on the biofilm exterior.

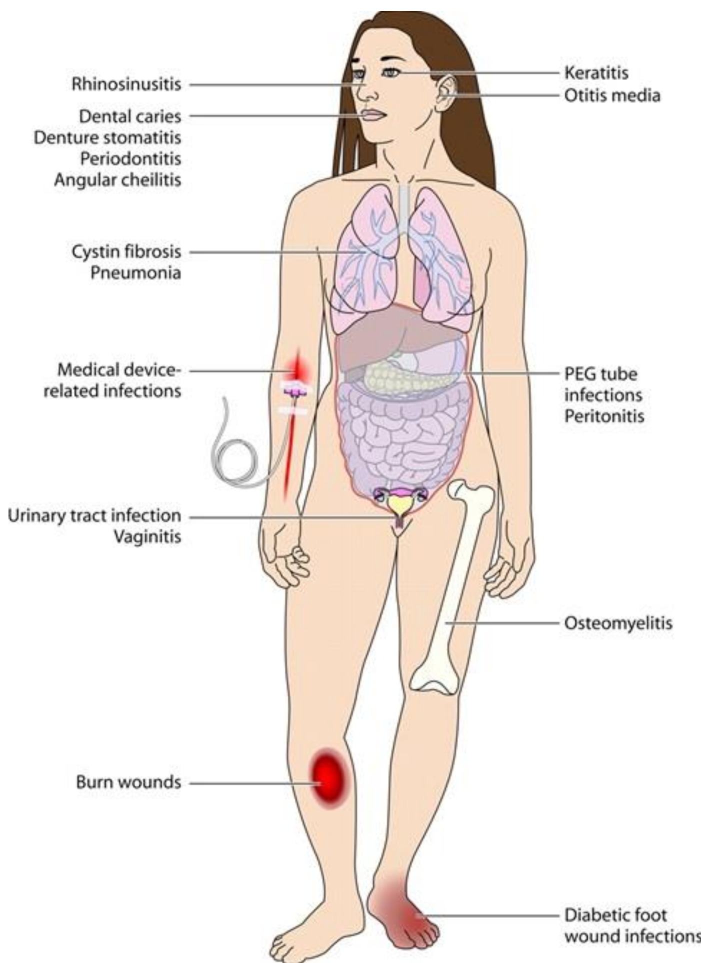


Fig.1.6: Schematic highlighting the sites and types of poly-microbial diseases commonly located throughout the human body.

After the transition from the planktonic (or free-floating) state to the sessile state, as mentioned before, attached microorganisms begin to radically change their gene and protein expressions toward biofilm formation (25). In an environment that is highly competitive for space and nutrients, biofilm formation and co-aggregative effects allow competing microbes to maximize the colonization surface area and intimately position themselves near potential sources of nutrition (88, 89). An example of the complexity of co-aggregation may be the range of inter-generic co-aggregations occurring between the oral fungal pathogen *Candida albicans* and other oral species that may play an important role in the colonization of the oral cavity by *C. albicans* (90).

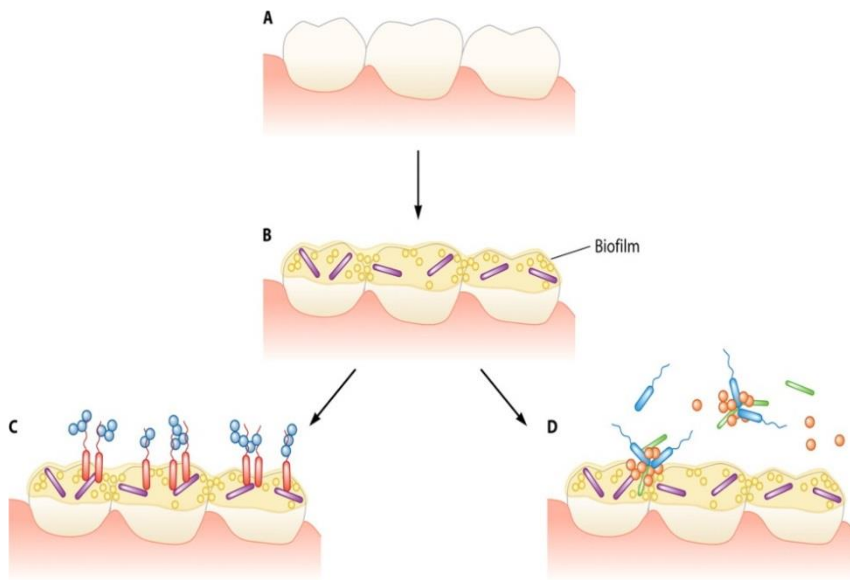


Fig. 1.7: Poly-microbial biofilm formation is thought to proceed in several distinct phases. (A) Uncolonized biotic surface (e.g., teeth) lacking any biofilm formation. (B) Deposition of a conditioning layer promotes the adherence of early colonizers that begin the co-aggregation cascade. The co-aggregative development of the poly-microbial biofilm can occur via two possible methods.(C) Early colonizing bacteria may directly support the binding of late colonizers that then facilitate the attachment of several other microbial species. (D) Specific planktonic inter-microbial interactions can lead to phenotypic changes that support the attachment of pre-aggregated clusters of cells; however, non-aggregated cells remain unable to attach (89).

Indeed, diversity of ligand-receptor interactions govern co-aggregation on both bacterial and fungal surfaces (90). So, many common infectious diseases can be initiated by a single pathogen or virulence factor but likely a complex milieu of microorganisms maybe involved. These consortia of microbes typically coexist as combinations of highly structured communities of bacteria, viruses, protozoans, and fungi attached to biotic and abiotic surfaces, with their architectures facilitated by specific inter-microbial and host interactions (90, 91).

1.7-Interaction between biofilm and viruses

1.7.1-Interaction between water biofilms and viruses

So far, most of the studies related to biofilms and viruses have been undertaken for viruses in drinking-water biofilms. These studies mostly have focused on involving virus detection in and virus elution from drinking water biofilm (93-95).

According to water biofilm, it is supposed that viruses can pass through the porous matrix of a biofilm (96-97). The structure of a biofilm can vary based upon physical parameters, source water constituents, flow conditions, surface properties, and component microorganisms (98). If a virus comes into contact with a biofilm, a number of interactions can occur, depending on the virus and constituents of the biofilm. If the virion is a bacteriophage and has a polysaccharide-degrading enzyme, or causes cell lysis, the biofilm may be destroyed (98). However, co-existence of the virion and bacteria may also occur within the biofilm.

Bacteriophages may have a role in controlling biofilm (99). In fact, they may produce polysaccharide depolymerases that can hydrolyze polymeric substances in the biofilm matrix. The effect of bacteriophages on biofilms has been studied in vitro (100,101). A bacteriophage was engineered to express a biofilm-degrading enzyme and simultaneously attack bacterial cells in the biofilm and the biofilm matrix (102). The engineered enzymatic bacteriophage demonstrated greater *in vitro* efficacy than the non-enzymatic bacteriophage against biofilms. Use of bacteriophages to prevent colonization and subsequent infection may be applicable to urinary or intravascular catheters. In an *in vitro* model, pretreatment of hydrogel-coated silicone catheters with a single *Staphylococcus epidermidis* bacteriophage (103) or a

cocktail of five *Pseudomonas aeruginosa* bacteriophages (104) reduced biofilm formation.

Many biofilms have an open-channel type structure, which may allow the virus access to the interior of the biofilm (98). If the phage is unable to infect the host cell, the cell may consume the virus as a nutrient source. Co-existence of the phage and host bacteria may also occur within the matrix of biofilms (98). Biofilms have been evidenced to entrap and concentrate viral-sized particulates up to 100-fold over concentrations in surrounding solution. The fate of captured infectious viruses will depend on the time of their immobilization as well as on their release as single or biofilm-associated particles (105).

1.7.2-Possible attachment and detachment of viruses to/from biofilm

Biofilms display large and variable surfaces (106, 107), including sorption sites that facilitate the binding and subsequent accumulation of colloidal matter (108). Therefore enteric viruses, which are considered to be small bio-colloids with particle sizes ranging from 20 to 100nm (109), can potentially interact with natural biofilms. Moreover, Vanden Bossche confirmed the presence of infectious enteroviruses within the water biofilm for the first time (110).

It has been hypothesized that biofilms may play a role in the accumulation, protection, and dissemination of pathogens through drinking water distribution networks (111). It has been shown that viruses can attach to biofilms (112, 113), and detachment of pieces of biofilm by shearing forces of the moving water has been observed (112,114).

Virological typing and quantification showed Echovirus and Coxsackie B virus counts of 5.4 log10 infectious doses per milliliter of eluted 10 g pooled scrapings of internal organic coating from several tubes used for

conveying finished water into the distribution system. This finding indicates that these viruses may be able to accumulate in biofilms (110).

1.7.3-Can viruses form a biofilm?

It is commonly accepted that the spread of most viruses occurs via the diffusion of 'cell-free' viral particles. This mode of viral dissemination requires high numbers of stable viral particles released by the infected cell into the extracellular environment, such as the host bodily fluids. Free viral particles were associated with a variety of components, such as lipids or proteins, which might reinforce virus envelop integrity and prevent envelope glycoprotein shedding (115,116). However, for other viruses, few viral particles are released, or they are poorly infectious when separated from infected cells. In such cases, virus propagation largely requires the presence of infected cells, implying that cell contacts mediate viral spread (117). This type of dissemination is mainly reported for enveloped viruses, such as some herpes viruses and some retroviruses, particularly delta retroviruses such as the human T-cell leukemia virus type 1 (HTLV-1). Many aspects of this mode of virus dissemination are largely unknown, such as the mechanism for forming cellular junctions that mediate cell–cell virus spread and the nature of the infectious material transferred. Both these factors depend on the virus and the type of infected cells (118).

The recent finding that the human T-cell leukemia virus type 1 (HTLV-1) encases itself in a sort of carbohydrate-rich adhesive extracellular cocoon, which enables its efficient and protected transfer between cells, unveiled a new infectious entity and a novel mechanism of viral transmission. These HTLV-1 structures are observed at the surface of T cells from HTLV-1-infected patients and are similar to bacterial biofilms (Fig. 1.8). The virus controls the synthesis of the matrix, which surrounds the virions and attaches

them to the T cell surface. It is proposed that, reminiscent to bacterial biofilms, '**viral biofilms**' could represent **'viral communities'** with enhanced infectious capacity and improved spread compared with 'free' viral particles, and might constitute a key reservoir for chronic infections (Fig.1.9) (119).

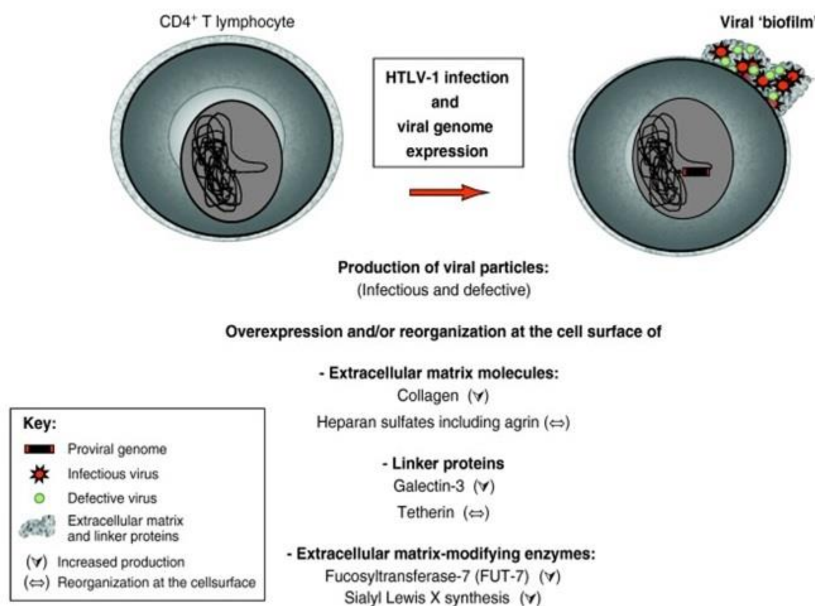


Fig. 1.8: Model of **'viral biofilm'** development on HTLV-1 infected lymphocytes. Viral-genome expression drives both the production of viral particles and of a matrix enriched in certain carbohydrate components (e.g. agrin and collagen), and some linker proteins (e.g. galectin-3 and tetherin). Together, infectious and defective viral particles embedded in the carbohydrate-rich matrix form the infectious structure we name a 'virus biofilm (118)

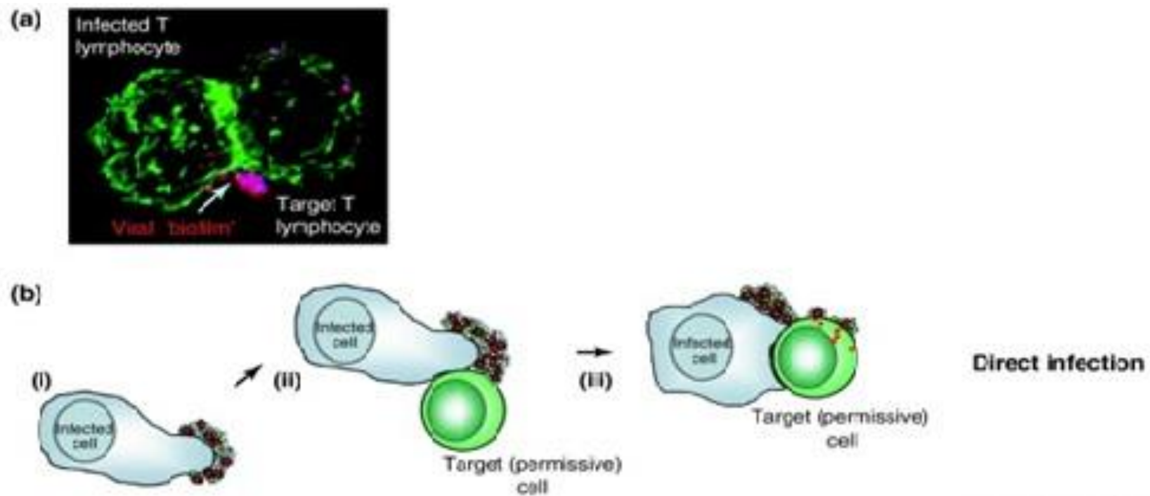


Fig. 1.9: **Viral biofilms**, a new mode of virus dissemination. (a) Transfer of viral 'biofilms' at the contact site between two primary CD4+ T cells (b) Model of viral transmission through viral 'biofilms', including (i) viral biofilm formation at the surface of infected cells, (ii) transfer of these infectious adhesive structures through dynamic interactions between infected and uninfected cells (permissive cells). The rupture and adhesion of biofilm fragments to the surface of these cells might lead to direct infection of permissive target cells (iii) (119)

1.8-Rationale for the present study

In present PhD study to investigate in vitro interaction between *Candida albicans* biofilm and pathogenic viruses, we used two viruses, Herpes simplex virus type 1(HSV-1) and Coxsakievirus Type B5 (CVB5), as two models of enveloped and non-enveloped viruses.

Herpes virus-infected cells can reduce the host defense to invade the host more efficiently giving rise to overgrowth of pathogenic bacteria and fungi (120). Furthermore, recent studies have quantified the herpes viruses in periodontal pockets to show their positive association with severity of periodontal disease. The prevalence and number of herpes viruses in periodontal pockets may vary according to type of periodontal disease (121). Biofilm formation is very frequent in dental and laryngeal prostheses and in other biomedical devices that are used in contact with skin or mucosal surfaces (66). Despite the strong association of the "Red Complex" perio-

pathogens with periodontitis, especially in the severe form of periodontitis pockets, it can harbor a great variety of microorganisms, including yeasts (122). The presence of enteric *Staphylococcus aureus* and *Candida sp*. should be expected, especially in patients with systemic disorders, i.e. diabetes mellitus (123), neutropenia, agranulocytosis and AIDS (124).

On the other hand, reprocessing and reuse of cardiac electrophysiology catheters has been a subject of considerable interest among the general public, healthcare professionals, and regulatory authorities. This non-lumen, multi electrode catheters, which are passed from a peripheral blood vessel into the heart during investigation of patients with cardiac rhythm disturbances, are broadly categorized into diagnostic catheters. Electrophysiology catheters have no deleterious effects on patient outcome, which is an important issue associated with the reprocessing of any invasive medical device is the potential for subsequent transmission of infectious viruses (125). Of particular interest is Enteroviruses such as those of the Coxsackie genus, which may infect the myocardium (125).

1.9-Herpes simplex virus- type 1

1.9.1-Taxonomy

Herpes simplex viruses (HSV) are important human pathogens belonging to the *Herpesviridae* family and causing diseases in a variety of different tissues and animal species (126). There are two antigenic types, HSV-1 and HSV-2, with HSV-1 being most often transmitted non-sexually and HSV-2 most usually sexually transmitted (126,127).

1.9.2-HSV-1 Structure

Studies have shown that all Herpes virions consist of four elements: DNA core, capsid, tegument and glycoprotein-containing envelope (129). A schematic presentation is given in Figure.1.10. The HSV genome contains

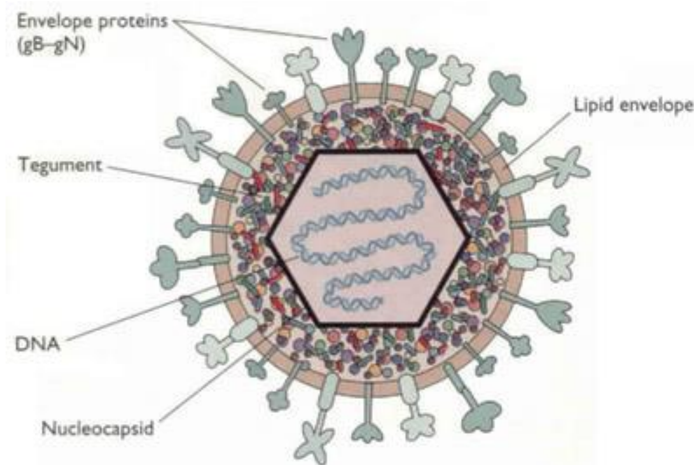


Fig. 1.10: Schematic picture of HSV-1

Approximately 152-kbp (130). The DNA of HSV-1 and HSV-2 consist of two covalently linked segments called the L (long) and S (short), with unique sequences-UL (unique long) and US (unique short), flanked by large inverted repeat sequences which are designated terminal and internal repeats of the long (TRL and IRL) and short (TRS and IRS) unique sequences, respectively (131). Additionally, the unique L and S components can invert relative to one another, yielding four linear isomers, and each of the four is equally virulent (functionally equivalent) in the host cell (31,132). The genes of the long and short unique sequences are designated UL1 to UL56 and US1 to US12, respectively (133). The capsid is a structurally well-defined icosahedron. It is 125 nm in diameter and approximately 15 nm thick, and it is organized into 162 capsomers, of which 150 are hexavelent capsomers (hexons), and 12 pentavalent capsomers (pentons) (134, 135). All capsomers

are connected in groups of three by trigonal nodules or triplexes on the capsid surfaces (136, 137). The tegument is the least understood component of the virion in relation to its structure and function, its role in virus entry, and mechanisms of its assembly and incorporation into virions. However, cryoelectron micoroscope analyses showed that at least the innermost part of the tegument that is located adjacent to the capsid may also exhibit icosahedral symmetry (138).

On the outer side of the tegument is the envelope, which is a lipid bilayer derived from the host cell. HSV specifies at least 12 glycoproteins designated gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gJ and gN (131,139). These glycoproteins function in several important roles, including pH-independent virus entry via fusion of the virion envelope with cellular membranes (140).

1.9.3-Entry of HSV into the host cell

The entry of HSV requires binding of virus to receptors on the cell surface and fusion of the virion envelope with the cell plasma membrane (130,131). The initial attachment is mediated through viral glycoprotein C (gC) and/or gB to cell surface heparan sulfate proteoglycans. The fusion of the viral envelope with the plasma membrane requires gB, gD, gH, and gL (131).

1.9.4-Herpes infections and human diseases

HSV is natural pathogens for humans, with particular affinity for the nervous tissue. The virus spreads from person to person by infected secretions, classically oral secretions for HSV-1 and genital secretions for HSV-2. There are three types of herpetic infections: lytic infection, latent infection and transforming infection. In a lytic infection, virus multiplies inside the nucleus of the infected cell. This is followed by production of

infectious virions before lysis of infected cells, partly due to suppression of host protein synthesis by a structural protein named virus host shutoff (vhs) protein, encoded by the UL41 gene (131, 135). In latent infection, viral DNA is maintained in a non- replicative state and persists in the nucleus as an episome for the entire life of the individual (Whitley and Roizman, 2001). Virus may reactivate following a variety of local or systemic stimuli to cause recurrent disease (141). During latency, the viral lytic genes are extremely repressed and only a single transcription unit encoding the latency-associated transcripts (LATs). The possibility that herpes simplex virus has transforming potential has been a focus of interest. Numerous studies have shown that, both HSV-1 and HSV-2 are able to transform the morphological phenotype of rodent cells. Transformation by HSV-1 does not require the entire viral genome, but is attributed to a region located between map units 0.31 and 0.42 designated as morphological transforming region of HSV-1 (mtr-I) (142). Failure to detect viral DNA in transformed cells led to the hit-and-run hypothesis of HSV-1 transformation (143). HSV-1 and HSV-2 are common human pathogens that can cause primary and recurrent infections of mucous membranes. Primary HSV infections are usually symptomatic but may be sub clinical. Recurrent infections are generally less severe than the primary infection. The most commonly seen clinical manifestations include oro-facial and genital lesions. Ocular infections may include any part of the eye including the retina, conjuctiva, cornea and eyelids
(144,145). Meningitis is usually benign, but the HSV encephalitis has been associated with high mortality (146). In addition, immunocompromised patients are at risk of developing more severe disseminated HSV infections through blood stream.

1.10-Coxsackievirus type B5

1.10.1-Taxonomy

Picornaviruses are a group of small, non-enveloped animal viruses with single-stranded RNA genome of positive polarity ('pico' Greek: very small – RNA viruses) (147). Nowadays they comprise the most common infections of humans in the developed world (148). Picornaviruses were previously classified according to their physicochemical properties (particle density, pH-sensitivity) and serological relatedness. More recently, the classification has been based on nucleotide sequence comparisons. The Enterovirus genus is further divided into five species, Poliovirus and Human enterovirus (HEV) A-D together with the eight subgroups (based on pathogenesis in experimental animals) and serotypes (149,150)

Coxsackieviruses belong to the genus of Enteroviruses and share many characteristics with poliovirus. With control of poliovirus infections in much of the world, more attention has been focused on understanding the non-polio Enteroviruses such as Coxsackievirus (149, 151). They are divided into group A and group B viruses based on early observations of their pathogenicity in mice (149).

1.10.2-Coxsackievirus structure

The CVB genome is 7,400 nucleotides in length. The single open reading frame encodes 11 proteins and is flanked by non-translated regions. The 5'-terminal nucleotide is linked to a virus-encoded protein, and the 3' terminus is completed with a polyadenosine tail (152). The structure of the B3 virus determined to 3.5 A resolution, (153) shows each of the 12 pentamers on the icosahedron surrounded by a canyon which is the binding site for specific cellular receptor molecules (154). The capsid is made up of 60 identical building units, each containing one copy of the four structural

proteins VP1-VP4. The folding pattern of polypeptides VP1-VP3 is similar, resulting in an eight- stranded antiparallel b-barrel structure (Fig. 1.11).

Once translation begins, host-cell protein synthesis is rapidly shut off, and the viral genome is translated into a large polyprotein. These include the proteins destined for incorporation into new virus capsids, as well as the RNA -dependent RNA polymerase, other enzymes involved in genome replication and a specific protease for polyprotein cleavage (155).

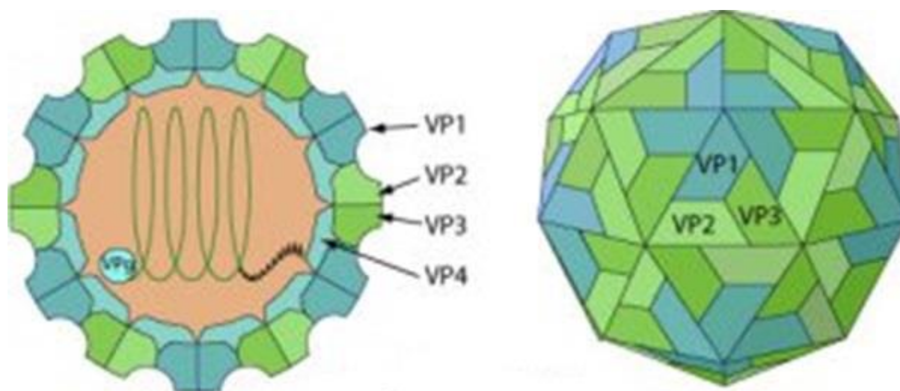


Fig. 1.11: Schematic picture of Coxsackievirus B5

1.10.3-Coxsacievirus B infections and human diseases

Division in group A and B viruses was based on the different disease caused in mice. Group A Coxsackieviruses cause a flaccid paralysis following generalized myositis, while group B Coxsackieviruses cause a spastic paralysis due to focal muscle injury and degeneration of neuronal tissue. At least 23 serotypes (1-22, 24) of group A and 6 serotypes (1-6) of group B are recognized (155,156).

Infections caused by CVBs are often asymptomatic but may occasionally result in severe diseases of the heart, pancreas, and central nervous system (157) and less severe pathologies of mucosal and skin

surfaces and respiratory tract. One of these serotypes, CVB5, is now believed to be virtually identical to swine vesicular disease virus (SVDV) which is now classified as a subspecies of CVB5, based on genetic relationships between the virus strains (158).

There is also good evidence that CVB5 plays a putative role in the development of chronic diseases, such as cardiomyopathy, insulin-dependent diabetes, and metabolic myopathy (155).

1.11-The present study focused on the following four points:

1.11.1-Evaluating whether *Candida albicans* biofilm can be a reservoir for HSV-1 and/or CVB5

We exposed *C. albicans* biofilm to HSV-1 or to CVB5 to assess whether these viruses can be encompassed in biofilms retaining their infectivity and then can be released into culture medium. Biofilms were grown in cell culture plates, on polyvinyl chloride (PVC) or silicon catheters and on silicon membranes. Moreover, immunofluorescence studies as well as electron microscope investigations were carried out to localize viruses inside the biofilm.

1.11.2-Studying virus interactions with Candida biofilm in terms of virus sensitivity to disinfectants and to neutralizing antibodies

C. albicans biofilms exposed to HSV-1 and CVB5 were treated with hypochlorite and the residual infectivity of viruses entrapped in extracellular matrix (ECM) determined. Similarly, we incubated the two viruses encompassed in Candida biofilm with neutralizing antibodies to assess whether virus-biofilm interactions can prevent virus neutralization by antibodies.

1.11.3-Assessing the ability of *Candida albicans* biofilm to hold non adhering HSV1-infected T lymphocytes

HSV1-infected JJHAN cells were added to *C. albicans* biofilms and then the ability of biofilm to retain them was evaluated as well the amount of infectious virus released by the cells possibly entrapped in biofilm.

1.11.4-Determining whether the biofilm presence can impact the activity of antivirals on HSV1-infected cells encompassed in the biofilm

On the basis of literature data showing that microorganisms grown as biofilm display a reduced sensitivity to antimicrobials, we assessed the inhibiting dose 50 of acyclovir and foscarnet on HSV1-infected JJHAN cells embedded in Candida biofilm.

Chapter two
Materials and Methods

2.1-*Candida albicans* strains

Two strains of *C. albicans* were used: namely the highly virulent, biofilm producer strain 50vr isolated from a clinical case of invasive fungal infection, highly virulent in an infection model in *Galleria mellonella* (159) and the agerminative low virulent strain PCa_2 as a planktonic, biofilm non-producer strain (160). Stock cultures of these strains were prepared on Sabouraud dextrose agar plates, stored at 4 °C and weekly subcultured. Before biofilm preparation, the two Candida strains were overnight cultured on Yeast Peptone Dextrose (YPD).

2.2-Virus strains

HSV-1 and CVB5 were clinical isolates, identified by monoclonal antibodies (161,162). Both strains were laboratory adapted through serial passages (>50) on VERO cells over many years. The virus inocula employed in the experiments consisted of cell-free virus suspensions, obtained from centrifuged lysates of virus-infected VERO cells.

2.3-Cell lines

The epithelial cell line Vero, used to propagate and titrate HSV-1 and CVB5, was maintained in Eagle's Minimal Essential Medium (MEM) with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C and 5% CO_2. Additionally, the human T cell lymphoblast line JJHAN was used for experiments on the interaction between biofilm and cells in suspension. This cell line was maintained in RPMI 1640 added with same components as for MEM. Both cell lines were maintained by passages in fresh medium twice a week.

2.4-Biofilm formation and detachment

For biofilm production, Candida strain 50vr was grown overnight at 37°C in Yeast Peptone Dextrose (YPD), harvested, washed with phosphate-buffered saline (PBS), and resuspended to 1×10^6 cells/ml in RPMI-10% FCS; 100 µl were seeded in duplicate in polystyrene, flat-bottom 96-well plates (Euroclone S.p.A., Pero, Mi), Italy) and incubated at 37°C to allow biofilm formation, according to reported studies (163, 164).

For biofilm detachment, 3 different methods were tested and compared.

First, a mechanical treatment: the culture wells were washed 3 times with 100 µl of PBS and then, after adding 50 µl of PBS, were energetically scraped for 2 minutes. Second, an enzymatic detachment according to Al-Fattani and Douglas (84): after 3 washings with 100 µl of PBS, the culture wells were added with 100 µl of proteinase K (PK) solution (50 µg/ml in Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5) and the plates incubated for 2 additional hours at 37 °C. Afterwards, proteinase K was blocked by adding 50 µl of phenyl methane sulfonyl fluoride (25 mM) (Sigma-Aldrich Srl - Milan - Italy).

Third, a thermal detachment: the samples were washed 3 times with 100 µl of PBS and then, after adding 50 µl of PBS, the plate was heated at 55 °C for 15 minutes. At the end of each detachment method, the rescued materials were transferred into microtubes, centrifuged at 14000 rpm for 10 minutes and then the cell-free supernatants were processed for virus titration as detailed below.

Candida biofilm was also grown on nasal and urinary catheters made by silicone and PVC in order to evaluate whether biofilm grows differently on different materials. Both types of tube were cut as 5 mm high discs, sterilized and placed inside 24 wells of cell culture plate. The two strains of Candida

were then added as above described for biofilm formation in cell culture plastic plates. Moreover, Candida biofilm was cultured also on silicone disks from non-reinforced 0.5mm sheets (Bio-plexus Inc., Vernon, CT, USA). In this case, pre-treatment for 30’ with 50 µmol/ml poly-L- lysin (Sigma-Aldrich Srl - Milan - Italy) was used to increase yeast adhesion and biofilm formation. After incubation of the silicon disks for 30’ at 37°C non-bounded poly-l-lysin was washed out twice and Candida cells seeded for biofilm formation as already described.

2.5-XTT assay

The XTT colorimetric tetrazolium assay was used to determine fungal cell viability as an indirect quantification of Candida biofilm grown in culture wells as well as of biofilm remained in the wells after detachment (165). A commercial kit (AppliChem GmbH, Darmstadt, Germany) was employed following the manufacturer’s instructions. Briefly, wells containing Candida biofilm, planktonic pCa2 Candida cells or controls (medium only) were washed 3 times with 200 µl of PBS and then 100 µl of the colorimetric solution were added: this solution contained 1 part of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) and 1 part of menadione 10mM in acetone. After 2h incubation in darkness at 37°C the absorbance of the colored reduction product was measured by a spectrophotometer (Sunrise, Tecan Group Ltd, Männedorf, Switzerland) at 450nm. Each experimental group consisted of 6 replicates.

2.6-Crystal violet assay

Crystal Violet (CV) staining was used to quantify the biofilm grown in the presence of JJHAN cells (166): XXT was not used in this case since it

stains all viable cells and does not allow to distinguish biofilm mass from JJHAN cells. Briefly, wells containing Candida biofilm, planktonic pCa2 Candida cells or controls (medium only) were washed 3 times with 200 µl of PBS and then air dried for 5'. After fixation by 100 µl of methanol for 20', samples were stained with 100 µl of 1% CV solution for 5 min. Afterwards, each well was washed 3 times with 200 µl of distilled water and added with 33% acetic acid (100 µl/well). After 10 min, the optical densities (OD) were measured at 540 nm by a microplate reader (Sunrise, Tecan Group Ltd, Männedorf, Switzerland). Each experimental group consisted of 6 replicates.

2.7-Exposure of biofilms to cell-free virus *inocula*

Twenty-four hours after Candida seeding in culture plates, 100 µl of each virus inoculum were added to biofilm, planktonic and control samples. The final virus concentration in the culture wells was 10^7 PFU/ml for HSV-1 and 10^4 $TCID_{50}$/ml for CVB5. The samples were incubated with the virus *inocula* for 48h. Then, after washing with PBS and detachment by scraping, as described above, the viral load of the rescued materials was determined by end-point titration on VERO cells. In other sets of experiments performed to assess whether biofilms exposed to viral *inocula* release infectious virus, after incubation of biofilms with the viruses for 48h, the culture wells were washed three times and instead of harvesting the samples, fresh growth medium was added: after further 48h incubation, virus in centrifuged supernatants was titrated on VERO cells.

In experiments aimed at evaluating the influence of the virus presence on biofilm formation, the virus *inoculum* was added to Candida cells at the same time of cell seeding into the culture plate: after 48h the amount of biofilm was quantified by XTT assay.

2.8-Virus titration

To quantify the amount of virus, end-point titration was used. After centrifugation of the rescued materials, 10-fold dilutions of the supernatants in MEM with 5% FCS and 50mg/ml Amphotericin B were seeded in duplicate onto 24 h-old VERO cell cultures in 96-well plates. After a 3 day incubation at 37°C, the virus titre of each sample was read as the highest dilution showing the typical viral cytopathic effect. The results, expressed as $TCID_{50}$/ml, were calculated using the Reed and Muench formula (167).

2.9-Double staining for virus detection inside the biofilm

In order to localize virus inside the biofilm, biofilms exposed for 24 to HSV-1 were double stained using Uvitex 2B, a fluorescent dye binding to the chitin in fungal wall, and a FITC-conjugated monoclonal antibody (MAb) against a HSV-1 envelope glycoprotein (Argene S.R.L., Segrate, Italy).

Candida biofilms grown for 24h on silicone discs put in a 96-well tissue culture plates were added with 100µl of Uvitex 2B working solution for 30´ at 37°C. After two times washing with PBS, the wells were fixed with 200 µl of paraformaldehyde 4% for 30 minutes at 4°C. Then, the wells were washed two times for 10´ and then 10 µl of MAb anti HSV-1 were added for 30´. After 2 final washes with PBS, the silicone discs were mounted on a glass slide and observed by a fluorescence microscope (Nikon, Eclipse, 80i).

2.10-Effect of biofilm presence on virus sensitivity to sodium hypochlorite

Biofilms, planktonic samples and controls, exposed to each virus for 48h as above described, were treated or not with sodium hypochlorite (final dilution 1:400), further incubated for 30’ at 37°C, washed and then scraped as

already detailed. The rescued materials were centrifuged and the viral load of the cell-free supernatants assessed by end-point titration.

2.11-Effect of biofilm presence on virus neutralization by antibodies

Biofilms, planktonic samples and controls, exposed to each virus for 48h, were treated or not with human polyclonal purified immunoglobulins (IgVena 50g/l, Kedrion, Pascoli Barga, Lu, Italy) (final dilution 1:10), further incubated for 15’ at 37°C, washed and then scraped as detailed above. The rescued material was centrifuged and the viral load of the cell-free supernatants assessed by end-point titration.

2.12-Exposure of HSV-1 infected JJHAN cells to Candida biofilm

JJHAN cells were infected with HSV-1 at a multiplicity of infection of 10 PFU/cell and 24 h later were added to Candida biofilms, Candida planktonic samples and controls (5×10^5 infected cells/well). After 3h and 8h at 37°C, wells were washed 3 times with 200 µl PBS; 0.2% Trypan Blue solution was added (50µl/well) and then scraping performed. The viable cells in the rescued materials were counted on a Bürker chamber. In parallel groups, the plates were frozen-and-thawed after washing and scraping to lyse the infected JJHAN cells. Virus titration was then performed as described above. Moreover, in order to evaluate the influence on the presence of cells on the stability and viability of biofilm, after 8h incubation of biofilms with HVS1-infected and –uninfected JJHAN cells, the CV assay was carried out to quantify the amount of biofilm.

2.13-Localization of infected JJHAN cells inside candida biofilm

In order to localize HSV1-infected JJHAN cells inside the biofilm, biofilms grown on silicone disks and exposed for 3h to HSV1-infected and un-infected JJHAN were double stained using Uvitex 2B, and a FITC-conjugated monoclonal antibody (MAb) against a HSV-1 envelope glycoprotein. 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to A-T rich regions in DNA, was used to better visualize JJHAN cells inside the biofilm as a counterstain. One hundred µl/well of DAPI solution (5 mg/ ml) were added for 30 minutes at 37°C to JJHAN cells infected 24h earlier and then the stained cells were added to biofilms for 3 hours and then the fluorescence double staining was carried out as described above except for using acetone instead of paraformaldehyde 4%.

2.14-Influence of the biofilm presence on the antiviral activity of Acyclovir and Foscarnet

VERO cells were infected with HSV-1 (multiplicity of infection1 PFU/Cell) and incubated at 37°C for 90´. After washing with PBS, 2×10^5 cells/well were added to 24h old biofilms grown in 96 well tissue culture plates. Together with the VERO cells, acyclovir (50 µM, Recordati S.p.A., Milan, Italy) or foscarnet (0.6 µM, Clinigen, Burton-on-Trent, UK) were added to the cultures. After 24h incubation at 37°C, plates were frozen and thawed and the viral titer of each well was determined by end-point titration on VERO cells. In parallel, HSV-1infected VERO cells without biofilm were treated with the two antivirals.

In another set of experiments, 2-fold scalar dilutions of each drug were added to cultures of infected VERO cells in the presence and in the absence of 24-old Candida biofilm. The viral yield of each culture was titrated 24h

later by plaque assay. For acyclovir, concentrations ranging from 3µM to 48µM were assayed, while for foscarnet from 0.018µM to 2.4µM. A dose-response curve was then elaborated and the inhibiting dose 50 (ID50) was determined for each drug with or without Candida biofilm as the dose hypothetically giving a 50% reduction of the plaque number.

2.15-Statistical analysis

The data reported in figures are the mean values (± standard deviation) from at least 3 different experiments performed. The results were analyzed by the two-tailed Student's t test and were considered significant when $p<0.05$.

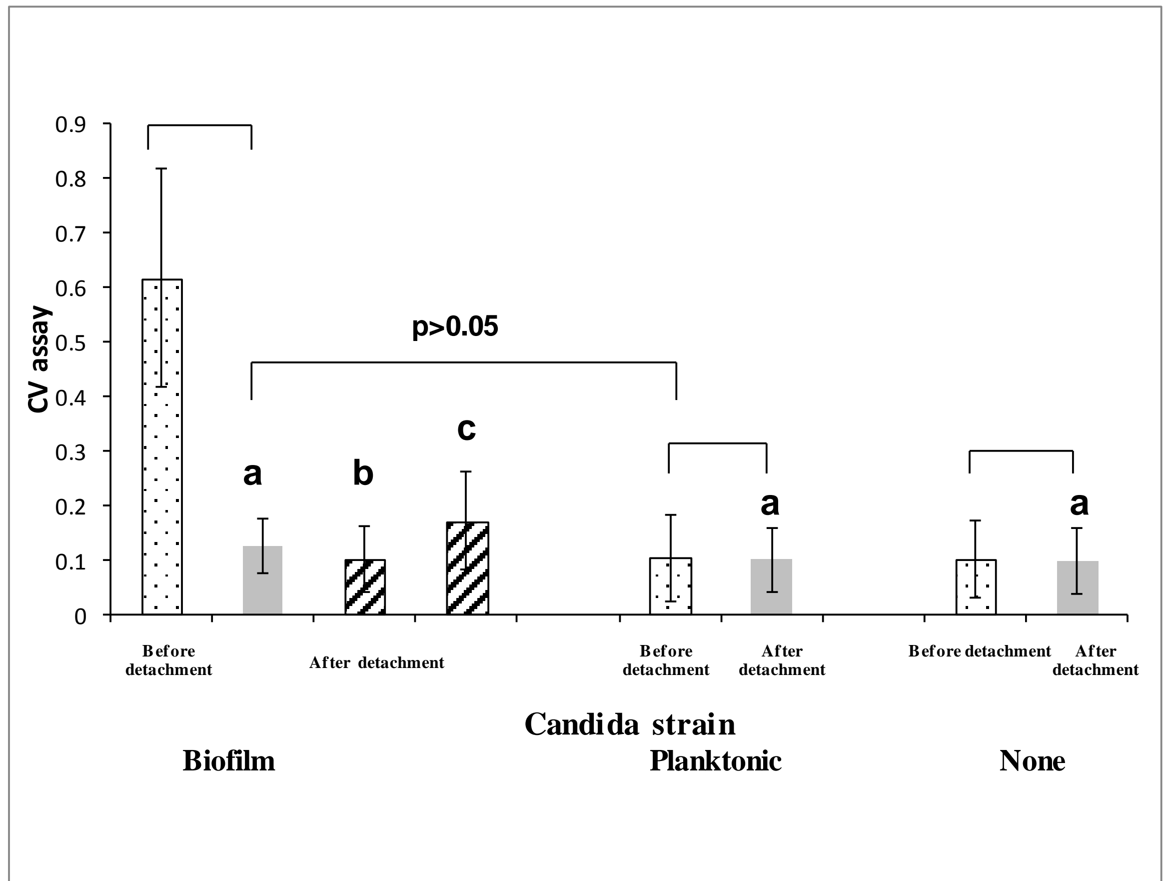
Chapter Three
Results

3.1- Candida biofilm formation and detachment

XTT assay was used to assess the amount of Candida biofilm produced in 96-well plates after 24h incubation in culture medium. As shown in Fig.3.1(dotted bars), the OD value in the wells containing the biofilm producer Candida strain (50vr) was 1.31, whereas that of planktonic Candida samples was significantly lower (0.12) and similar to the control's value (0.1). Since XTT measures cell viability and is therefore an index of the biofilm mass, these results indicate that, under the employed conditions, only the 50vr Candida strain produces a biofilm. Figure 3.2 shows the initial phase (3h after Candida seeding) and the late phase (24h) of Candida 50vr biofilm formation observed by light microscope.

Fig 3.1

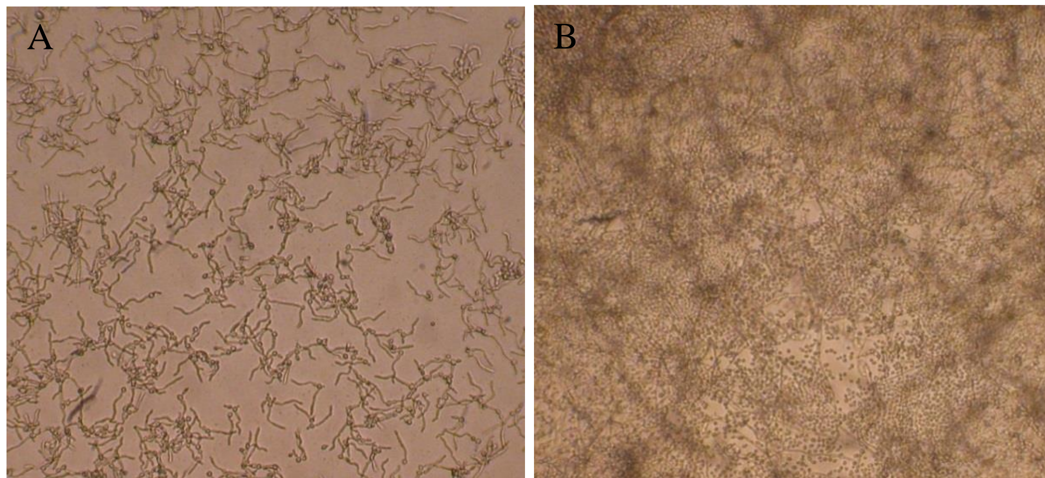
Evaluation of Candida biofilm formation and detachment



XTT assay was carried out on 24h old Candida biofilm before and after biofilm removal. Methods used to detach the biofilms: a) scrapping; b) Proteinase K treatment; c) heating. Samples of planktonic candida and controls were run in parallel.

Fig 3.2

Observation of Candida biofilm by light microscopy



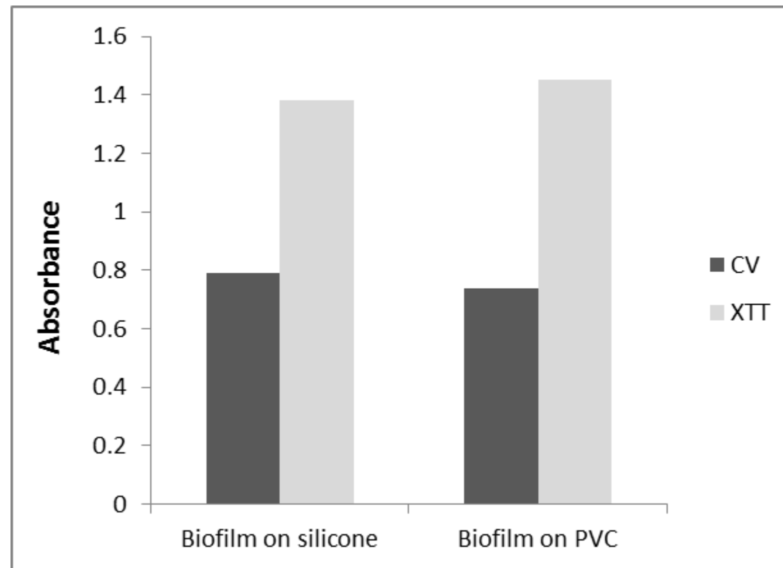
Biofilm formation observed at light microscope (20X): A early phase (3h after seeding of strain 50vr), B late phase (24h).

Candida biofilm formation was assessed also on the surface of two types of catheters, silicone and PVC (sterilized and put inside wells of 24 well cell culture plates). After 24h from Candida seeding, XTT and CV assays were used to determine the amount of biofilm mass. CV values were 1.145 and 1.38 on silicone and PVC. Absorbance values after XTT assay were 0.736 and 0.79 on silicone and PVC (Fig.3.3).

Fig 3.3

Candida biofilm formation

on silicone and PVC catheters



Candida biofilm was grown for 24h on silicone and PVC catheter located in a tissue culture plate and then the amount of biofilm was quantified by XTT and CV assays.

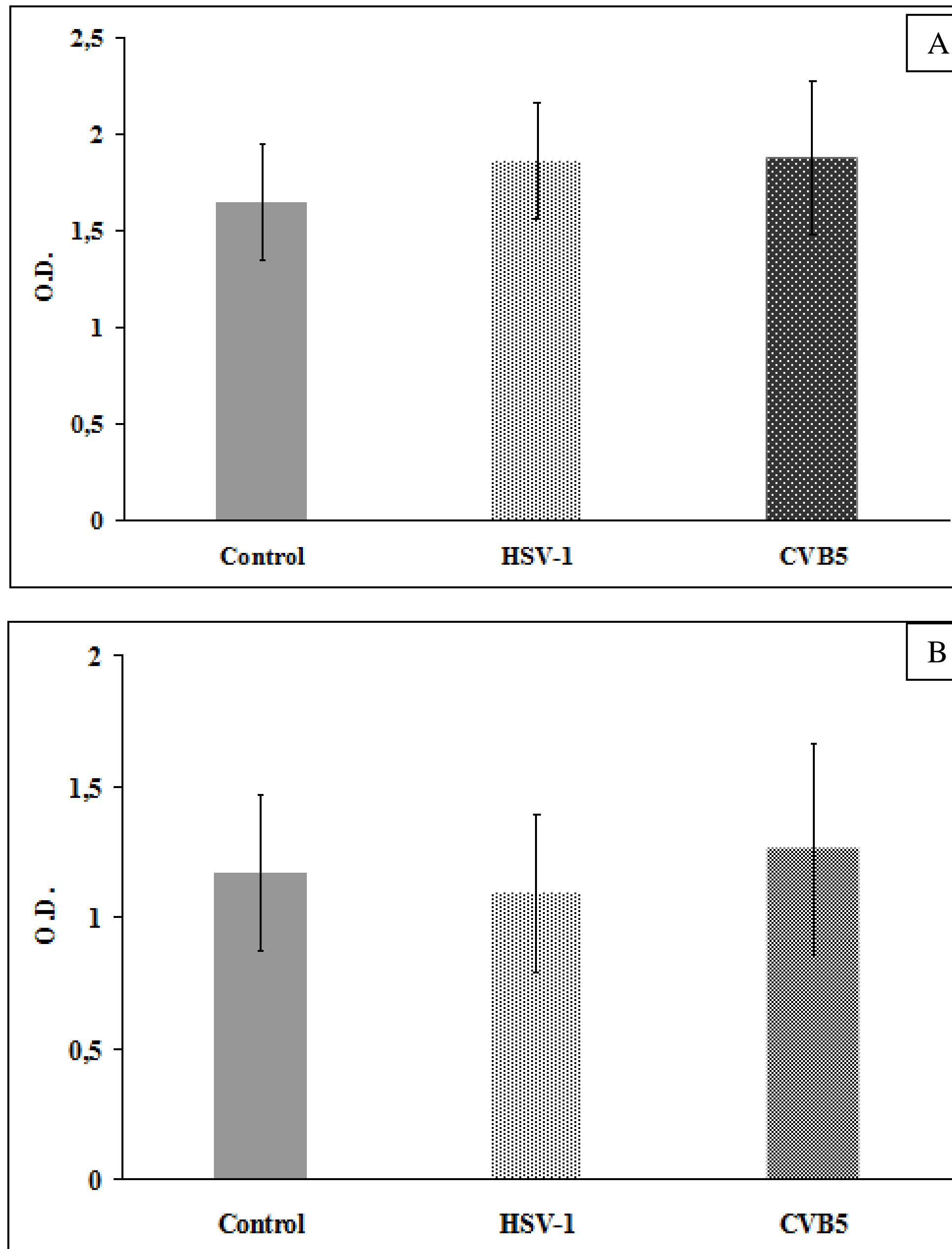
In order to determine whether virus particles were retained within biofilm, 3 methods for biofilm detachment was compared and their efficiency evaluated by XTT assay quantification of the remains in each well (Fig.3.1). The OD values in the cultures with 50vr Candida strain samples after biofilm removal by each method were rather similar (0.14 for scraping, 0.12 for PK digestion and 0.19 for heating): such values were comparable to the background value of the controls (P> 0.05), proving that the 3 protocols are able to efficiently remove biofilm.

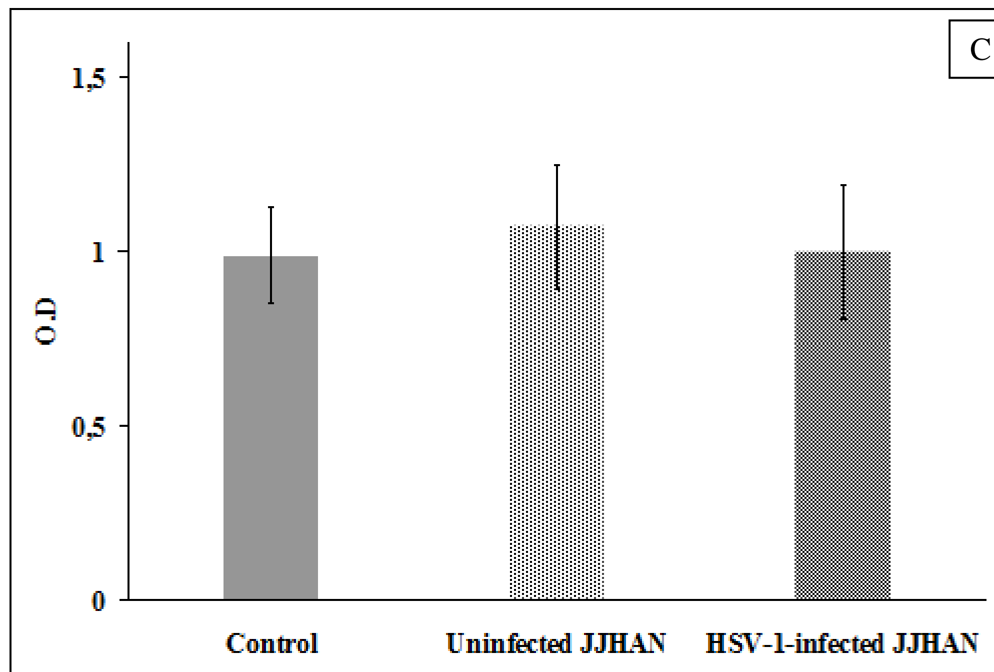
3.2- The virus presence affects neither Candida biofilm formation nor its maintenance

Figure 3.4 shows the results of the XTT assay on Candida biofilm grown in the presence of each of the two viruses under study, added at the same time of fungal seeding. The OD values of biofilms produced in the presence or in the absence of the viruses were similar (1.66 for controls without virus, 1.86 for biofilms + HSV-1 and 1.88 for biofilms + CVB5) suggesting that the presence of the virus does not affect biofilm production by Candida. In panel B of the same figure, the results of similar experiments in which the virus *inoculum* was added to mature biofilms are reported. Again, no significant differences were observed between virus exposed biofilms (1.09 for HSV-1 and 1.26 for CVB5) and controls without virus (1.17). Panel C of the figure 3.4 shows the results of experiments in which HSV-1-infected JJHAN cells were added to mature biofilms and after 8h incubation CV staining was used to determine the amount of biofilm the OD value of biofilms exposed to infected JJHAN cells (1.0) was not significantly different from those of the two types of controls consisting of biofilms with uninfected JJHAN (1.07) and biofilms without JJHAN cells (0.99). These results demonstrate that also cells entrapped in biofilm do not alter its viability and stability.

Fig 3.4

Virus presence does not alter Candida biofilm





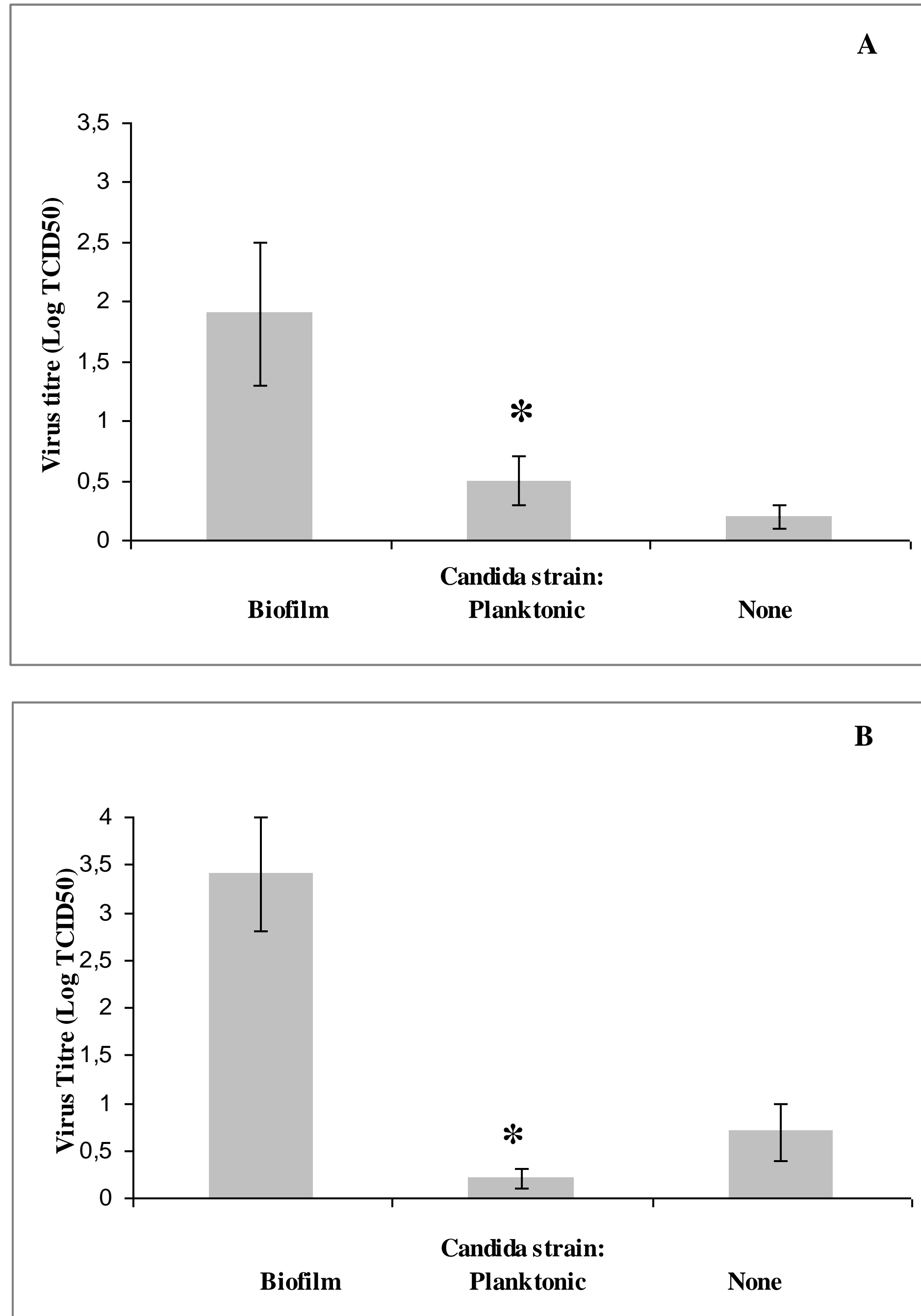
A: biofilms were grown in the presence of virus and after 48h, XTT assay was carried out to determine biofilm viability; B: biofilms were grown for 24h and then the viral *inoculum* was added and the cultures incubated for further 48h before XTT assay; C: biofilms were grown for 24h, then HSV-1 infected JJHAN cells were added for 8h and CV staining was performed. P>0.05 in all comparisons.

3.3- Virus particles are entrapped within Candida biofilm

Candida biofilms were exposed to virus inocula for 48h, then biofilms were thoroughly washed and the biomass was detached by scraping. The same treatment was carried out also in culture wells containing planktonic Candida or only medium (controls). The presence of infectious virus in the detached/rescued material was then end-point titrated on VERO cells. (Fig.3.5 panel A) shows the CVB5 titers in samples of Candida biofilm, planktonic Candida and controls, which were $10^{1.9}$ $TCID_{50}$, $10^{0.2}$ $TCID_{50}$ and $10^{0.5}$ $TCID_{50}$, respectively. As far as HSV-1, the amount of virus was $10^{3.4}$ $TCID_{50}$ for biofilm, $10^{0.2}$ $TCID_{50}$ for planktonic Candida and $10^{0.7}$ $TCID_{50}$ for the control (Fig. 3.5, panel B).

Fig 3.5

Viral load in Candida biofilm

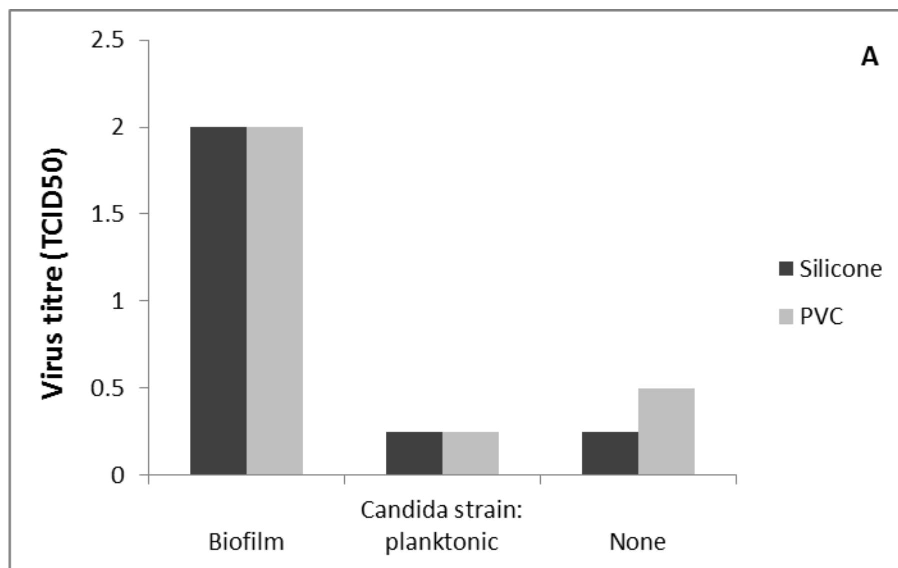


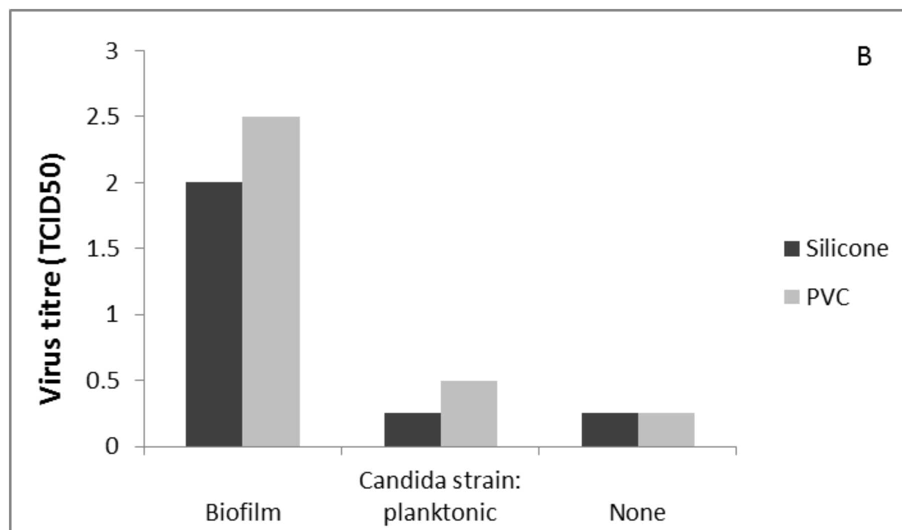
Twenty-four hours old Candida biofilms were exposed for 48h to virus *inocula* of CVB5 (panel A) or HSV-1 (panel B). Afterwards, samples were washed, scraped and the amount of virus remained in the wells end-point titrated. Planktonic Candida and controls were run in parallel. * $p<0.05$

Moreover, also Candida biofilms formed on the silicone and PVC catheters inside 24 wells plate were exposed to cell-free *inocula* of CVB5 and HSV-1. After 48h, the catheters were washed, transferred to new microtubes containing 100 µl PBS and the amount of infectious virus in the rescued material was end-point titrated on VERO cells. Figure 3.6 (panel A) shows the CVB5 titers from cultures of Candida biofilm, planktonic Candida and controls on silicone and PVC catheters: they were 10^2 $TCID_{50}$ for biofilm on both types of catheters, $10^{0.2}$ $TCID_{50}$ for planktonic Candida on both types of catheter and $10^{0.2}$ and $10^{0.5}$ $TCID_{50}$ for the controls on silicone and PVC catheter respectively. As far as HSV-1, the amount of virus was 10^2 $TCID_{50}$ for the biofilm on both types of catheter, $10^{0.25}$ and $10^{0.5}$ $TCID_{50}$ for the planktonic Candida on silicone and PVC catheter respectively, and $10^{0.2}$ $TCID_{50}$ for the controls on both types of catheter (Fig. 3.6, panel B).

Fig 3.6

Viral load of Candida biofilm grown on silicone and PVC catheters





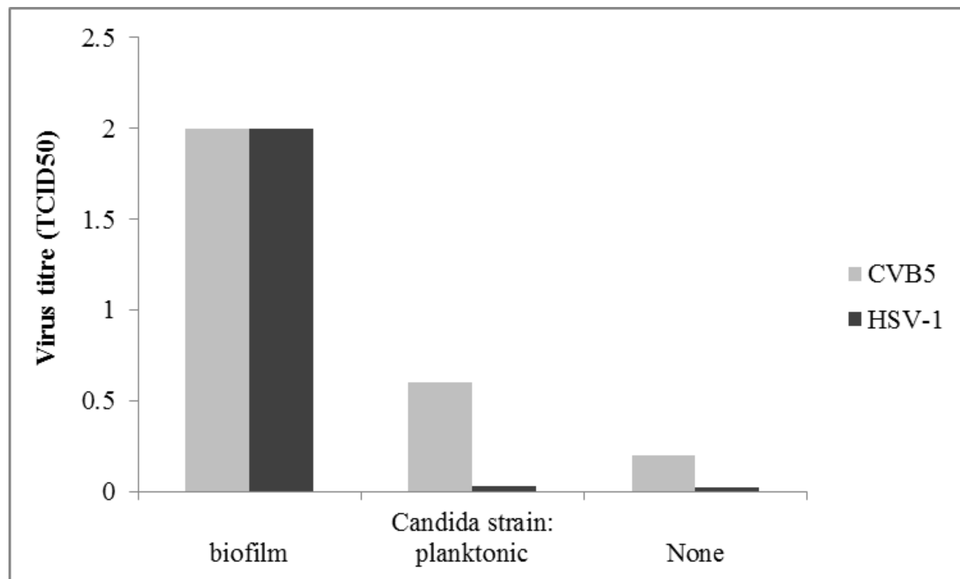
Twenty-four hours old Candida biofilms grown on catheters located in tissue culture plates were exposed for 48h to virus *inocula* of CVB5 (panel A) or HSV-1 (panel B). Afterwards, samples were washed and the amount of virus end-point titrated. Planktonic Candida and controls were run in parallel.

As far as Candida biofilm grown on silicone disks, the results were similar to those obtained with the other types of surfaces. As shown in the figure 3.7, titers of CVB5 (panel A) and HSV (panel B) in samples of Candida biofilm, planktonic Candida and controls on the silicone disks were 10^{2} vs 10^{2} $TCID_{50}$ for the biofilm, $10^{0.6}$ vs 10^{0} $TCID_{50}$ for the planktonic and $10^{0.2}$ vs 10^{0} $TCID_{50}$ for the control.

These data indicate that both viruses are entrapped within the Candida 50vr biofilm regardless of the material type used as adhering surface. The low levels of virus detected in the two types of controls (planktonic Candida and culture medium) represent the amount of virus spontaneously attached to the abiotic surfaces.

Fig 3.7

Vial load of Candida biofilm grown on silicone disks



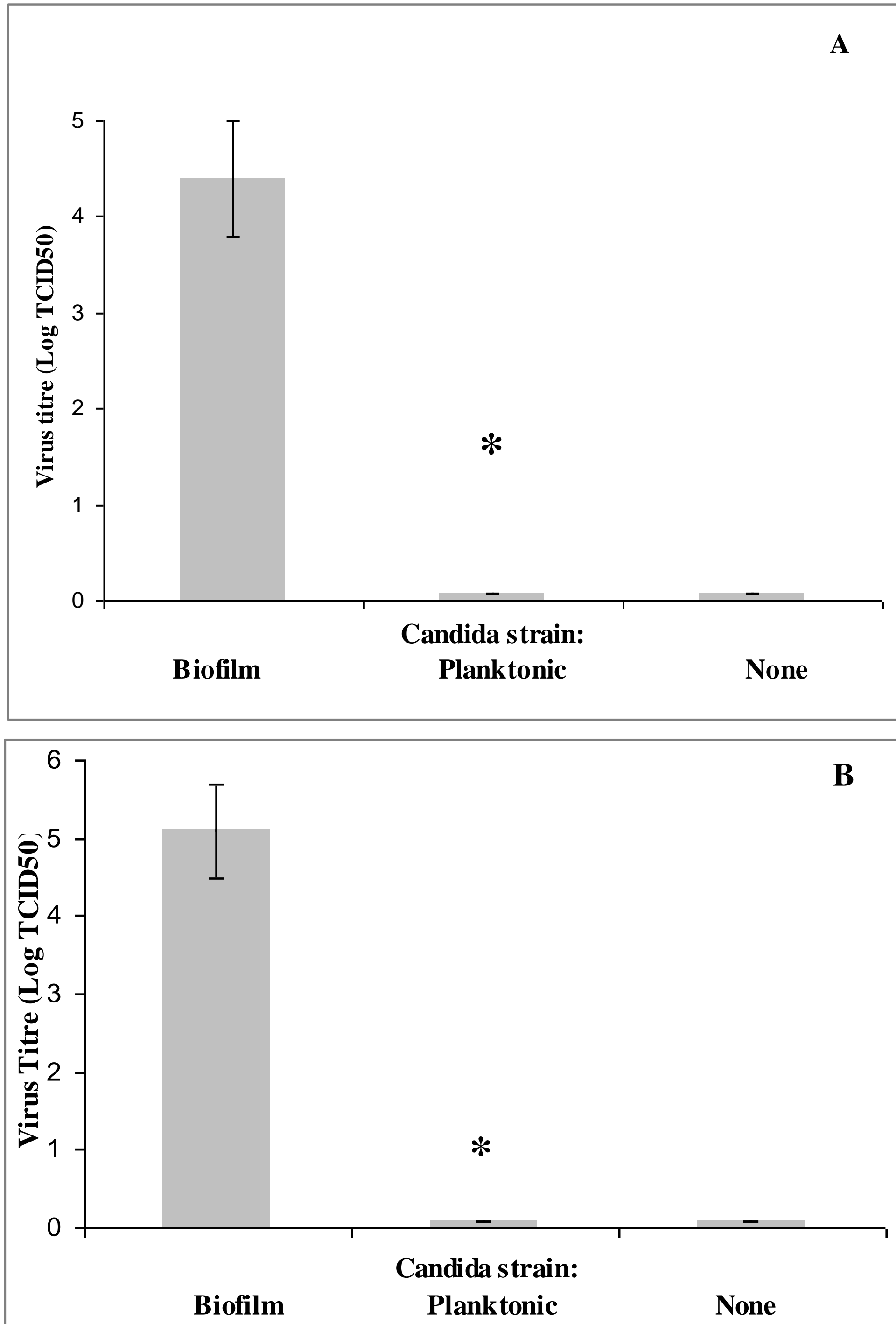
Twenty-four hours old Candida biofilms grown on silicone disks located in tissue culture plates were exposed for 48h to virus *inocula* of CVB5 or HSV-1. Afterwards, samples were washed and the amount of virus end-point titrated. Planktonic Candida and controls were run in parallel

3.4- Virus-exposed biofilms release infectious virus

Biofilms exposed to each of the two viruses under study for 48h were extensively washed, added with fresh medium and incubated for further 24h. Virus present in the supernatants was then titrated on VERO cells. Supernatants from biofilms exposed to CVB5 had a virus titre of $10^{4.4}$, while the relative controls (cultures with planktonic Candida and with medium only) had titres lower than 10^{1}(Fig.3.8 A). Similarly, the results obtained with HSV-1 were $10^{5.1}$ for biofilm and titers lower to 10^{1} for controls (Fig.3.8 B).

Fig 3.8

Viral load released by Candida biofilm

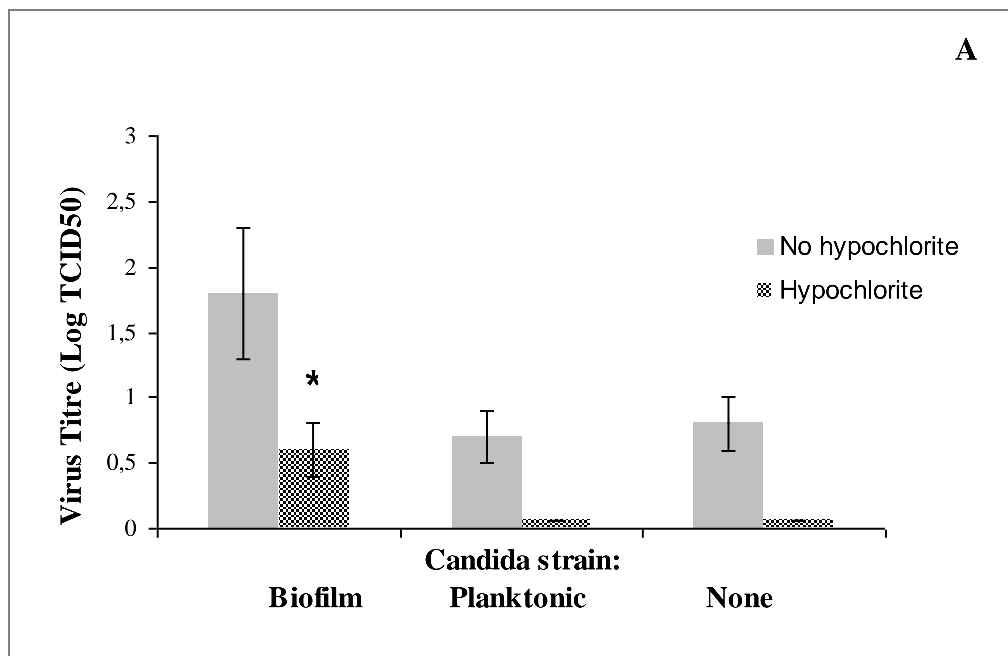


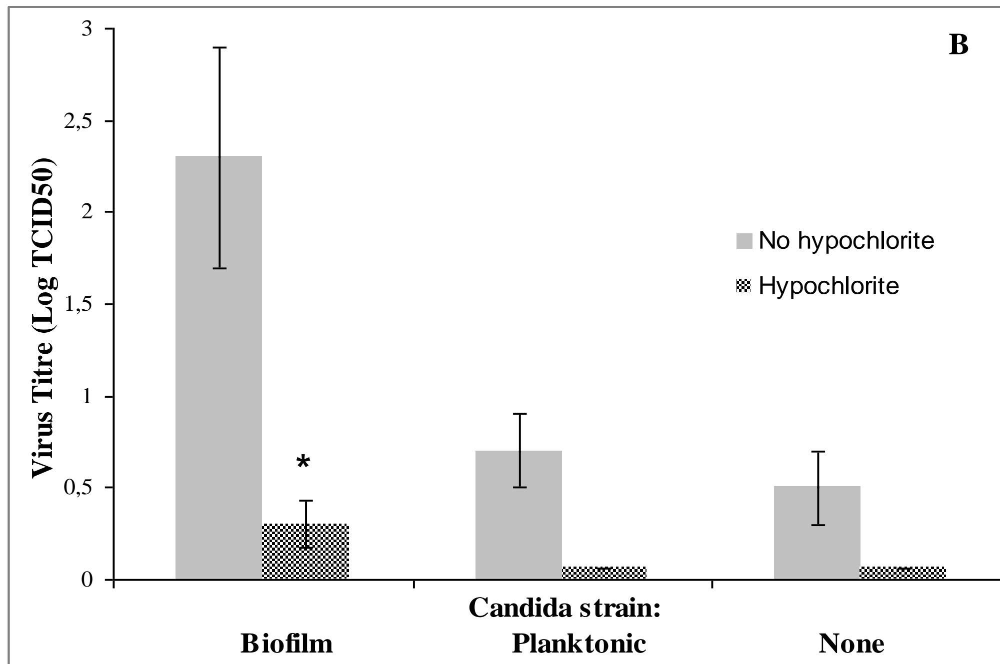
Twenty-four hours old Candida biofilms were exposed for 48h to virus *inocula* of CVB5 (panel A) or HSV-1(panel B). Afterwards, samples were washed, added with fresh medium, incubated for further 48h and then the amount of virus in the culture supernatants was end-point titrated. Planktonic Candida and controls were run in parallel. * $p<0.05$

3.5-Virus encompassment in Candida biofilm reduces the efficacy of virus inactivation by sodium hypochlorite

Biofilm impact on the virucidal activity of disinfectants was evaluated. Thus, after 48h incubation with virus, Candida biofilms were treated or not with sodium hypochlorite for 30', then thoroughly washed and detached by scraping. The load of infectious virus embedded in the detached/rescued biofilm was titrated and the results are depicted in figure 3.9. As for CVB5, the virus titers decreased, after hypochlorite treatment, from $10^{1.7}$ $TCID_{50}$ to $10^{0.7}$ $TCID_{50}$ in biofilms, from $10^{0.7}$ $TCID_{50}$ to 0 in the samples of planktonic Candida and from $10^{0.8}$ $TCID_{50}$ to 0 in controls. Similarly, for HSV-1, the virus titers decreased, after hypochlorite treatment, from $10^{2.3}$ $TCID_{50}$ to $10^{0.3}$ $TCID_{50}$ in biofilms, from $10^{0.7}$ $TCID_{50}$ to 0 in the samples of planktonic Candida and from $10^{0.5}$ $TCID_{50}$ to 0 in controls. Although for both viruses the rate of infectivity reduction was lower in the absence of biofilm (3-6 fold reduction) than in the biofilm cultures (10 fold for CVB5 and 200 fold for HSV-1), however in these last samples there was no complete neutralization of virus infectivity and significant, though low, titers of infectious virus were still detected after hypochlorite treatment. These findings demonstrate that biofilm presence significantly reduces the efficacy of hypochlorite treatment which loses its ability to completely inactivate viruses.

Figure 3.9
Virus inactivation by sodium hypochlorite is impaired by Candida biofilm





Twenty-four hours old Candida biofilms were exposed to virus inocula of CVB5 (panel A) or HSV-1(panel B). After 48h incubation, sodium hypochlorite was added to the samples, incubated for 30´ and then the culture wells were washed, scraped and the amount of virus remained in the wells end-point titrated. Planktonic Candida and controls were run in parallel. * $p<0.05$, ** $p<0.01$

3.6-Virus encompassment in candida biofilm does not prevent neutralization by antibodies

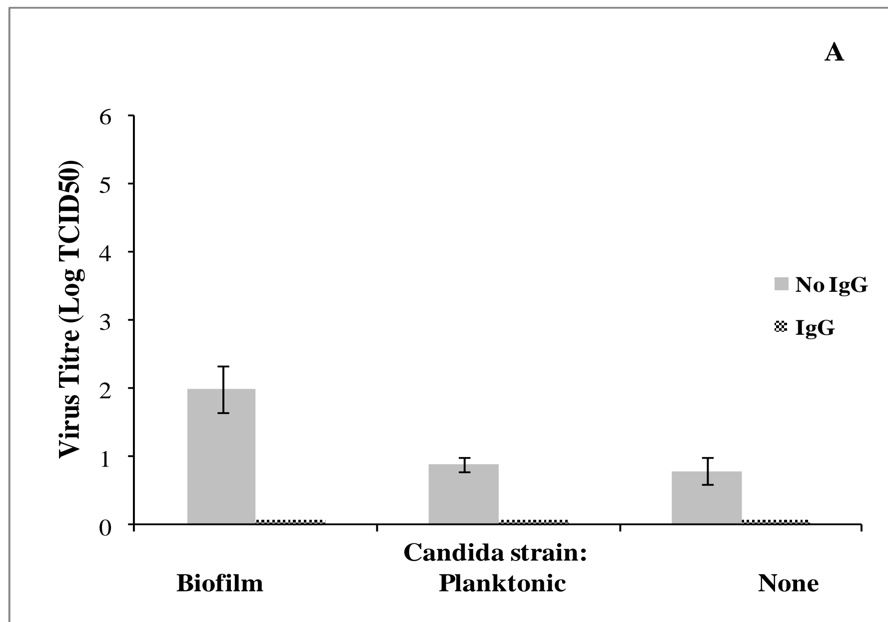
Next, we evaluated whether virus interaction with the biofilm could influence virus neutralization by antibodies. Thus, after 48h incubation with virus, Candida biofilms were exposed or not to neutralizing antibodies. Afterwards, washing and scraping were performed and the load of infectious virus in the detached/rescued biofilm was determined. CVB5 titer detected in biofilms decreased from $10^{1,7}$ $TCID_{50}$ to $10^{0,7}$ $TCID_{50}$ after treatment with immunoglobulin (Fig. 3.10); similarly, the titers decreased from $10^{0,7}$ $TCID_{50}$ to 0 in the samples of planktonic Candida and from $10^{0,8}$ $TCID_{50}$ to 0 for controls. As far as HSV-1, in the biofilms the titers decreased from 10^{3} $TCID_{50}$ to 0, while in planktonic from $10^{0,8}$ $TCID_{50}$ to 0 $TCID_{50}$ and in controls from $10^{0,7}$ $TCID_{50}$ to 0 $TCID_{50}$. Overall, these data indicate that

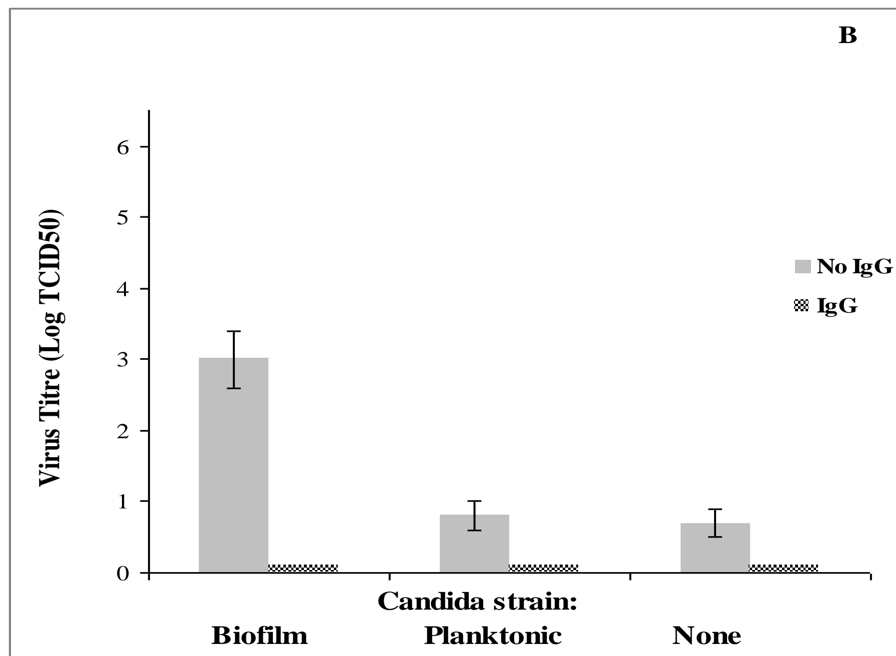
viruses embedded in Candida biofilm are still neutralized by antibodies.

Fig 3.10

Virus neutralization by antibodies

is not affected by Candida biofilm





Twenty-four hours old Candida biofilms were exposed to virus *inocula* of CVB5 (panel A) or HSV-1(panel B). After 48h incubation, human purified polyclonal IgG were added to the samples, incubated for 15´ and then the culture wells were washed, scraped and the amount of virus remained in the wells end-point titrated. Planktonic Candida and controls were run in parallel. P>0.05 in all comparisons

3.7-Hsv-1 infected JJHAN cells are encompassed by candida biofilm and release infectious virus

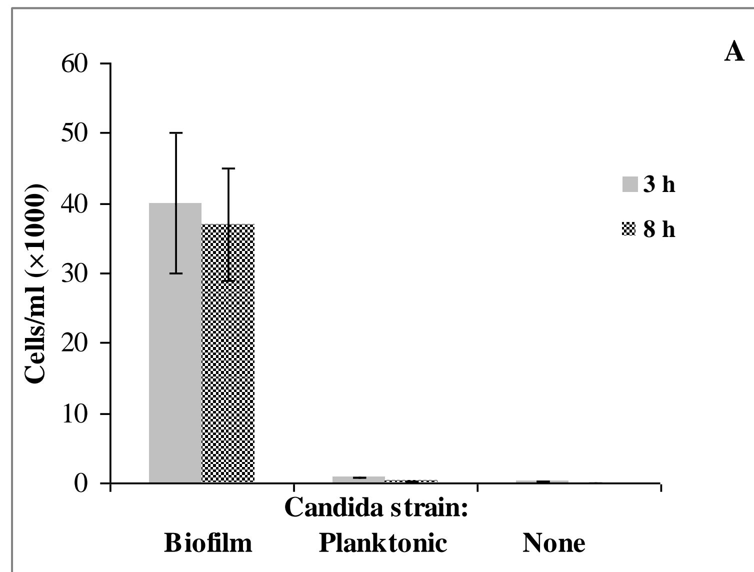
The ability of Candida biofilm to entrap virus-infected lymphoid cells was assessed. Twenty-four hours after infection with HSV-1, 5×10^5 infected JJHAN cells were added to 24h old Candida biofilms. Following additional 3 and 8 hours of incubation, washing and scraping were performed; the amount of viable JJHAN cells in the detached materials was determined by Trypan Blue exclusion test. The panel A of figure 3.11 shows the number of viable cells embedded in the biofilm: $4x10^4$ cell and $3,7x10^4$ cell, at 3h and 8h, respectively. In the culture wells with planktonic Candida, the values were $9x10^2$ cells and $3,5x10^2$ cells at 3h and 8h, respectively, and in the controls $3,5x10^2$ cells and 10^2 cells. The dramatic decrease in viable cells in the

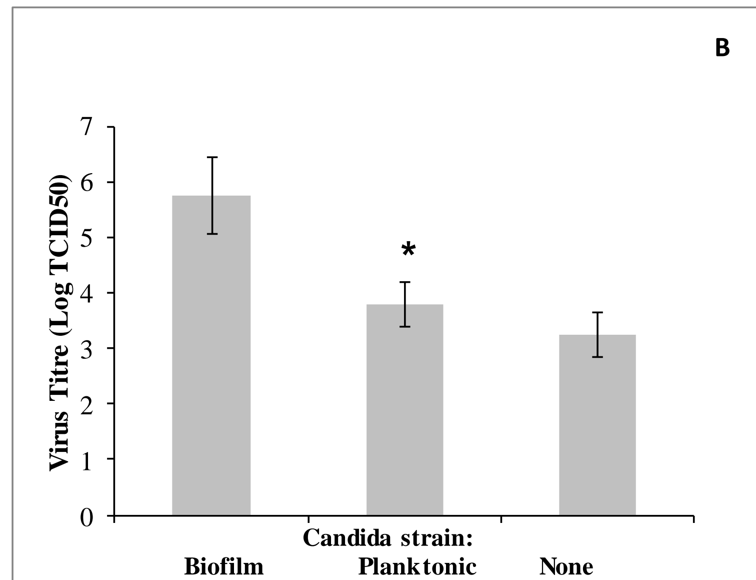
biofilm cultures between 3h and 8h incubation prevented us to prolong the incubation time: with longer incubation (12h or 24h) cells were almost totally dead (data not shown).

In parallel cultures, after washing and scraping, the plates were frozen-and-thawed and HSV-1 titrated on Vero cells: the panel B of the figure 3.11shows these results. Virus titer in biofilm was $10^{5,5}$ $TCID_{50}$, in the planktonic samples $10^{3,8}$ and in the controls $10^{3,3}$ $TCID_{50}$. These results show that HSV1-infected cells are embedded in Candida biofilm, where they remain viable and release virus.

Fig 3.11

Interaction between Candida biofilm and HSV-1 infected cells



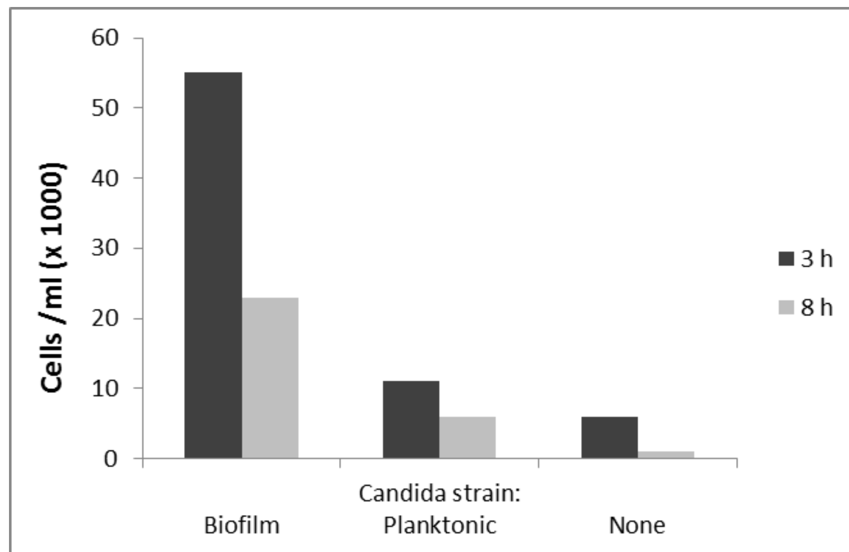


Twenty-four hours old Candida biofilms were exposed to HSV-1- infected JJHAN cells (5×10^5 /well). After 3h and 8h, samples were washed, scraped and the amount of cells remained in the wells counted by Trypan blue exclusion test (panel A). In parallel groups, only at 8h, after washing and scraping, the plates were frozen and thawed to lyse infected JJHAN and the released virus was end-point titrated (panel B). * p <0.05 biofilm vs planktonic Candida

The ability to retain HSV1-infected JJHAN was assessed also on biofilm grown on silicone disks. The figure 3.12 shows the number of viable cells embedded in the biofilm after 3h and 8h: on the silicone disks with biofilm $5.5x10^4$ cells and $2.3x10^4$ cells were counted, respectively at 3h and 8h incubation between biofilm and JHHAN cells. In the silicone disks with planktonic Candida, the values were 1.1×10^3 cells and 6×10^3 cells at 3h and 8h, respectively, and in the controls 6×10^3 cells and 10^3 cells.

Fig 3.12

Interaction between candida biofilm grown on silicone disks and HSV-1 infected cells



Twenty-four hours old Candida biofilms grown on silicone disks were exposed to HSV-1 infected JJHAN cells (5×10^5 /well). After 3h and 8h, samples were washed, and the amount of cells remained counted by Trypan Blue exclusion test

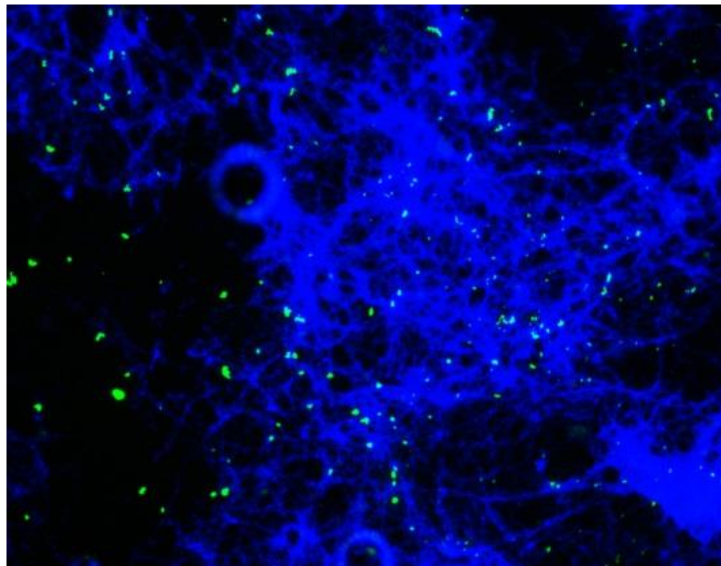
3.8- Localization of HSV-1 antigens and HSV1-infected cells inside Candida biofilm

The localization of viral end point inside biofilms grown on silicone disks and then exposed to either free-cell HSV-1 or HSV1-infected JJHAN cells was ascertained by a double fluorescent staining with Uvitex-B, a fluorescent dye specific for fungi, and with an anti-HSV-1 MAb. As shown in figure 3.13, aggregates of viral antigens can be seen irregularly dispersed inside the biofilm, prevalently not associated with fungal hyphae suggesting its localization within EPS matrix. On the contrary, JJHAN cells, either infected or uninfected, appear more abundant in areas with more compact

hyphae tangle, suggesting a possible interaction with fungi (Fig. 3.14).

Fig 3.13

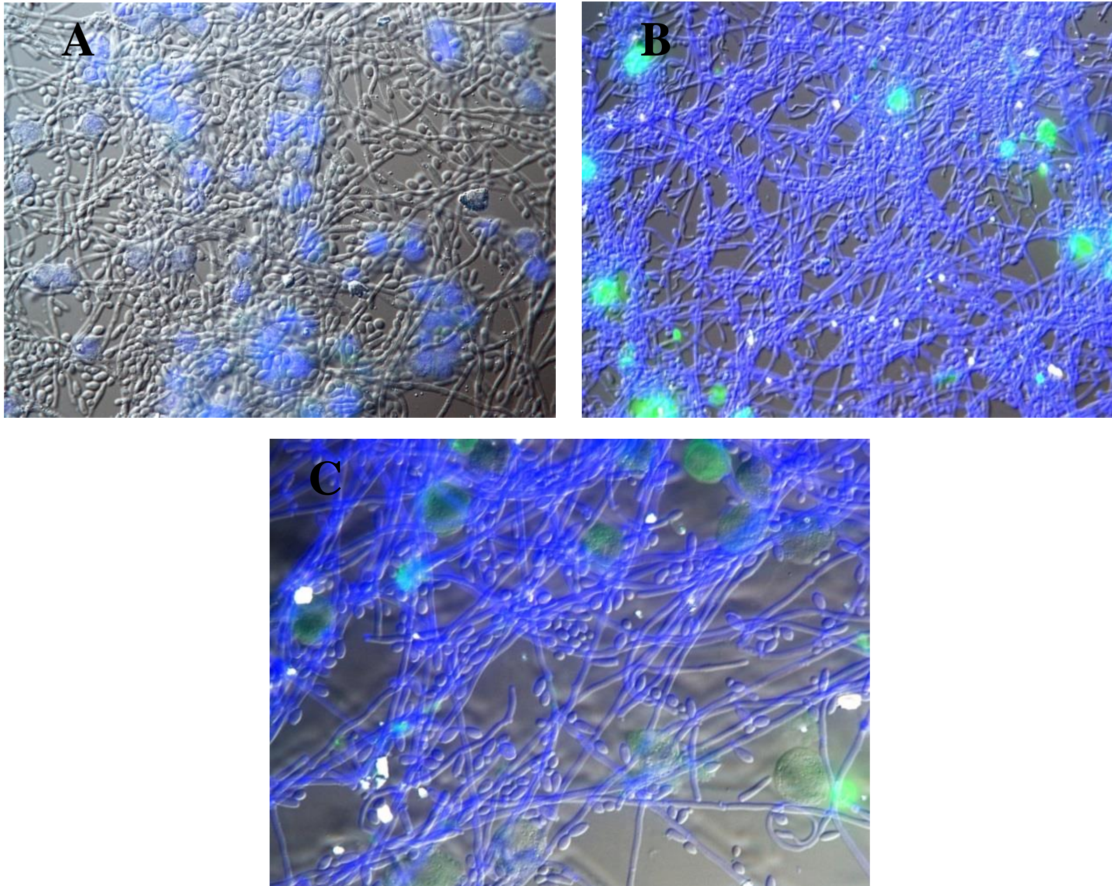
HSV-1 localization inside Candida biofilm



Twenty-four hours old Candida biofilms grown on silicone disks located in tissue culture plates were exposed for 48h to HSV-1 inocula. Afterwards disks were double-stained with Uvitex-B (specific for fungi which appear blue) and an FITC-conjugated anti- HSV-1 Mab. Aggregates of viral antigens can be observed dispersed inside the biofilm. Filters: DAPI and FITC; 20X magnification.

Fig 3.14

HSV-1 infected cells entrapped within Candida biofilm



Twenty-four hours old Candida biofilms grown on silicone disks were exposed to HSV-1- infected JJHAN cells (5×10^5 /well). After 8h, samples were stained with Uvitex-B, a FITC-conjugated anti HSV-1 MAb and DAPI. Samples with uninfected JJHAN were run in parallel. A: uninfected JJHAN stained by DAPI can be observed among Candida hyphae (filters: DAPI and DIC, 40X magnification); B and C: HSV-1 positive JJHAN are stained in fluorescent green while Uvitex-B fungi are stained in blue (filters: FITC, DAPI and DIC; 40 X magnifications in figure B and 60X in figure C)

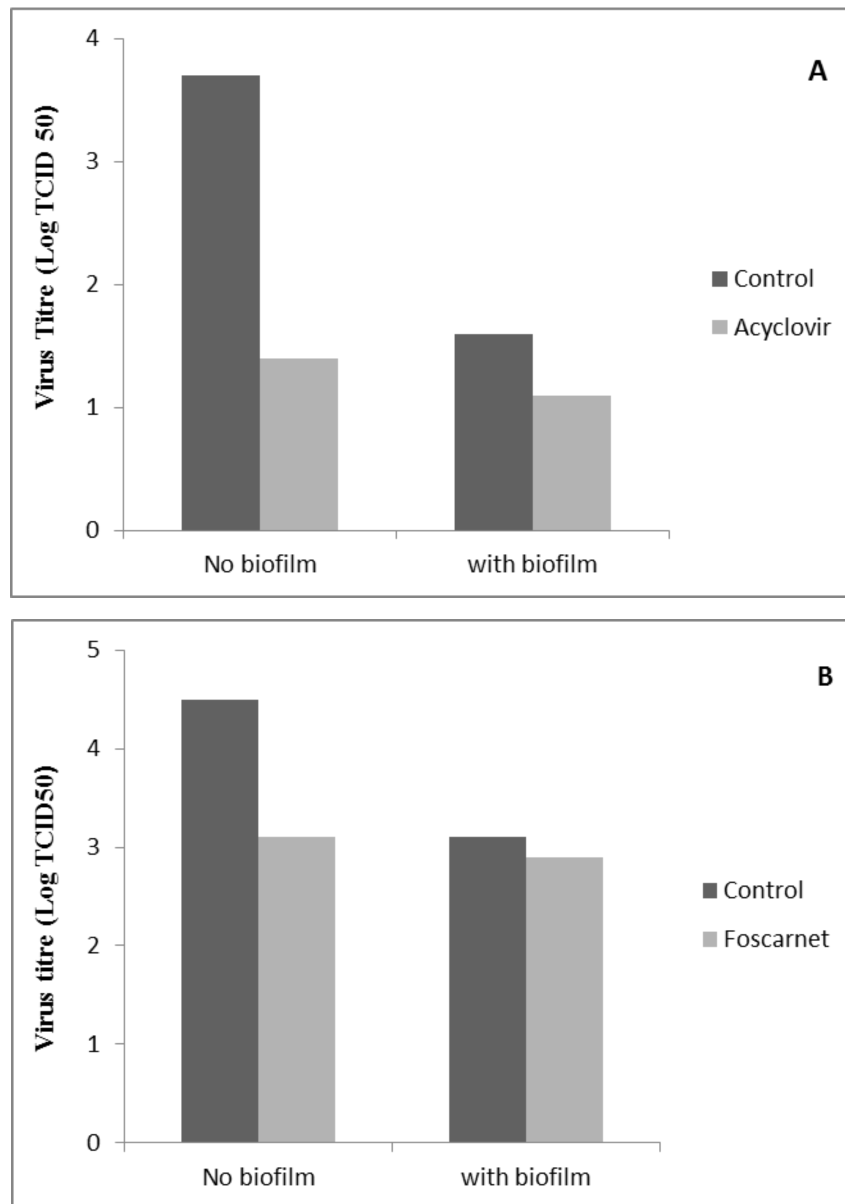
3.9-HSV-1 encompassment in Candida biofilm reduces the anti-herpes activity of acyclovir and foscarnet

HSV1-infected VERO cells were added to Candida biofilms, then exposed to acyclovir (50 μM) or foscarnet (0.6 μM) for 24h and the viral yield titrated. The comparison of virus replication inhibition between

VERO cells with Candida biofilm and VERO cells without biofilm showed a highly significant reduction of the antiviral activity of these two drugs when infected cells were embedded in biofilm. In fact, in the absence of biofilm acyclovir treatment caused a 2.3Log reduction of the viral titre ($10^{3,7}$ $TCID_{50}$ in the untreated controls vs $10^{1,4}$ $TCID_{50}$ in the samples treated with acyclovir) whereas when Candida biofilm was present the viral titre reduction was only 0.5Log ($10^{1,6}$ $TCID_{50}$ vs $10^{1,1}$ $TCID_{50}$) (Fig. 3.15A). Similar results were obtained with foscarnet: without biofilm the titre reduction was 2.4Log ($10^{4,5}$ $TCID_{50}$, vs $10^{3,1}$ $TCID_{50}$) while with biofilm the reduction was 0.2 Log ($10^{3,1}$ $TCID_{50}$ vs$10^{2,9}$ $TCID_{50}$) (Fig. 3.15 B). The ID_{50} of each drug in the presence and in the absence of biofilm was determined by adding 2-fold scalar concentrations of the two drugs. For both antivirals, the ID_{50} in the presence of biofilm was higher than without biofilm: for acyclovir they were 5,4 μM and 22,6 μM, without and with biofilm respectively, while for foscarnet 0,054 mM and 0,661 mM, respectively.

Figure 3.15

Anti HSV-1 activity of acyclovir and foscarnet in the presence and in the absence of candida biofilm



Twenty-four hours old Candida biofilms were exposed to HSV-1 infected VERO cells (5×10^5 /well) and either acyclovir (panel A) or foscarnet (panel B) were added to the culture medium. After 24h incubation, washing and scraping were performed, the plates were frozen and thawed to lyse infected VERO and the released virus was end-point titrated

Chapter Four
Discussion

Recently, microbial biofilms have gained prominence because of the increase in infections related to indwelling medical devices (IMD). *Candida albicans*, the pathogenic fungus which is a major cause of morbidity and mortality for blood stream infections, is the most common fungal pathogen isolated from patients with IMD-associated infections (66). Biofilm formation by *Candida albicans* is believed to contribute to invasiveness of these fungal species.

The aim of the present work was to assess whether *C. albicans* biofilm grown on cell culture plates and different materials of medical devices may mediate encompassment, retainment and dissemination of human pathogenic viruses.

The interaction between human viruses and biofilm has been poorly investigated so far and most studies are focused on aquatic biofilms inside pipelines. Although viruses are not involved in biofilm initial building, they might be involved in its later evolution and/or, in turn, be influenced in their own biology. Several mechanisms likely lead to virus inclusion in biofilms on both natural and artificial surfaces: link with extracellular matrix polymers, adhesion to host molecules responsible for the conditioning of abiotic surfaces, encompassment of virus-infected cells inside the biofilm. Biofilm matrix, having variable surfaces with many sorption sites, facilitate binding and accumulation of colloidal matters (107, 168-171). Those viruses that have a particle size ranging from 20-120 nm are considered as bio-colloids and, therefore, can be incorporated and retained into biofilm (172). On the other hand, some viruses are present in biological fluids associated with particular lipids or proteins which may enhance their capability to interact with biofim (116, 173).

For our study, we selected an enveloped and a non enveloped virus, HSV-1 and CVB5, respectively, which are widespread and important human pathogens. Although no literature data refer of an augmented infection risk for these viruses in patients with biofilms, we chose them as an *in vitro* model of easy growing viruses that can cause viremic infections with bloodstream dissemination, thus possibly interacting with biofilms potentially present in the host.

As a preliminary step, we assessed the biofilm formation *in vitro*, quantifying the biofilm mass by XXT assay and/or CV staining (165, 166, 174-176). We found that biofilm growth efficiently occurs in tissue culture plates (Fig. 3.1) and that the presence of virus impairs neither biofilm formation nor its viability and stability after maturation. Notoriously, biofilms tightly attach to surfaces so that their complete detachment is difficult. We compared 3 different detachment procedures, namely scraping, proteinase K treatment, and heating at 55°C and we assessed their efficacy in detaching fungal cells by CV staining of the remains in the culture wells, after treatment. In all cases, comparable results were observed, namely the OD values consistently resembled the background values of negative controls, implying that the 3 methods efficiently remove biofilm matrix. Hereafter, we used mechanical detachment by scraping, because it is faster than heating and more reproducible than enzymatic treatment (data not shown).

Here we show that when 24-h biofilms of *C. albicans* were exposed to HSV-1 or CVB5 as cell-free inocula for additional 48h, and then mechanically detached, the virus amounts detected in the rescued material are significantly higher for both viruses compared with non-biofilm counterparts (planktonic Candida and negative controls with only medium).

Interestingly, the same phenomenon is observed in experiments with a 6 days long incubation (data not shown), further emphasizing that, inside biofilm, virus particles are protected from degradation and retain their infectivity. We also demonstrated that virus-exposed biofilms can release virus: in fact, high virus titers were detected in the supernatants from virus–exposed biofilms re-cultured in fresh medium for 24h after extensive washes.

Similar results in terms of efficiency of biofilm formation (assayed by XTT and CV assays) and virus retainment were obtained with biofilms grown on silicone and PVC pieces of catheters, two common types of nasal and urinary catheters. These devices represent an ideal surface for biofilm formation. Depending on the kind of device, its insertion side, duration of insertion and the nature of infecting microorganism, biofilm constitutes a major reason for infections to occur and persists at various sites in the human body (177). XTT and CV assays showed that *C. albicans* efficiently form biofilms also on these surfaces. Moreover, when 24h old biofilms on catheters were exposed to cell free *inocula* of CVB5 and HSV-1, they retained both viruses with loads similar to those found in biofilms on tissue culture plastic.

Our finding on the ability of Candida biofilm to encompass and retain CVB5 is in agreement with previous studies showing that enteric viruses have the potential to accumulate within water biofilms, likely penetrating into micropores or channels (178). It has been proposed that aquatic biofilm provides embedded viruses a sort of shelter protecting them from disinfections (179). According to these observations, we evaluated whether the same phenomenon might occur also with human viruses embedded in Candida biofilm. In our experimental model, the treatment of the virus-containing biofilms with sodium hypochlorite significantly impairs virus

infectivity without completely abolishing it, implying that biofilm protection of such viruses from inactivation, is only partial. As predictable, CVB5 resulted less sensitive to the treatment (1 Log reduction) than HSV- 1 (2 Logs reduction). The discrepancy in comparison with the results reported by Quignon (179) might be due to the different composition of the biofilm models employed: unlike ours, the water environment-derived biofilms are polimicrobic and, therefore, have a different, and likely more complex, composition of the extracellular polymeric matrix. Possibly, also biofilm age, known to influence stiffness, thickness and volume of the matrix, might impact on chlorine inactivation effects. In water pipelines, biofilms persist for a long time, while, in our model, viruses were exposed to 24h old Candida biofilms.

A similar set of data was observed when assessing the efficacy of neutralizing antibodies on viruses embedded in biofilm. One possible reason hypothesized to explain immune escape of microrganisms grown as biofilm is that biofilm matrix can interfere with antigen recognition/linking. In our hands, both viruses were completely neutralized, irrespective of the presence or the absence of biofilm (fig.3.10). We can hypothesize that a 24h-old Candida biofilm is leaky and lax enough to allow antibody penetration inside its structure; thus, binding to virus particles embedded in the biofilm efficiently occurs, as already demonstrated with other types of biofilms (180). Moreover, it should be considered that full penetration of antibodies inside the biofilm may largely depend on the amount of IgG and on the incubation time.

Chandra and et al (182) studied the interactions between *C. albicans* biofilm and peripheral blood mononuclear cells (PBMC): SEM images revealed that the PBMCs were intact and appeared close to Candida biofilm

hyphae not exhibiting phagocitic activity, while PBMCs incubated with planktonic Candida for the same amount of time clearly demonstrate to phagocytose fungal cells. We therefore investigated the interactions between HSV1-infected lymphoblastic cells and Candida biofilm. Firstly, we demonstrated that the presence of infected JJHAN cells do not influence the maintenance of the total amount of biofilm produced (Fig. 3.4 C). Then we showed that Candida biofilm exhibits the capability to encompass HSV-1 infected T cells. Infected cells entrapped in the biofilm, in turn, release infectious virus at a titer 1.7 Log higher than infected cells exposed to the planktonic candida counterpart. This finding was not unexpected, since it has been previously demonstrated that, in T cells, Retroviruses can spread cell-to-cell by inducing either cell-cell nanotubes or specialized cell-cell contact areas (the so called virological synapses), where virions and receptors are concentrated (182, 183). Accordingly, enveloped virus-infected cells display a high tendency to clump. Moreover, recent findings suggest that HTLV-1 can form carbohydrate-rich adhesive extracellular viral assemblies for virus cell-cell transferring (184). Such extracellular assemblies consist of clusters of viral particles embedded in a matrix of highly glycosylated proteins, produced by the infected T cell upon virus induction: a sort of viral biofilm aimed to ensure virus protection, persistence in a specific environment and spread. Intriguingly, some components of bacterial biofilm were also found in such viral assemblies (119). Although this phenomenon has been demonstrated only for HTLV-1 so far, it is likely that a similar mechanism can be used also by other enveloped viruses, like Herpesviruses, known to spread mainly by cell-cell contact. Therefore, we may speculate that HSV-1-infected JJHAN cells, forming this sort of “viral biofilm” are easily

encompassed in Candida biofilm due to adhesive interactions between these two types of EPS sharing a similar composition.

Interestingly, the antiviral efficacy of two anti HSV-1 drugs, acyclovir and foscarnet, is dramatically decreased when infected cells are embedded in biofilm in comparison with cells not included in biofilm. We can hypothesize that EPS hampers drug diffusion by chemically complexing drug molecules. However, the role of EPS and influence of viruses inside the matrix still remains as an important question. The finding of a reduced sensitivity of HSV-1 to antiviral drugs suggests that, during viral infections, biofilm presence inside patient's body could form niches where circulating infected cells are protected from drugs, reducing the efficacy of the antiviral therapy. Retainment of virus particles and of HSV1-infected cells was observed also in Candida biofilm grown on silicone disks. Using this material we investigated by immunofluorescence double staining virus localization inside the biofilm. As shown in figure 3.13, aggregates of viral antigens can be seen irregularly dispersed inside the biofilm, prevalently not associated with fungal hyphae suggesting its localization within EPS matrix. On the contrary, JJHAN cells, either infected or uninfected, appear more abundant in areas with more compact hyphae tangle, suggesting a possible interaction with fungal components (Fig. 3.14).

Concluding, the present work is the first report investigating the interactions between *C. albicans* biofilm and human pathogenic viruses. Using different types of surfaces for biofilm formation, we demonstrated that:

1) *C. albicans* biofilm allows encompassment and release of infectious CVB5 and HSV-1 with partial protection from hypochlorite inactivation but not from antibody neutralization;

2) HSV1-infected cells are entrapped within Candida biofilm, releasing infectious virus without damaging the biofilm;
3) virus particles are diffusely dispersed inside the biofilm whereas HSV1-infected lymphocytes are mainly located in denser areas;
4) encompassment of HSV1-infected cells within the biofilm causes a dramatic decrease in antiviral efficacy of ayclovir and foscarnet.
On the whole, with all the limits of an *in vitro* study, these results suggest that, *in vivo, C. albicans* biofilm may represent also a reservoir of infectious virus. Circulating virus particles during viremic infections and/or circulating infected cells might be retained in the biofilm and, later on, released as either single particles or biofilm small fragments. Moreover, antiviral drug are reduced in their activity towards virus-infected cells entrapped in biofilm. Therefore Candida biofilm represents a further health risk especially for immunocompromised patients.

Chapter five
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Human pathogenic viruses are retained in and released by *Candida albicans* biofilm *in vitro*

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ABSTRACT

Candida albicans is the most prevalent human fungal pathogen associated with biofilm formation on indwelling medical devices. Under this form, Candida represents an infectious reservoir difficult to eradicate and possibly responsible for systemic, often lethal infections. Currently, no information is available on the occurrence and persistence of pathogenic viruses within *C. albicans* biofilm. Therefore, the aim of this study was to investigate whether *Herpes Simplex Virus type 1* (HSV-1) and *Coxsackievirus type* B5 (CVB5) can be encompassed in Candida biofilm, retain their infectivity and then be released. Thus, cell-free virus inocula or HSV-1-infected cells were added to 24 h-old fungal biofilm in tissue culture plates; 48 h later, the biofilm was detached by washing and energetic scratching and the presence of virus in the rescued material was end-point titrated on VERO cells. Planktonic Candida cultures and samples containing only medium were run in parallel as controls. We found that both HSV-1 and CVB5 free virus particles, as well as HSV-1 infected cells remain embedded in the biofilm retaining their infectivity. As a second step, the influence of biofilm on virus sensitivity to sodium hypochlorite and to specific neutralizing antibodies was investigated. The results showed that virus encompassment in fungal biofilm reduces virus sensitivity to chemical inactivation but does not affect antibody neutralization. Overall, these data provide the first *in vitro* evidence that viruses can be encompassed within Candida biofilm and then be released. Thus, it may be speculated that Candida biofilm can be a reservoir of viruses too, posing a further health risk.

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1. Introduction

Biofilms are structured communities of microorganisms in which the cells bind to a surface and become embedded in a self-produced matrix of extracellular polymeric substances (Costerton et al., 1987; Hall-Stoodley et al., 2004; Lynch and Robertson, 2006; Flemming and Wingender, 2010). Biotic as well as abiotic surfaces, including mucosal tissues, indwelling medical devices, water piping systems or natural aquatic eco-systems, may all provide a useful support for biofilm building (Donlan, 2002).

Clinically, biofilm production accounts for up to approximately 65% of microbial infections (Donlan, 2001; Douglas, 2002; Hall-Stoodley et al., 2012). Most of them are implant-related infections, being located on the surface of devices such as catheters, prostheses, and heart valves (Donlan, 2001; Douglas, 2002, 2003). Also, about 50% of nosocomial infections are associated with medical devices (Kojic and Darouiche, 2004; Cauda, 2009); notoriously, such infections are difficult to be treated and often device removal becomes mandatory (Chandra et al., 2001; Donlan, 2001, 2002; Douglas, 2002, 2003; Kojic and Darouiche, 2004; Kumamoto and Vinces, 2005; Cauda, 2009). Overall, biofilm-related infections are a common complication for many hospitalized subjects and they represent an additional significant cost for medical management of these patients.

Candida spp, especially *Candida albicans*, is known as one of the major agents of hospital-acquired infections worldwide causing both mucosal and deep seated infections associated with candidemia (Pfaller and Diekema, 2007). Recent data estimate that *C. albicans* ranks as the fourth most common cause of bloodstream infection and is responsible for a significantly increasing number of device-related infections. The ability of *C. albicans* to form biofilms on medical devices has a great impact on its pathogenicity and it is related to most cases of invasive candidiasis (IC) (Sutherland et al., 2004; Blankenship and Mitchell, 2006; Nett and Andes, 2006). IC

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is associated with a high mortality rate, which is estimated to be about 40% in the United States and raging from 30% to 50% in other countries. Besides, treatment costs for IC are very high. (Mermel et al., 2001; Ramage et al., 2005; Blankenship and Mitchell, 2006; Nett and Andes, 2006).

The development of Candida biofilm occurs in three phases over a period of 24–48 h (Hughes et al., 1998; Hanlon et al., 2001; Donlan, 2002). The initial phase begins when single yeast cells that adhere to an abiotic or a biotic surface. In a second phase, cells proliferate and begin to switch to their hyphal form. In the final step Candida cells produce an extracellular polymeric matrix encompassing a dense network of filamentous forms (pseudohyphae and hyphae) and yeast cells. During this late phase, single Candida cells or biofilm fragments can detach from the biofilm itself. It follows that biofilm-infected devices serve as reservoirs of Candida that, once released into the bloodstream, may gain access to distant places and cause deep-seated fungal infection.

Several studies describe the effect of phages against bacterial biofilms showing that they can provide impressive strategies as anti-biofilm agents for their ability to kill bacteria (Duran et al., 2003; Donlan, 2009). Furthermore, many phages induce production of depolymerases that hydrolyze extracellular polymers of biofilm (Hughes et al., 1998). Conversely, there are only few investigations on the interactions between human pathogenic viruses and biofilm and these are mainly focused on water biofilms. Enteroviruses have been detected in biofilms inside water pipelines, stably attached with high resistance to water flow pressure and disinfection. These viruses encompassed in biofilm represent a risk for human health as a source of waterborne infections (Quignon et al., 1997; Storey and Ashbolt, 2003; Skraber et al., 2005; Wingender, 2011; Wingender and Flemming, 2011). Currently, there are no studies on the interplay between viruses and biofilms produced in humans.

By the present *in vitro* study, we examined the interactions between *C. albicans* biofilm and two human pathogenic viruses, namely, Herpes Simplex Virus-type 1 (HSV-1) and Coxsackievirus type B5 (CVB5). We assessed whether free viral particles could be encompassed in Candida biofilm, retaining their infectivity, and possibly be released. We also investigated the influence of biofilm on the virus sensitivity to hypochlorite and to neutralizing antibodies, as well as the ability of Candida biofilm to harbour HSV-1-infected cells.

2. Materials and methods

2.1. *Candida albicans strains*

Two strains of *C. albicans* were used: namely the highly virulent, biofilm producer strain 50vr isolated from a clinical case of invasive fungal infection, highly virulent in an infection model in *Galleria mellonella* (Cirasola et al., 2013) and the agerminative low virulent strain PCa_2 as a planktonic biofilm non-producer strain (Kurtz et al., 1996).

2.2. *Virus strains*

HSV-1 and CVB5 were clinical isolates, identified by monoclonal antibodies (Cermelli, 2002 and Cermelli, 2009). Both strains were laboratory adapted through serial passages (>50) on VERO cells over many years. The virus inocula employed in the experiments consisted of cell-free virus suspensions, obtained from centrifuged lysates of virus-infected VERO cells.

2.3. *Cell lines*

The epithelial cell line Vero, used to propagate and titrate HSV-1 and CVB5, was maintained in Eagle's Minimal Essential Medium (MEM) with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C and 5% CO_2. Additionally, the human T cell lymphoblast line JJHAN was used for experiments on the interaction between biofilm and cells in suspension. This cell line was maintained in RPMI 1640 added with same components as for MEM. Both cell lines were maintained by passages in fresh medium twice a week.

2.4. *Biofilm formation and detachment*

For biofilm production, Candida strain 50vr was grown overnight at 37 °C in Yeast Peptone Dextrose (YPD), harvested, washed with phosphate-buffered saline (PBS), and resuspended to 1×10^6 cells/ml in RPMI-10% FCS; 100 μl were seeded in duplicate in polystyrene, flat-bottom 96-well plates (Euroclone S.p.A., Pero (Mi), Italy) and incubated at 37 °C to allow biofilm formation, according to reported studies (Chandra et al., 2008; Pierce et al., 2008).

For biofilm detachment, 3 different methods were tested and compared.

First, a mechanical treatment: the culture wells were washed 3 times with 100 μl of PBS and then, after adding 50 μl of PBS, were energetically scraped for 2 min.

Second, an enzymatic detachment according to Al-Fattani and Douglas (2006): after 3 washings with 100 μl of PBS, the culture wells were added with 100 μl of proteinase K solution (50 μg/ml in Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5) and the plates incubated for 2 additional hours at 37 °C. Afterwards, proteinase K was blocked by adding 50 μl of phenyl methane sulfonyl fluoride (25 mM) (Sigma–Aldrich Srl – Milan – Italy).

Third, a thermal detachment: the samples were washed 3 times with 100 μl of PBS and then, after adding 50 μl of PBS, the plate was heated at 55 °C for 15 min.

At the end of each detachment method, the rescued materials were transferred into microtubes, centrifuged at 14,000 rpm for 10 min and then the cell-free supernatants were processed for virus titration as detailed below.

2.5. *XTT assay*

The XTT colorimetric tetrazolium assay was used to determine fungal cell viability as an indirect quantification of Candida biofilm grown in culture wells as well as of biofilm remained in the wells after detachment (Kuhn et al., 2003). A commercial kit (AppliChem GmbH, Darmstadt, Germany) was employed following the manufacturer's instructions. Briefly, wells containing Candida biofilm, planktonic pCa2 Candida cells or controls (medium only) were washed 3 times with 200 μl of PBS and then 100 μl of the colorimetric solution were added: this solution contained 1 part of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) and 1 part of menadione 10 mM in acetone. After 2 h incubation in darkness at 37 °C the absorbance of the coloured reduction product was measured by a spectrophotometer (Sunrise, Tecan Group Ltd, Männedorf, Switzerland) at 450 nm. Each experimental group consisted of 6 replicates.

2.6. *Crystal violet assay*

Crystal Violet (CV) staining was used to quantify the biofilm grown in the presence of JJHAN cells (Jin et al., 2003): XXT was not used in this case since it stains all viable cells and does not allow to distinguish biofilm mass from JJHAN cells. Briefly, wells containing Candida biofilm, planktonic pCa2 Candida cells or controls (medium only) were washed 3 times with 200 μl of PBS and then air dried for 5′. After fixation by 100 μl of methanol for 20′,

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samples were stained with 100 μl of 1% CV solution for 5 min. Afterwards, each well was washed 3 times with 200 μl of distilled water and added with 33% acetic acid (100 μl/well). After 10 min, the optical densities (OD) were measured at 540 nm by a microplate reader (Sunrise, Tecan Group Ltd, Männedorf, Switzerland). Each experimental group consisted of 6 replicates.

2.7. Exposure of biofilms to viruses

Twenty-four hours after Candida seeding in culture plates, 100 μl of each virus inoculum were added to biofilm, planktonic and control samples. The final virus concentration in the culture wells was 10^7 PFU/ml for HSV-1 and 10^4 $TCID_{50}$/ml for CVB5. The samples were incubated with the virus *inocula* for 48 h. Then, after washing with PBS and detachment by scraping, as described above, the viral load of the rescued materials was determined by end-point titration on VERO cells. In other sets of experiments performed to assess whether biofilms exposed to viral *inocula* release infectious virus, after incubation of biofilms with the viruses for 48 h, the culture wells were washed three times and instead of harvesting the samples, fresh growth medium was added: after further 48 h incubation, virus in centrifuged supernatants was titrated on VERO cells.

In experiments aimed at evaluating the influence of the virus presence on biofilm formation, the virus *inoculum* was added to Candida cells at the same time of cell seeding into the culture plate: after 48 h the amount of biofilm was quantified by XTT assay.

2.8. Virus titration

To quantify the amount of virus, end-point titration was used. After centrifugation of the rescued materials, 10-fold dilutions of the supernatants in MEM with 5% FCS and 50 mg/ml Amphotericin B were seeded in duplicate onto 24 h-old VERO cell cultures in 96-well plates. After a 3 day incubation at 37 °C, the virus titre of each sample was read as the highest dilution showing the typical viral cytopathic effect. The results, expressed as $TCID_{50}$/ml, were calculated using the Reed and Muench formula (Lennette, 1964).

2.9. Effect of biofilm presence on virus sensitivity to sodium hypochlorite

Biofilms, planktonic samples and controls, exposed to each virus for 48 h as above described, were treated or not with sodium hypochlorite (final dilution 1:400), further incubated for 30′ at 37 °C, washed and then scraped as already detailed. The rescued materials were centrifuged and the viral load of the cell-free supernatants assessed by end-point titration.

2.10. Effect of biofilm presence on virus neutralization by antibodies

Biofilms, planktonic samples and controls, exposed to each virus for 48 h, were treated or not with human polyclonal purified immunoglobulins (IgVena 50 g/l, Kedrion, Pascoli Barga (Lu), Italy) (final dilution 1:10), further incubated for 15′ at 37 °C, washed and then scraped as detailed above. The rescued material was centrifuged and the viral load of the cell-free supernatants assessed by end-point titration.

2.11. Exposure of HSV-1 infected JJHAN cells to Candida biofilm

JJHAN cells were infected with HSV-1 at a multiplicity of infection of 10 PFU/cell and 24 h later were added to Candida biofilms, Candida planktonic samples and controls (5×10^5 infected cells/well). After 3 h and 8 h at 37 °C, wells were washed 3 times with 200 μl PBS; 0.2% Trypan Blue solution was added (50 μl/well) and then scraping performed. The viable cells in the rescued materials were counted on a Bürker chamber. In parallel groups, the plates were frozen-and-thawed after washing and scraping to lyse the infected JJHAN cells. Virus titration was then performed as described above. Moreover, in order to evaluate the influence of the presence of cells on the stability and viability of biofilm, after 8 h incubation of biofilms with HVS1-infected and–uninfected JJHAN cells, the CV assay was carried out to quantify the amount of biofilm.

2.12. Statistical analysis

The data reported in figures are the mean values (± standard deviation) from at least 3 different experiments performed. The results were analyzed by the two-tailed Student's *t* test and were considered significant when $p < 0.05$.

3. Results

3.1. Candida biofilm formation and detachment

XTT assay was used to assess the amount of Candida biofilm produced in 96-well plates after 24 h incubation in culture medium. As shown in Fig. 1, the OD value in the wells containing the biofilm producer Candida strain (50vr), was 1.31, whereas that of planktonic Candida samples was significantly lower (0.12) and similar to the control's value (0.1). Since XTT measures cell viability and is therefore an index of the biofilm mass, these results indicate that, under the employed conditions, only the 50vr Candida strain produces a biofilm.

In order to determine whether virus particles were retained within biofilm, 3 methods for biofilm detachment were compared and their efficiency evaluated by XTT assay quantification of the remains in each well (Fig. 1). The OD values in the cultures with 50vr Candida strain samples after biofilm removal by each method were rather similar (0.14 for scraping, 0.12 for PK digestion and 0.19 for heating): such values were comparable to the background value of the controls ($P > 0.05$), proving that the 3 protocols are able to efficiently remove biofilm.

3.2. The virus presence affects neither Candida biofilm formation nor its maintenance

Fig. 2A shows the results of the XTT assay on Candida biofilm grown in the presence of each of the two viruses under study, added at the same time of fungal seeding. The OD values of biofilms produced in the presence or in the absence of the viruses were similar (1.66 for controls without virus, 1.86 for biofilms + HSV-1 and 1,88 for biofilms + CVB5) suggesting that the presence of the virus does not affect biofilm production by Candida. In panel B of the same figure, the results of similar experiments in which the virus *inoculum* was added to mature biofilms are reported. Again, no significant differences were observed between virus exposed biofilms (1.09 for HSV-1 and 1.26 for CVB5) and controls without virus (1.17). Panel C of Fig. 2 shows the results of experiments in which HSV-1-infected JJHAN cells were added to mature biofilms and after 8 h incubation CV staining was used to determine the amount of biofilm (Fig. 2C): the OD value of biofilms exposed to infected JJHAN cells (1.0) was not significantly different from those of the two types of controls consisting of biofilms with uninfected JJHAN (1.07) and biofilms without JJHAN cells (0.99). These results demonstrate that also cells entrapped in biofilm do not alter its viability and stability.

3.3. Virus particles are entrapped within Candida biofilm

Candida biofilms were exposed to virus inocula for 48 h, then biofilms were thoroughly washed and the biomass was detached

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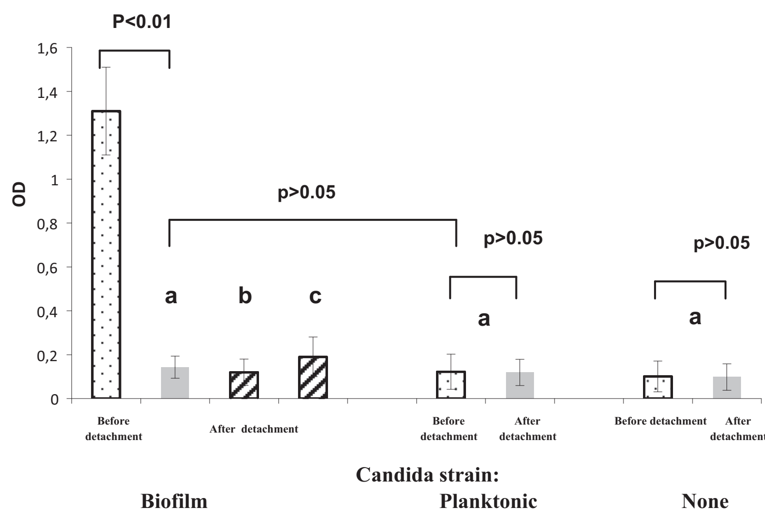

Fig. 1. Evaluation of Candida biofilm formation and detachment.

by scraping. The same treatment was carried out also in culture wells containing planktonic Candida or only medium (controls). The presence of infectious virus in the detached/rescued material was then end-point titrated on VERO cells. Fig. 3 (panel A) shows the titres of CVB5 in samples of Candida biofilm, planktonic Candida and controls, which were $10^{1.9}$ $TCID_{50}$, $10^{0.2}$ $TCID_{50}$ and $10^{0.5}$ TCID50, respectively. As far as HSV-1, the amount of virus was $10^{3.4}$ $TCID_{50}$ for biofilm, $10^{0.2}$ $TCID_{50}$ for planktonic Candida and $10^{0.7}$ $TCID_{50}$ for the control (panel B). These data indicate that both viruses are entrapped within the Candida 50vr biofilm, the low levels of virus detected in the two types of controls representing the amount of virus attached to the plastic of the cell culture plate.

3.4. Virus-exposed biofilms release infectious virus

Biofilms exposed to each of the two viruses under study for 48 h were extensively washed, added with fresh medium and incubated for further 24 h. Virus present in the supernatants was then titrated on VERO cells. Biofilms exposed to CVB5 had a virus titre of $10^{4.4}$, while the relative controls (cultures with planktonic Candida and with medium only) had titres lower to 10^1(Fig. 4A). Similarly, the results obtained with HSV-1 were $10^{5.1}$ for biofilm and titres lower to 10^1 for controls (Fig. 4B).

3.5. Virus encompassment in Candida biofilm does not prevent inactivation by sodium hypochlorite

Biofilm impact on the virucidal activity of disinfectants was evaluated. Thus, after 48 h incubation with virus, Candida biofilms were treated or not with sodium hypochlorite for 30′, then thoroughly washed and detached by scraping. The load of infectious virus embedded in the detached/rescued biofilm was titrated and the results are depicted in Fig. 5. As for CVB5, the virus titres decreased, after hypochlorite treatment, from $10^{1.7}$ $TCID_{50}$ to $10^{0.7}$ $TCID_{50}$ in biofilms, from $10^{0.7}$ $TCID_{50}$ to 0 in the samples of planktonic Candida and from $10^{0.8}$ $TCID_{50}$ to 0 in controls. Similarly, for HSV-1, the virus titres decreased, after hypochlorite treatment, from $10^{2.3}$ $TCID_{50}$ to $10^{0.3}$ $TCID_{50}$ in biofilms, from $10^{0.7}$ $TCID_{50}$ to 0 in the samples of planktonic Candida and from $10^{0.5}$ $TCID_{50}$ to 0 in controls. Although for both viruses the rate of infectivity reduction was lower in the absence of biofilm (3–6 fold reduction) than in the biofilm cultures (10 fold for CVB5 and 200 fold for HSV-1), however in these last samples there was no complete neutralization of virus infectivity and significant, though low, titres of infectious virus were still detected after hypochlorite treatment. These findings demonstrate that biofilm presence significantly reduces the efficacy of hypochlorite treatment which looses its ability to completely inactivate viruses.

3.6. Virus encompassment in Candida biofilm does not prevent neutralization by antibodies

Next, we evaluated whether virus interaction with the biofilm could influence virus neutralization by antibodies. Thus, after 48 h incubation with virus, Candida biofilms were exposed or not to neutralizing antibodies. Afterwards, washing and scraping were performed and the load of infectious virus in the detached/rescued biofilm was determined. CVB5 titre detected in biofilms decreased from $10^{1,7}$ $TCID_{50}$ to $10^{0,7}$ $TCID_{50}$ after treatment with immunoglobulin; similarly, the titres decreased from $10^{0,7}$ $TCID_{50}$ to 0 in the samples of planktonic Candida and from $10^{0,8}$ $TCID_{50}$ to 0 for controls. As far as HSV-1, in the biofilms the titres decreased from 10^3 $TCID_{50}$ to 0, while in planktonic from $10^{0,8}$ $TCID_{50}$ to 0 $TCID_{50}$ and in controls from $10^{0,7}$ $TCID_{50}$ to 0 $TCID_{50}$ (Fig. 6). Overall, these data indicate that viruses embedded in Candida biofilm are still neutralized by antibodies.

3.7. HSV-1 infected JJHAN cells are encompassed by Candida biofilm and release infectious virus

The ability of Candida biofilm to entrap virus-infected lymphoid cells was assessed. Twenty-four hours after infection with HSV-1, 5×10^5 infected JJHAN cells were added to 24 h old Candida biofilms. Following additional 3 and 8 h of incubation, washing and scraping were performed; the amount of viable JJHAN cells in the detached materials was determined by Trypan Blue exclusion test. The panel A of Fig. 7 shows the number of viable cells embedded in the biofilm: 4×10^4 cell and $3{,}7 \times 10^4$ cell, at 3 h and 8 h, respectively. In the culture wells with planktonic Candida, the values were 9×10^2 cells and $3{,}5 \times 10^2$ cells at 3 h and 8 h, respectively, and in the controls $3{,}5 \times 10^2$ cells and 10^2 cells. The dramatic decrease in viable cells in the biofilm cultures between 3 h and 8 h incubation prevented us to prolong the incubation time: with longer incubation (12 h or 24 h) cells were almost totally dead (data not shown).

In parallel cultures, after washing and scraping, the plates were frozen-and-thawed and HSV-1 titrated on Vero cells: the panel B of the Fig. 7 shows these results. Virus titre in biofilm was $10^{5,5}$

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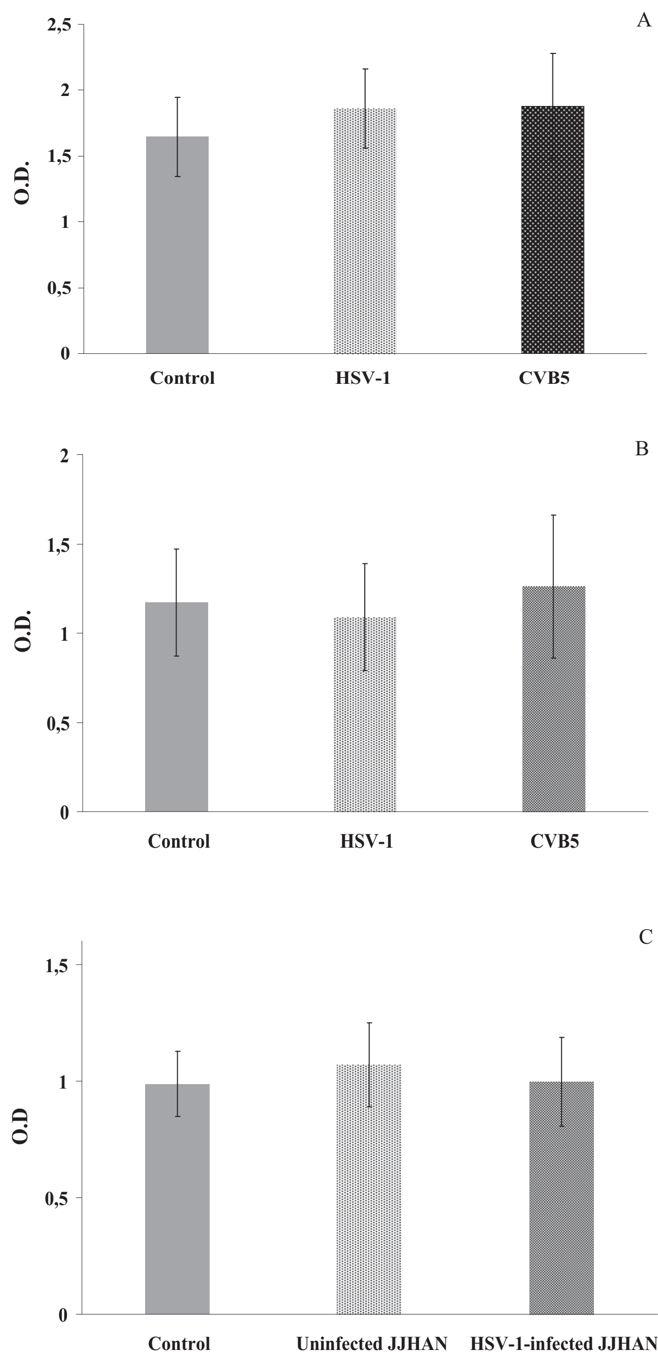

Fig. 2. Virus presence does not alter Candida biofilm.

$TCID_{50}$, in the planktonic samples $10^{3.8}$ and in the controls $10^{3.3}$ $TCID_{50}$. These results show that HSV1-infected cells are embedded in Candida biofilm, where they remain viable and release virus.

4. Discussion

The aim of the present work was to assess whether *C. albicans* biofilm may mediate encompassment, retainment and dissemination of pathogenic viruses. The importance of biofilms on medical devices as a source of infection has been highly regarded. Candida biofilm is responsible for a significantly increasing number of device-related infections causing severe invasive candidemia, often lethal (Kojic and Darouiche, 2004). The interaction between eukaryotic viruses and biofilm has been poorly investigated so far and most

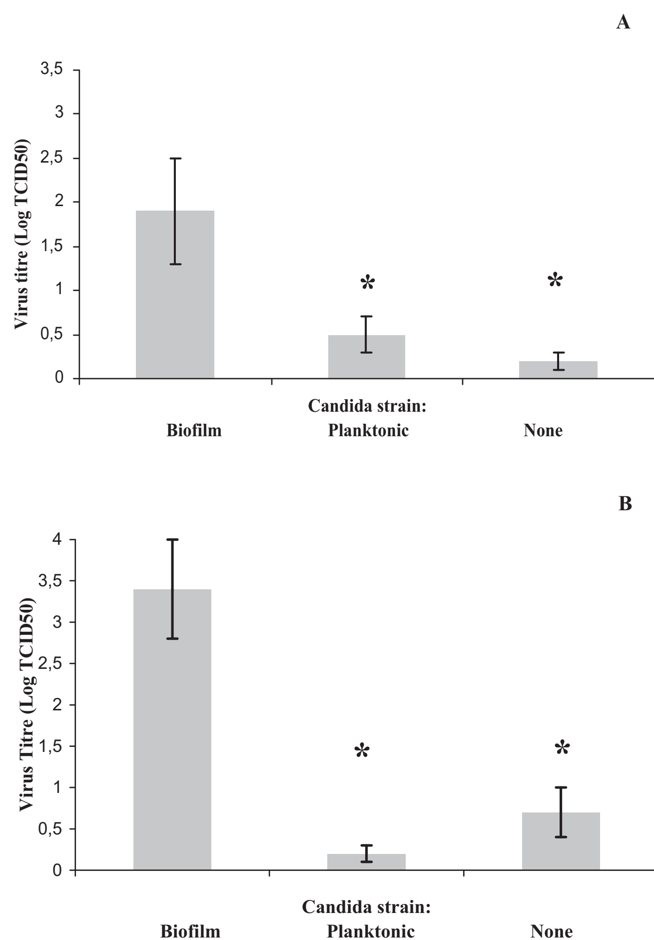

Fig. 3. Viral load in Candida biofilm.

studies are focused on aquatic biofilms inside pipelines. Although viruses are not involved in biofilm initial building, they might be involved in its later evolution or be influenced in their own biology. Several mechanisms likely lead to virus inclusion in biofilms on both natural and artificial surfaces: link with extracellular matrix polymers, adhesion to host molecules responsible for the conditioning of abiotic surfaces, encompassment of virus-infected cells inside the biofilm. Biofilm matrix, having variable surfaces with many sorption sites, facilitate binding and accumulation of colloidal matters (Flemming, 1995; Wimpenny, 2000; Menaia et al., 2003; Wingender, 2011). Those viruses that have a particle size ranging from 20 to 120 nm are considered as bio-colloids and, therefore, can be incorporated and retained into biofilm (Leclerc et al., 2002). On the other hand, some viruses are present in biological fluids associated with particular lipids or proteins which may enhance their capability to interact with biofim (Lindenbach et al., 2006; Munch et al., 2007).

For our study, we selected an enveloped and a non enveloped virus, HSV-1 and CVB5, respectively, which are widespread and important human pathogens. Although no literature data refer of an augmented infection risk for these viruses in patients with biofilms, we chose them as an *in vitro* model of easy growing viruses that can cause viremic infections with bloodstream dissemination, thus possibly interacting with biofilms potentially present in the host.

As a preliminary step, we assessed the biofilm formation *in vitro*, quantifying the biofilm mass by XXT assay and/or CV staining (Stepanovic et al., 2000; Li et al., 2003; Jin et al., 2003; Kuhn et al., 2003; Peeters et al., 2008). We found that biofilm growth efficiently occurs in tissue culture plates (Fig. 1) and that the presence of virus

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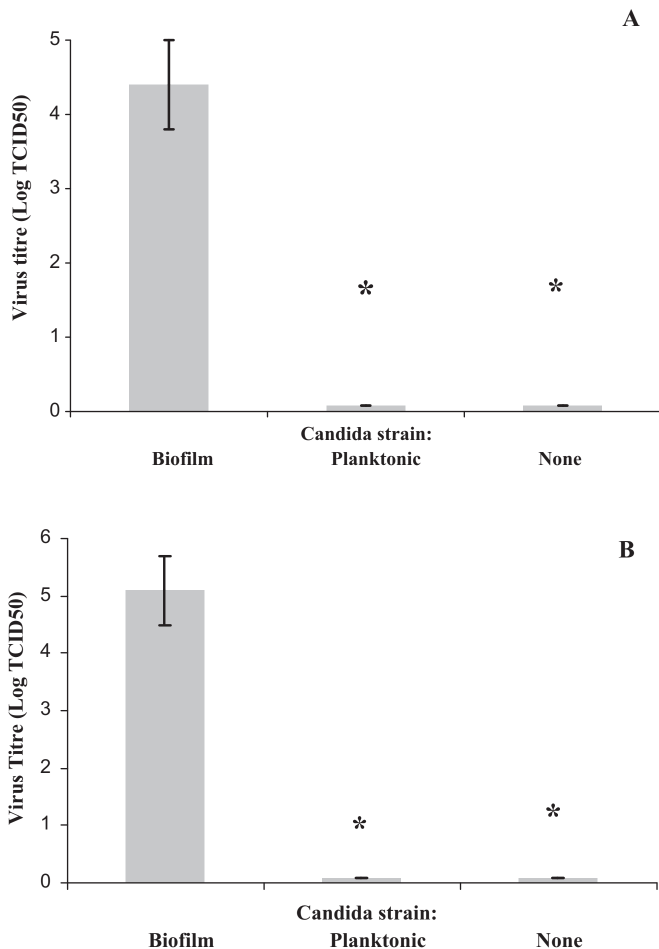

Fig. 4. Viral load in Candida biofilm.

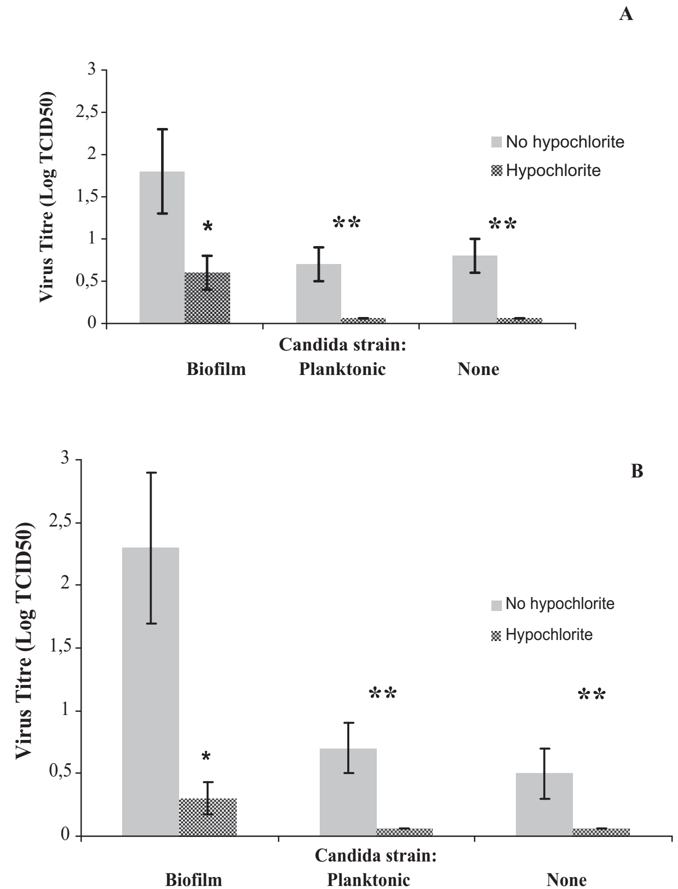

Fig. 5. Virus inactivation by sodium hypochlorite is impaired by Candida biofilm.

impairs neither biofilm formation nor its viability and stability after maturation (Fig. 2A and B). Notoriously, biofilms tightly attach to surfaces so that their complete detachment is difficult. We compared 3 different detachment procedures, namely scraping, proteinase K treatment, and heating at 55 °C and we assessed their efficacy in detaching fungal cells by XTT assay staining of the remains in the culture wells, after treatment. In all cases, comparable results were observed, namely the OD values consistently resembled the background values of negative controls, implying that the 3 methods efficiently remove biofilm matrix. Hereafter, we used mechanical detachment by scraping, because it is faster than heating and more reproducible than enzymatic treatment (data not shown).

Here we show that when 24 h biofilms of *C. albicans* were exposed to HSV-1 or CVB5 as cell-free *inocula* for additional 48 h, and then mechanically detached, the virus amounts detected in the rescued material are significantly higher for both viruses compared with non-biofilm counterparts (planktonic Candida and negative controls with only medium). Interestingly, the same phenomenon is observed in experiments with a 6 day long incubation (data not shown), further emphasizing that, inside biofilm, virus particles are protected from degradation and retain their infectivity. We also demonstrated that virus-exposed biofilms can release virus: in fact, high virus titres were detected in the supernatants from virus–exposed biofilms re-cultured in fresh medium for 24 h after extensive washes. Although in this study we did not performed any experiments to localize virus in the biofilm, we can hypothesize that it is deeply dispersed inside the matrix as suggested by the persistence of virus after washes, scraping and centrifugation. Preliminary results of a study by immunofluorescence assay aimed to detect where virus is located, show a diffuse amount of viral antigens inside the biofilm hyphal architecture. Our finding on the ability of Candida biofilm to encompass and retain CVB5 is in agreement with previous studies showing that enteric viruses have the potential to accumulate within water biofilms, likely penetrating into micropores or channels (Storey and Ashbolt, 2001). It has been proposed that aquatic biofilm provides embedded viruses a sort of shelter protecting them from disinfections (Quignon et al., 1997). According to these observations, we evaluated whether the same phenomenon might occur also with human viruses embedded in Candida biofilm. Unexpectedly, in our experimental model, the treatment of the virus-containing biofilms with sodium hypochlorite significantly impairs virus infectivity without completely abolishing it, implying that biofilm protection of such viruses from inactivation, is only partial. As predictable, CVB5 resulted less sensitive to the treatment (1 Log reduction) than HSV-1 (2 Logs reduction). The discrepancy in comparison with the results reported by Quignon (1999) might be due to the different composition of the biofilm models employed: unlike ours, the water environment-derived biofilms are polimicrobic and, therefore, have a different, and likely more complex, composition of the extracellular polymeric matrix. Possibly, also biofilm age, known to influence stiffness, thickness and volume of the matrix, might impact on chlorine inactivation effects. In water pipelines, biofilms persist for a long time, while, in our model, viruses were exposed to 24 h old Candida biofilms. A similar set of data was observed when assessing the efficacy of neutralizing antibodies on viruses embedded in biofilm. One possible reason hypothesized to explain immune escape of microorganisms grown as biofilm is that biofilm matrix can interfere with antigen recognition/linking. In our hands,

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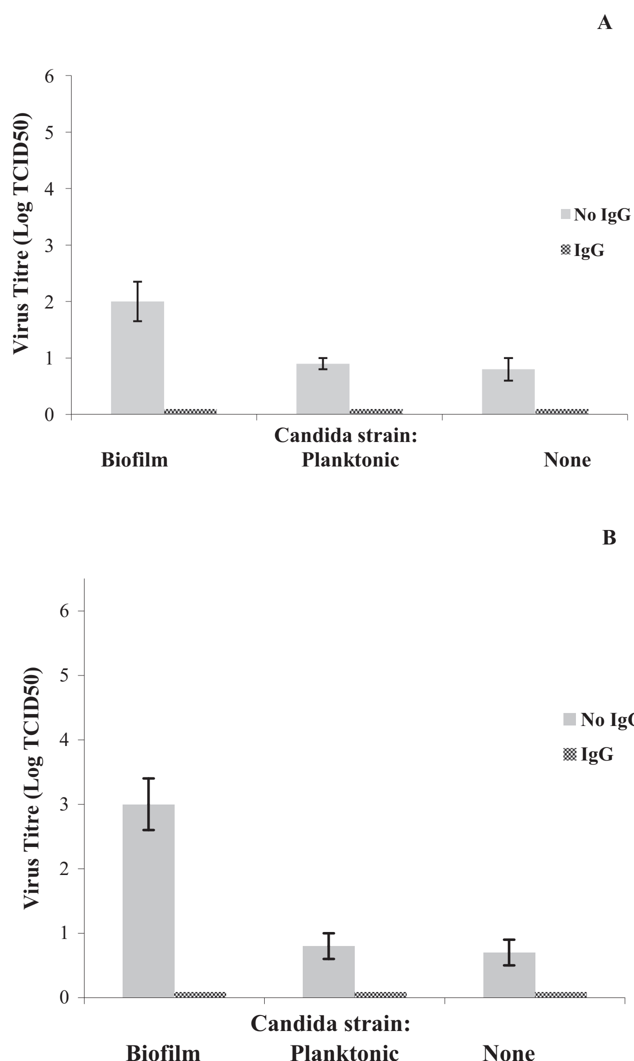

Fig. 6. Virus neutralization by antibodies is not affected by Candida biofilm.

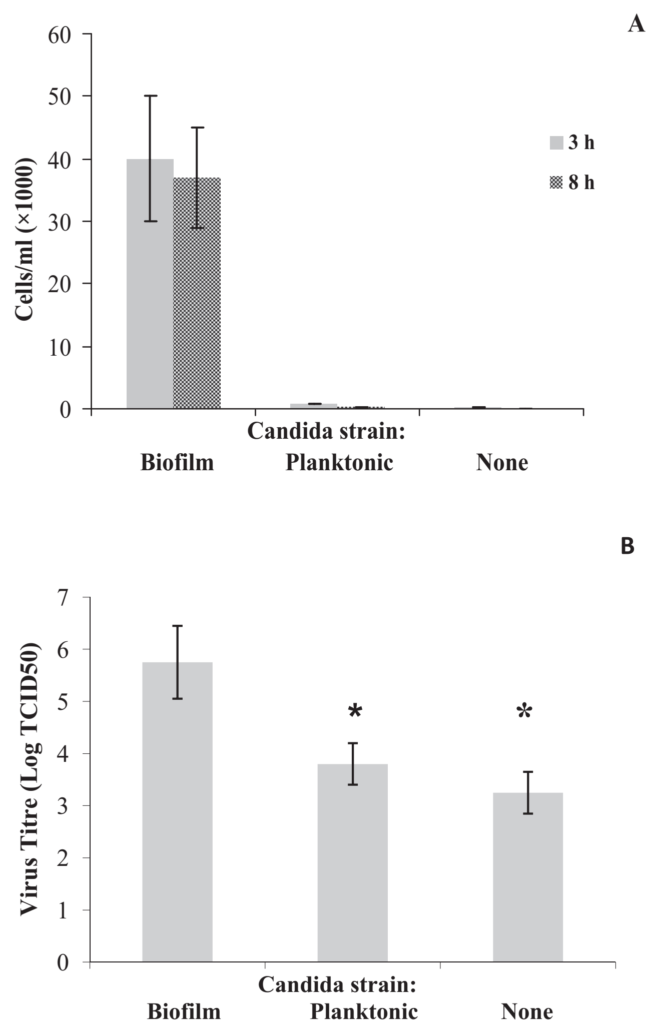

Fig. 7. Interactions between Candida biofilm and HSV-1-infected cells.

both viruses were completely neutralized, irrespective of the presence or the absence of biofilm. We can hypothesize that a 24 h-old Candida biofilm is leaky and lax enough to allow antibody penetration inside its structure; thus, binding to virus particles embedded in the biofilm efficiently occurs, as already demonstrated with other types of biofilms (Zhu et al., 2001). Moreover, it should be considered that full penetration of antibodies inside the biofilm may largely depend on the amount of IgG and on the incubation time.

Not only *C. albicans* biofilm is capable of retaining viral particles, but it also exhibits the capability to encompass HSV-1 infected T cells (Fig. 7A) which do not impact biofilm maintenance (Fig. 2). Infected cells entrapped in the biofilm, in turn, release infectious virus (Fig. 7B) at a titre 1.7 Log higher than infected cells exposed to the planktonic candida counterpart. This finding was not unexpected, since it has been previously demonstrated that, in T cells, retroviruses can spread cell-to-cell by inducing either cell-cell nanotubes or specialized cell-cell contact areas (the so called virological synapses), where virions and receptors are concentrated (Jolly and Sattentau, 2004; Nejmeddine et al., 2005). Accordingly, enveloped virus-infected cells display a high tendency to clump. Moreover, recent findings suggest that HTLV-1 can form carbohydrate-rich adhesive extracellular viral assemblies for virus cell-cell transferring (Pais-Correia et al., 2010). Such extracellular assemblies consist of clusters of viral particles embedded in a matrix of highly glycosylated proteins, produced by the infected T cell upon virus induction: a sort of viral biofilm aimed to ensure virus protection, persistence in a specific environment and spread. Intriguingly, some components of bacterial biofilm were also found in such viral assemblies (Thoulouz and Alcover, 2011). Although this phenomenon has been demonstrated only for HTLV-1 so far, it is likely that a similar mechanism can be used also by other enveloped viruses, like Herpesviruses, known to spread mainly by cell-cell contact. Therefore, we may speculate that HSV-1-infected JJHAN cells, forming this sort of "viral biofilm" are easily encompassed in Candida biofilm due to adhesive interactions between these two types of extracellular matrix sharing a similar composition.

5. Conclusions

The present work is the first report investigating the interactions between *C. albicans* biofilm and human pathogenic viruses. We demonstrated that, *in vitro*, *C. albicans* biofilm allows: 1) encompassment and release of infectious CVB5 and HSV-1 with partial protection from hypochlorite inactivation but not from antibody neutralization; 2) entrapment of HSV-1 infected cells, which continue to release infectious virus without damaging the biofilm. On the whole, with all the limits of an *in vitro* study, these results suggest that, *in vivo*, *C. albicans* biofilm may represent also a reservoir of infectious virus. Circulating virus particles during viremic infections and/or circulating infected cells might be retained in the

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biofilm and later on released, representing a further risk especially for immunocompromised patients.

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PROGRAMME and ABSTRACT BOOK

047

IDENTIFICATION OF RABBIT HAEMMORRAGIC DISEASE VIRUS "Fra2010" AS CAUSE OF AN EUROPEAN BROWN HARE SYNDROME - LIKE DISEASE IN THE SARDINIAN HARE: IS "Fra2010" A NEW MEMBER OF THE LAGOVIRUS GENUS?

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Background: Rabbit Hemorrhagic Disease (RHD) is a highly contagious, severe acute hepatitis that specifically afflicts rabbits (*Oryctolagus cuniculus*). Since its emergence in 1984, RHD has resulted in the deaths of nearly a quarter billion rabbits. The etiological agent of RHD is a virus member of the family *Caliciviridae*. European brown hare syndrome virus (EBHSV) causes an RHD-like disease in brown hares (*Lepus europaeus*) and, together RHDV, is classified in the *Lagovirus* genus. RHDV "Fra2010" is a new consistent genetic variant of RHDV detected on mid 2010 in France and characterized by an apparent reduced pathogenicity.
Methods: anatomopathological observations of dead animals, ELISA test for diagnosis and subtyping and gene amplification (PCR) and sequencing.
Results: starting from October 2011, in Sardinia, an contemporaneous increasing in RHD incidence in rabbits and in EBHSV-like disease in Sardinian hares (*Lepus capensis*) was noted. Six outbreaks in rabbits and 7 in hares were registered. Histopatological examinations of the livers showed massive necrosis with moderate mononuclear inflammatory infiltrate and little fatty degeneration. First ELISA RHDV positive rabbit samples were confirmed as positive for RHDVFra2010 using a specific panel of MAbs and phylogenetic analysis. Similarly, all hare samples were positive for RHDVFra2010.
Conclusion: RHD is endemic in Sardinia since at least 2000 but RHDV has been never identified in autochthonous hares. The finding that RHDVFra10 causes an EBHSV-like disease in Sardinian hares, in addition to RHD in rabbits, is a further indication that RHDVFra2010 could be a new viral entity and not a simply RHDV variant.

048

INVESTIGATION OF THE MECHANISMS ADOPTED BY THE FELINE IMMUNODEFICIENCY VIRUS ENVELOPE GLYCOPROTEIN IN OVERCOMING RESTRICTION BY FELINE TETHERIN

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Feline Immunodeficiency Virus (FIV), a non-primate lentivirus, is widespread in feline populations and it causes an AIDS-like illness in domestic cats similar to the one induced in humans by human immunodeficiency virus type-1 (HIV-1) infection. FIV resembles HIV-1 in many molecular and biochemical properties, thus representing an attractive model for AIDS research. In this context, understanding the interplay between viral and host factors plays a crucial role for elucidating the molecular mechanisms involved in virus pathogenicity and for the development of effective therapeutic and vaccine approaches. Recently it has been reported that Tetherin (BST2) is the host cell factor that blocks the particle release of some enveloped viruses. In this context, we identified the feline orthologue of the human protein BST2/tetherin, renamed cBST2 or feline tetherin, and showed that it impairs HIV-1 and other lentiviruses particle release. Moreover, we demonstrated that the FIV envelope glycoprotein represents a tetherin antagonist. Starting from this findings, we set out to examine the molecular mechanisms at the basis of FIV ability to overcome cBST2. Our results might contributed to determine the relevance of tetherin antagonism in the viral pathogenesis.

049

IN VITRO INTERACTIONS BETWEEN VIRUSES AND CANDIDA BIOFILM

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BACKGROUND *Candida albicans* is known as one of the major cause of infections related to biofilms forming on medical devices such as catheters, artifical vales, prostheses which become a source invasive candidiasis with a high mortality rates (30-50%). So far, only few studies investigated the interactions between human pathogenic viruses and biofilms, mainly focused on water biofilms. To our knowledge, there are no studies on the interplay between biofilms in humans and viruses. In this study, we studied whether Herpes Simplex Virus type 1 (HSV-1) and Coxsackievirus type B5 (CoxB5) can be encompassed in Candida

biofilm, retaining their infectivity, and then be released. Moreover, we investigated the ability of Candida biofilm to hold non adhering HSV-1 infected cells within the matrix.
METHODS *Candida albicans* biofilms were grown in tissue culture microplates and then exposed to HSV-1 or CoxB5 for 48h: after deep washing and energetic scapring of the wells to remove the matrix, the residual presence of virus was end-point titrated on VERO cells. In parallel, wells with a strain of non-biofilm producer *Candida albicans* (planktonic) and negative controls with only medium were processed at the same way. Alternatively, Candida biofilms were exposed to non adhering HSV-1-infected cells and then, after washing and scraping, the number of living cells attached to the biofilms and virus titer were determined.
RESULTS AND DISCUSSION Both free virus particles of HSV-1 and CoxB5 and HSV-1 infected cells remained embedded in the biofilmf with a significantly higher load than in the presence of planktonic Candida or in the negative controls. Candida biofilm can be a reservoir for viruses.

050

UPREGULATION OF MSRV/ HERV-W IN PATIENTS AFFECTED BY INFECTIOUS MONONUCLEOSIS

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Background: Eight per cent of human DNA consists of retroelements, remnants of ancestral infections by exogenous retroviruses. The founder member of the HERV-W family of human endogenous retroviruses is MSRV (Multiple Sclerosis Associated Retrovirus). It has been identified in multiple sclerosis (MS) patients, and it is released by cells of these patients, as virus-like particles. Another HERV-W element is ERVW-1, able to produce only the Syncytin-1 env protein. MSRVenv and Syncytin-1 proteins have neuro-pathogenic and immuno-pathogetic properties, as demonstrated *in vitro* and in humanized or transgenic animal models. We have shown recently that EBV is able to transactivate HERV-W/MSRV/Syncytin-1 in cultured cells (Ref PLOSone in press). Therefore, we planned to study whether the infection with EBV could activate HERV-W/MSRV/Syncytin-1 also *in vivo*, during the symptomatic phase of a delayed primary EBV infection, i.e. during the course of infectious mononucleosis (IM).
Methodology: 30 consecutive IM patients hospitalysed in the Infectious Disease Section of the *Department of Experimental Medicine*, Univ. of Sassari, and 10 EBV-negative healthy controls were enrolled. Peripheral blood mononuclear cells (PBMCs) were purified and processed for polyA+RNA extraction, and retro-transcription. The MSRVenv and Syncytin-1 transcripts were detected by discriminatory real time RT-PCR assays. Routine clinical and serological parameters were collected and all data recorded in a database.
Results: The data show that in patients with acute EBV infection (antiVCA IgM serum titers >1:160) there is higher expression of MSRVenv and Syncytin-1 transcripts, with respect to EBV-negative healthy individuals. Notably, IM patients aging >21years show higher MSRVenv and Syncytin-1 expression than IM patients aging <21years.
Conclusions: The activation of HERV-W retroelements during EBV infection occurs also *in vivo*, and it is higher with age of patients. This is in line with our hypothesis of interactions among the two proposed MS-cofactors, HERV-W and EBV, and that this interaction could contribute to the development of MS, years later, possibly in the presence of a predisposing genetic background.

(Ref PLOSone in press) Expression and Activation by Epstein Barr Virus >of Human Endogenous Retroviruses-W in Blood >Cells and Astrocytes: Inference for Multiple Sclerosis .Mameli et al..........A Dolei.2012
Supported by grants awarded by FISM to Prof.ss. Antonina Dolei n° 2010/R/16

051

INHIBITION OF HUMAN ENDOGENOUS RETROVIRUS TYPE W IN BLOOD DURING NATALIZUMAB THERAPY.

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Background: Multiple sclerosis (MS) is an immune-mediated complex disease of the central nervous system (CNS), triggered by environmental/infectious factors operating in a predisposing genetic background. The

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PROGRAMME AT A GLANCE

Wednesday 11th of September

- 10h30–13h: Registration
- 13h–13h30: Opening - Welcome session
- 13h30–15h45: **Plenary 1 – FROM BASIC VIROLOGY TO THERAPY** – Paul DUPREX (USA) - ESCV Gardner Lecture; Johan NEYTS (Belgium); Esteban DOMINGO (Spain); Marina CAVAZZANA-CALVO (France) – p. S37
- 15h45–16h15: Coffee break
- 16h15–18h15: WS 8 Antiviral therapy and resistance to acute infection (p. S41); WS 3 Innate immunity against viruses (p. S39); WS 24 Typing of viruses (p. S43); WS 27 Neurotropic viruses (p. S45)
- 18h15–22h: Get together - Cheese and Wine party; Poster

Thursday 12th of September

- 8h30–10h30: WS 25 Respiratory viral infections (p. S54); WS 17 Vector borne viral infections (p. S51); WS 5 Viral (immuno) pathogenesis (p. S47); WS 12 Viral gene expression (p. S49)
- 10h30–11h: Coffee break
- 11h–13h15: **Plenary 2 – INTERACTION OF VIRUSES WITH PATHOGENS OR ENDOSYMBIONTS** – Julie PFEIFFER (USA); Marilyn ROOSSINCK (USA); Nicolas FASEL (Switzerland); Steven SINKINS (UK) – p. S56
- 13h30–14h45: Lunch; Symposium BioMérieux (p. S58)
- 15h–17h15: WS 23 Viral diagnosis in chronic infections and during pregnancy (p. S67); WS 18 Emerging diseases in veterinary virology (p. S64); WS 4 Adaptive immunity and vaccines (p. S59); WS 14 Virus structure, dynamic imaging and trafficking (p. S62)
- 17h30–18h: Jan Felix DREXLER (Germany) – Heine Medin AWARD
- 18h–20h: General Assembly ESCV; Poster - Cheese and Wine party
- 22h–21h: Lyon City Hall reception

Friday 13th of September

- 8h30–10h30: WS 15 Highly pathogenic viruses (p. S75); WS 10 Virus entry: envelope glycoproteins, receptors, endocytosis (p. S72); WS 6 Restriction factors of viral infection, interfering RNA & immune response (p. S70); WS 28 Gastro-intestinal viral infections (p. S77)
- 10h30–11h: Coffee break
- 11h–13h15: **Plenary 3 – HOST RESTRICTION AND BARRIERS TO VIRAL INFECTION** – Soren PALUDAN (Denmark); Maria-Carla SALEH (France); Frank KIRCHHOFF (Germany); Ari HELENIUS (Switzerland) - ESV AWARD – p. S80
- 13h30–14h45: Lunch; Symposium Qiagen (p. S81); Symposium Sanofi Pasteur (p. S82)
- 15h–17h15: WS 9 Antiviral therapy and resistance to chronic infection (p. S86); WS 13 Viral replication strategies (p. S89); WS 7 Cellular factors controlling viral infection and role of host genetics (p. S83); WS 16 Zoonotic viruses (p. S92)
- 17h30–18h: Michaela GACK (USA) – ESV Junior AWARD
- 18h–20h: General Assembly ESV; Poster and Wine party
- 22h–00h: Gala dinner - Lyon Convention Centre

Saturday 14th of September

- 8h30–10h30: WS 26 Viral infections in transplant and immuno-compromised patients (p. S101); WS 19 Viral evolution and quasispecies (p. S99); WS 11 Virus exit: assembly, maturation and release (p. S97); WS 2 Oncolytic viruses and gene therapy (p. S95)
- 10h30–11h: Coffee break
- 11h–13h: **Plenary 4 – THE UNIVERSE OF VIRUSES** – Dennis BAMFORD (Finland); Christian DROSTEN (Germany); Richard ELLIOTT (UK); Ron FOUCHIER (Netherland) – p. S104
- 13h15–14h30: Lunch; Symposium Inst. Pasteur Fond.Mérieux Networks; Symposium Finovi Rhône-Alpes Viral hepatitis
- 14h45–16h45: WS 22 Innovative methods in viral diagnosis (p. S115); WS 20 Virus epidemiology (p. S111); WS 21 Virus discovery and metagenomics (p. S113); WS 1 Oncogenic mechanisms of viruses (p. S109)
- 16h45–17h: Final words - Next meeting presentation

Locations: Amphitheater; Room Gratte-ciel 1,2,3; Room Prestige Gratte-Ciel; Room Tête d'Or; Forum 5&6 - Poster Area

10. VIRUS ENTRY: ENVELOPE GLYCOPROTEINS, RECEPTORS, ENDOCYTOSIS

Posters: REF 147 to REF 168

REF 147

Candida albicans biofilm can retain and release Human Herpes Simplex Virus type 1 in vitro

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Microorganisms universally attach to surfaces and produce extracellular polymeric saccharides (EPS), resulting in the formation of a biofilm. Biofilms can pose a serious problem for public health because of the increased resistance of biofilm associated organisms to antimicrobial agents and the potential for these organisms to cause infections in patients with indwelling medical devices. Moreover, involvement of enteric viruses with a variety of biofilms has been reported, although very little is known about this phenomenon. The presence of some pathogenic viruses in water biofilms underlines the ability of viruses to attach and cling to biofilms retaining their infectivity. No information is available so far on interactions between pathogenic viruses and Candida albicans biofilm. This biofilm is responsible for severe device related disseminated infections causing invasive candidemias with a very high rate of mortality. The aim of this in vitro study was to ascertain whether Herpes Simplex Virus type 1 (HSV 1) can be encompassed in Candida biofilm produced in cell culture plates and/or on silicone and PVC catheters. HSV 1 was added to mature biofilms and the amount of infectious virus embedded in biofilm matrix detached by washing and energetic scratching was titrated on VERO cells 24 48 h later. Experiments with planktonic Candida were carried out in parallel, as well as in the absence of Candida. According to our results, free virus particles of HSV 1, as well as HSV 1 infected cells, remain embedded in Candida biofilm on tissue cell culture plates as well as on both types of catheter with a significantly higher load than in the presence of planktonic Candida or in the negative controls. These results provide the first evidence that infectious viruses, after being entrapped in Candida biofilms, can retain their infectivity and be released posing a health risk for patients with implanted medical devices. Interactions between HSV 1 embedded in Candida biofilm and disinfectants as well as neutralizing antibodies and drugs are discussed.

Key words: pathogenic viruses, Candida albicans biofilm, Extracellular matrix, infectious reservoir

REF 148

Function of Andes Hantavirus Gn and Gc glycoproteins in viral self assembly and entry into the cell

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Current efforts to understand the basic processes of the replicative cycle of hantaviruses have been in part hampered by the lack of a reverse genetic system andthe biosafety requirements. Recombinant Hantaan virus like particles (VLPs) have been previously described being prepared by co expression of the viral Gn/Gc glycoproteins and the nucleoprotein. Our current research is focused on understanding the participation of viral proteins in the formation of Andes virus (ANDV) and other hantavirus particles and on the hantavirus cell entry mechanism. ANDV VLPs were obtained from purified supernatants of transfected cells and characterized by Western blots, electron microscopy and dynamic light scattering. The results show that ANDV Gn and Gc are sufficient to self assemble into VLPs without the need of other viral components. Further, we investigated the function of Gn and Gc during cell entry. Specifically, we discuss results related with cell binding, glycoprotein activation and multimerization, membrane interaction and fusion.

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REF 149

Thermodynamics Tune the Paramyxovirus Membrane Fusion Machinery

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The morbillivirus cell entry machinery consists of a fusion (F) trimer that drastically refolds to mediate membrane fusion following receptor induced conformational changes in its binding partner, the tetrameric attachment (H) protein. To investigate the molecular determinants that control F refolding, we initially generated F chimera between measles virus (MeV) and canine distemper virus (CDV) and identified a central pocket within the morbillivirus F's globular head domain that regulates the intrinsic thermal energy of the metastable, prefusion state. Most mutants of this "pocket" were de stabilized, a phenotype, which, depending on the mutant and triggering system (receptor type and H's origin) resulted in membrane fusion activation or inhibition. Strikingly, under specific triggering conditions, some F mutants exhibited resistance to a broadly active morbillivirus cell entry inhibitor; a molecule known to enhance thermostability of prefusion F complexes. By exploring the intrinsic thermal energy of each F mutants, in the presence and absence of the antiviral compound, we found that de stabilized F trimers were able to adapt to the fusion inhibitor as a result of low inherent thermal stabilities that could compensate for the prefusion state's stabilizing effect exerted by 3 g. Finally, our data additionally revealed that the nature of the F triggering system was directly impacting the ability of F trimers to resist, or not, to the antiviral molecules. In summary, our results not only demonstrate how thermodynamics contribute to antiviral drug adaptation but shed light on how the triggering of the morbillivirus membrane fusion machinery is finely regulated.

REF 150

Biophysical and structural characterization of Bunyavirus membrane fusion

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Bunyaviruses are enveloped, mostly arthropod borne viruses that can cause infections in vertebrate hosts, including humans. Symptoms may