Video Article

Determination of Biofilm Initiation on Virus-infected Cells by Bacteria and Fungi

Balbina J. Plotkin¹, Ira M. Sigar¹, Vaibhav Tiwari¹, Scott Halkyard¹

¹Department of Microbiology and Immunology, Midwestern University

Correspondence to: Balbina J. Plotkin at BLOTK@midwestern.edu

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Abstract

The study of polymicrobial interactions across the taxonomic kingdoms that include fungi, bacteria and virus have not been previously examined with respect to how viral members of the microbiome affect subsequent microbe interactions with these virus-infected host cells. The co-habitation of virus with bacteria and fungi is principally present on the mucosal surfaces of the oral cavity and genital tract. Mucosal cells, particularly those with persistent chronic or persistent latent viral infections, could have a significant impact on members of the microbiome through virus alteration in number and type of receptors expressed. Modification in host cell membrane architecture would result in altered ability of subsequent members of the normal flora and opportunistic pathogens to initiate the first step in biofilm formation, *i.e.*, adherence. This study describes a method for quantitation and visual examination of HSV's effect on the initiation of biofilm formation (adherence) of *S. aureus* and *C. albicans*.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54162/>

Introduction

The human microbiome includes diverse organisms from multiple taxonomic kingdoms that share geographic regions in the body. Adherence to cell surfaces is an essential first step in biofilm formation, which is part of the microbiome colonization process. Included in the microbiome can be viruses that cause chronic and persistent infections. The chronic cell infection by these viruses can cause an alteration in putative receptor availability.^{1,2} In addition, cell entry by intracellular pathogens could also affect host membrane fluidity/hydrophobicity which in turn may alter attachment of other microbiome members, including bacteria and fungi. In order to understand the interactions that can occur between these multiple pathogens that co-localize in the same geographic regions of the human host, we must be able to study the interaction of pathogens that represent the spectrum of taxonomic kingdoms present at the mucosal surface.

The Herpesviridae are a family of microbes present in 100% of humans as permanent members of the microbiome^{3,4}. In addition they can also be persistently shed both in the presence and absence of symptoms. Specifically, herpes simplex virus-1 and herpes simplex virus-2 (HSV-1 and HSV-2, respectively) are permanent members of the microbiome in the oronasopharynx and genital tract. In immune-competent individuals, both HSV-1 and HSV-2 cause gingivostomatitis, as well as genital herpes⁵⁻⁸. At these sites, HSV causes a latent infection characterized by chronic persistent asymptomatic viral shedding⁹. Entry of HSV into cells results in alterations in surface expression of nectins, heparan sulfate, lipid rafts and herpesvirus entry mediator/tumor necrosis factor receptor (HVEM/TNFR)¹⁰⁻²⁵. These potentially represent shared receptors for some bacteria and fungi, *e.g.* *S. aureus* and *C. albicans*, which while opportunistic pathogens, can also reside as members of the mucosal microbiome of the oronasopharynx^{26,27}. Within the oronasopharynx *S. aureus* and *C. albicans* occupy two distinct sites of colonization. In hosts with natural teeth, the oral mucosa is shared by HSV-1 and *C. albicans*, while the anterior nasal nares are occupied by *S. aureus*²⁸. However, despite *in vitro* findings that *S. aureus* adheres to mouth epithelial cells,^{29,30} *S. aureus* is infrequently isolated from oral specimens when normal tissue is present^{29,30}. Little is known concerning genital tract co-colonization niches beyond the clinical findings that *S. aureus* is associated with aerobic vaginitis, characterized by genital inflammation, discharge and dyspareunia, while *C. albicans* produces mucosal lesions similar to that observed in the oral cavity³¹⁻³⁵. Thus, although these members of the oral and genital microbiome cross taxonomic kingdoms little is known concerning their interaction as it impacts their ability to initiate biofilm formation through adherence to the host cell surface⁵. This protocol has been effectively applied to determine the functional consequences of co-colonization/infection.

Protocol

1. HSV Strains and Handling

Note: Recombinant non-spreading HSV-1(KOS) gL86 and HSV-2 (KOS) 333gJ¹ with beta-galactosidase reporter activity used were provided by V. Twiani^{36,37}.

1. Use virus from a single lot and store at -80 °C at a 1:1 ratio of Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS) and skim milk until use. Before viral lot storage, determine virus concentration by *o*-nitrophenyl- β -D-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) assay.
2. Determine virus viability and multiplicity of infection (MOI) by X-Gal staining for each experimental assay run using reporter virus entry assay, as previously described (**Figure 1**)¹⁴.
3. Dilute virus (Opt-MEM) to desired MOI. Fix monolayers (paraformaldehyde; 0.5 ml/well) before staining. Place virus viability controls in a separate microtiter plate in parallel with polymicrobial assay plates.

2. HeLa 299 Cell Handling

1. Grow at 37 °C, 5% CO₂ in DMEM with 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (FBS), gentamicin (50 μ g/ml) and L-glutamine. Passage cells at 80% confluence with Trypsin solution (ethylenediaminetetraacetic acid, 0.53 M EDTA; 0.05% Trypsin; 5 ml/flask).

3. *C. albicans* Handling

Note: *C. albicans* obtained from a clinical laboratory source is stored at -80 °C in Remmel skim milk 2x medium.

1. Culture frozen stock onto Sabouraud Dextrose Medium (37 °C). After 24 hr subculture the *C. albicans* onto Fungisel medium (37 °C; 48 hr) for use.
2. Generate germ tube (GT) forms (pick representative colonies; 3 ml FBS; 3 hr; 37 °C; Abs₆₀₀, 0.3). After incubation, wash GT (HBSS; 2x; 4,000 x g). Add washed GT to warmed HBSS (37 °C; 0.32 Abs₆₀₀). GT forms should be 99% of cells observed as determined by hemocytometer count.
3. Make yeast form (YF) stock suspensions by picking representative Fungisel colonies (HBSS, 3 ml; 0.32 Abs₆₀₀). Count the number of YF forms/ml microscopically using a hemocytometer. YF forms should be 99% of cells observed as determined by hemocytometer count.
4. Make working fungal stock (250 μ l of GT or YF stock in 25 ml HBSS; 37 °C; 10⁵ CFU/ml)

4. *S. aureus* Handling

1. Store *S. aureus* ATCC 25923 (-80 °C; Remmel skim milk 2x). Culture onto sheep blood agar (5%; 37 °C; 24 hr). Pick representative colonies and transfer to mannitol salts medium within 2 days for stock (37 °C; 18 hr).
2. Make *S. aureus* stock suspension (3 ml HBSS; 1.32 Abs₆₀₀; 10⁸ CFU/ml)
 1. Make working *S. aureus* stock (100 μ l of the stock in 25 ml HBSS; 10⁵ CFU/ml).

5. *Candida* and *S. aureus* Suspensions

1. Make mixed *C. albicans* and *S. aureus* suspension (250 μ l YF or GT stock and 100 μ l *S. aureus* stock in 25 ml HBSS).

6. Polymicrobial Biofilm Assay

1. Seed 96 well plates with 200 μ l of 2 x 10⁵ HeLa cells/ml (85% confluence level). Rock plates (30 - 45 min; 37 °C) before incubation (37 °C; 5% CO₂ incubator; 18 hr). Wash monolayers (1x; Opt-MEM) then seed with HSV (HSV-1 (KOS) gL86 or HSV-2 (KOS) 33 gJ¹ in 100 μ l Opt-MEM; MOI 50 and 10). Incubate plates (3 hr; 37 °C; 5% CO₂). Use only one viral strain per day.
2. Wash infected monolayers (1x; phosphate buffered saline (PBS) with Mg⁺² and Ca⁺²; 100 μ l). Replace PBS with warm HBSS leaving 25 μ l in each well.
 1. Add YF, GT and/or *S. aureus* working suspensions (100 μ l; target to cell ratio =5:1; n=16) as indicated in **Table 1**. Incubate plates (static; 30 min; 37 °C; 5% CO₂).
3. After incubation, aspirate one column at a time immediately refilling with 300 μ l PBS with Mg⁺² and Ca⁺². Repeat this step twice then add radio-immunoprecipitation assay lysis buffer (RIPA; filter sterilized; 200 μ l of a 1:50 dilution).
4. Rapidly triturate the HeLa cell lysate then place 50 μ l onto mannitol salts (MS) and/or Fungisel (F) media (**Figure 2**). Spread the lysate using a glass rod bent at a 90° angle. Incubate the plates (18 hr at 37 °C). Manually count the number of colonies per plate. Controls consist of *S. aureus* and/or *C. albicans* adherence to HSV-uninfected HeLa cells.

7. Imaging Studies

1. Wash each round glass coverslip (12 mm; 50 ml acetone in 100 ml beaker). Dry and sterilize coverslips (Kimwipes; glass petri dishes). Place dry sterile coverslips into the wells of sterile 24 well plates with alcohol flame sterilized forceps.
2. Add HeLa cells (1 ml; 5x volume used for 96 well plates) to the wells of 24 well plates containing the washed sterile round glass coverslips. Add the virus, bacteria and fungi according to the template (**Table 2**) at 5x the volume used for the 96 well plates, then incubate and process as described in steps 6.2.1 to 6.4 above.
3. After the final wash, fix the cells for microscopy by flooding the slide with methanol and allowing it to evaporate. Store the plates at RT until staining.
4. For bright field microscopy (1,000x final original magnification) fill the wells containing methanol-fixed coverslips with deionized water. Immediately aspirate water. Cover each coverslip with Grams crystal violet. Wash coverslips free of non-bound stain (deionized water). Dry coverslips *in situ* then adhere them with hard set mounting medium to a labeled slide (**Figure 3**).
5. For fluorescent microscopy (100x objective; 1,000x final original magnification) wash coverslips free of methanol essentially as described in step 7.4. Dry the coverslips in the wells.
 1. After drying, remove the cover slips and place them on labeled slides. Then add a sufficient amount of 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated Herpes Simplex Virus Type 1 + 2 gD antibody to cover the coverslip (1 - 5 μ l).
 2. Incubate the slides in a moisture chamber at 37 °C for 30 min. After incubation, wash the coverslips in 4 changes of PBS.
 3. After the final wash, return the coverslip to the labeled slide. Stain with 4',6-diamidino-2-phenylindole (DAPI; moisture chamber; 37 °C for 30 min). After incubation wash the coverslips in 4 changes of PBS, then affix to the labeled slide with hard set mounting medium.
 4. Allow the mounting medium to cure for 24 hr at RT in the dark. Examine the coverslips under oil objective on either a bright field microscope or fluorescent microscope with FITC and DAPI cutoff filters. Examine pictures of at least 50 fields (100 cells per organism minimum) for co-localization (**Figure 4**).

Representative Results

The level of robustness of data obtainable from system described in this report is shown in **Figure 2 a-f**³⁸. Through the use of this system the modulation of staphylococcal and fungal interaction with virally infected cells and their effect on each other's adherence can be delineated. These types of studies require microscopic examination of the interaction as shown in **Figures 3 and 4**³⁸ in order to determine whether the polymicrobial interaction is occurring on the same cells. In this study differential cell interaction is observed as a result of HSV-modulation of staphylococcal and fungal adherence that is viral species specific.

S. aureus and *C. albicans* (GT and YF) adhered to the same HSV-uninfected HeLa control cells. This co-localization on cells indicates a lack of physical inference with each other's HeLa cell adherence and that the measured levels of differential adherence measured were likely HSV-mediated (**Figure 3A, A1**)³⁸. However, upon HSV-1 or HSV-2 infected HeLa cells no co-localization of staphylococci and *C. albicans* was observed. (**Figures 3B, B1, B2, B3, C1, C2**). Using fluorescent microscopy (FITC-conjugated anti-HSV-gD monoclonal antibody) further confirmed that *S. aureus* did not appear to co-localize with *C. albicans* nor HSV-1 or HSV-2 (**Figures 4A, A1, A2, A3, A4, B1, B2, B3**). This co-operation between HSV and *Candida* extended to both yeast and germ tube forms (**Figures 2A-F**) of *C. albicans*. This specificity of association between the triad of microbes reflects the specificity of colonization seen on a much broader scale in the host oronasopharynx mucosa.

Figure 1. X-gal Staining Pictures of Dosage Dependent HSV-1 Infection in HeLa Cells. HeLa cells infected with HSV-1 at various MOI and X-Gal stained. (A) HeLa cells in well of 96 well plate with X-Gal stained mock-infected HeLa cell control; 20x initial magnification; (B) HeLa cells in well of 96 well plate with X-Gal stained HeLa cell infected with HSV-1 at an multiplicity of infection (MOI) of 10; 20x initial magnification; (C) HeLa cells in well of 96 well plate with X-Gal stained HeLa cell infected with HSV-1 at an multiplicity of infection (MOI) of 50; 20x initial magnification; scale bar applies to all. [Please click here to view a larger version of this figure.](#)

Figure 2. Effect of HSV-1 (panels A, C, E) and HSV-2 (panels B, D, F) at Multiplicities of Infection (MOI) of 50 and 10 on Adherence of *S. aureus* and/or *C. albicans* to HeLa Cells. (A) *S. aureus* (Sa) binding to HSV-1 infected cells in the presence of *C. albicans* germ tubes (GT) or yeast forms (YF); (B) *S. aureus* (Sa) binding to HSV-2 infected cells in the presence of *C. albicans* germ tubes (GT) or yeast forms (YF); (C) *C. albicans* germ tubes (GT) binding to HSV-1 infected cells in the presence of *S. aureus* (Sa); (D) *C. albicans* germ tubes (GT) binding to HSV-2 infected cells in the presence of *S. aureus* (Sa); (E) *C. albicans* yeast forms (YF) binding to HSV-1 infected cells in the presence of *S. aureus* (Sa); (F) *C. albicans* yeast forms (YF) binding to HSV-2 infected cells in the presence of *S. aureus* (Sa). All data points are Mean $\pm$ SEM, n= 16 normalized to virus-free control. * = significantly different ($p < 0.05$) from uninfected HeLa cell control. # = significantly different ($p < 0.05$) from paired point indicated by bracket; scale bar applies to all.³⁸ [Please click here to view a larger version of this figure.](#)

Figure 3. Lack of *S. aureus* and *C. albicans* Interactions on HSV-1 and HSV-2 Infected HeLa Cells. HSV-1 and HSV-2 infected (MOI 50) HeLa cell monolayers with *S. aureus* and *C. albicans* (5:1 target to cell). For bright field microscopy, cell monolayers were stained with Gram's crystal violet, then examined by light microscopy. Cells that were positive for *Candida* or *S. aureus* (100 individual cells per microbe signal/per coverslip) were secondarily scanned for the presence of additional microbe co-localization signals (1,000x initial magnification). (**A & A1**) *S. aureus* (Sa) and *C. albicans* yeast forms (YF) or germ tube forms (GT) co-localize on uninfected HeLa cells; (**A2, insert**). Percent of HeLa cells with co-localized or individual microbes; (**B - B3**). Lack of *S. aureus* and *C. albicans* co-localization in the presence of HSV-1; (**C - C2**) Lack of *S. aureus* and *C. albicans* co-localization in the presence of HSV-2. Mean $\pm$ SEM; scale bar applies to all.³⁸ [Please click here to view a larger version of this figure.](#)

Figure 4. Lack of Co-localization of *S. aureus* with *C. albicans* on HSV-1 or HSV-2 Infected HeLa Cells. HSV-infected HeLa cell monolayers challenge with *S. aureus* and *C. albicans* (5:1 target to cell) stained (FITC-conjugated anti HSV gD antibody, and DAPI). Pictures are representative of findings from screening of cells that were signal positive for HSV then scanned for *Candida* and *S. aureus* (100 individual cells per microbe signal per coverslip) that were then secondarily scanned for the presence of additional microbe co-localization signals (1,000x initial magnification; Nikon). (**A - A4**) *C. albicans* (**A2, insert**; DAPI staining) co-localize with HSV-1; (**B - B3**) *C. albicans* co-localized with HSV-2 (**B1, insert**, *C. albicans* DAPI staining). Mean $\pm$ SEM; scale bar applies to all.³⁸ [Please click here to view a larger version of this figure.](#)

	1	2	3	4	6	7	8	9	10	11	12						
Tubes	GT	YF	MSSA	GT/MSSA		YF/MSSA		GT	YF	MSSA	GT/MSSA		YF/MSSA				
MEDIA	F	F	MS	MS	F	MS	F	F	F	MS	MS	F	MS	F			
A																	
B																	
C																	
D																	
E																	
F																	
G																	
H																	

Table 1.
General Template Determination of Microbial Adherence in Polymicrobial Interactions. GT= *Candida albicans* germ tube phenotype; YF= *C. albicans* yeast form phenotype; MSSA= methicillin sensitive *Staphylococcus aureus*; MS= mannitol salts medium; F= Fungisel medium.
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Plate 1	1	2	3	4	5	6
Control	HeLa cells only	HeLa + GT	HeLa + YF	HeLa + Sa	HeLa + GT+Sa	HeLa + YF + Sa
A						
B						
C						
D						
Plate 2						
HSV	HeLa cells + HSV	HSV + HeLa + GT	HSV + HeLa + YF	HSV + HeLa + Sa	HSV + HeLa + GT + Sa	HSV + HeLa + YF + Sa
A						
B						
C						
D						

Table 2.
General Template for Visual Analysis of Polymicrobial Interactions with HSV-infected and Uninfected HeLa Cells. GT= *Candida albicans* germ tube phenotype; YF= *C. albicans* yeast form phenotype; SA= methicillin sensitive *Staphylococcus aureus*; HSV=herpes simplex virus.
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Discussion

Currently no information is available on complex interactions between permanent to semi-permanent members of the host microbiome that cross multiple taxonomic domains, i.e., prokaryotic, eukaryotic and viral. Therefore we developed a novel *in vitro* model system to study biofilm initiation by *S. aureus* and *C. albicans* on HSV-1 or HSV-2 infected HeLa 229 (HeLa) cells³⁸. The HeLa cell model system presents a unique advantage. This is due to their lack of surface fibronectin expression, which serves as a receptor for both *S. aureus* and *C. albicans*³⁹⁻⁴¹. Since the apical⁴² surface of mucosal epithelia normally lacks fibronectin, this system more closely mimics that observed in natural infection and colonization⁴³⁻⁴⁷. Thus, we are able to more directly examine the role specific viral entry receptor turnover plays in subsequent adherence by other members of the microbiome.

Using entry proficient non-spreading HSV-1 and HSV-2, these findings show that cell entry of HSV-1 or HSV-2 renders cells refractory to super-infection with *S. aureus*, while enhancing *C. albicans* adherence in a virus concentration dependent manner (**Figure 4**). Interestingly, the effects of HSV-1 on GT forms vs. YF, was the reverse of that measured for HSV-2, a finding which may have a significant impact on promotion of vaginal candidiasis in clinical presentations⁴⁸⁻⁵¹. From a pathogenesis perspective, it is generally accepted that the GT phenotype of *C. albicans* is the pathogenic form, with the YF the commensal state⁵¹⁻⁵⁴. Adherence of *C. albicans* GT that were co-incubated with *S. aureus* showed an altered pattern of binding to HSV-infected HeLa cells, as compared to that measured for YF - *S. aureus* binding. HSV-1 enhanced adherence of GT to HeLa cells, but to a significantly lesser extent ($p < 0.05$) than that measured for YF adherence, as discussed above, i.e., 270 % control for YF adherence and 190% control for GT adherence. In addition, while *S. aureus* had no effect on HSV-1 enhancement of GT binding, the coccus negated the enhanced adherence mediated by the presence of HSV-2. The reverse pattern was observed for *S. aureus* effect on YF interaction with virus infected cells. This predilection for different fungal forms by HSV-1 (YF) vs. HSV-2 (GT) may play a role directing maintenance of the commensal state *in vivo*. With regards to the effect of the GT form on *S. aureus* binding, the GT almost completely abolished the HSV-1 inhibition

of staphylococcal adherence to HeLa cells. In contrast, although the ability of GT to associate with HSV-2 infected cells was significantly increased as compared to HSV-1, the presence of *S. aureus* blocked the HSV-2 mediated increase in adherence.

The question of whether any changes in microbe adherence are the result of altered specific putative receptors present, or due to mechanical steric hindrance can be answered in this model. Through the combined use of quantitation of microbe-cell interactions (CFU count) and microscopic examination of the interactions, we were able to determine that HSV-1 and HSV-2 appear to block *S. aureus*-cell interaction, since *S. aureus* adhered solely to uninfected HeLa cells. Furthermore, microscopic examination of cells show that *Candida* and HSV-1 and HSV-2 co-localized. Used together here the findings from this study parallel the *in vivo* observations site specificity for colonization indicating the utility of this model for the study of polymicrobial interactions.

Effective use of the protocol described herein is dependent on a variety of factors. First, the use of spread deficient virus. This enables a clean examination of the effect viral entry and cell signaling has on subsequent microbe interactions, without the complication of changes that can occur due to envelop-acquisition prior to leaving the cell. In addition, use of defective virus allows for a safer environment for the handling of Biosafety Class Two Pathogens. Secondly, this protocol allows for the use of both viral and microbe adherence variants. Use of variants that only bind to specific receptors permits delineation of shared receptors between microbes, or, alternatively the detection of novel receptors. Through the use of monoclonal antibodies to the receptors, there is the potential for visualization of adhesin localization on the microbe surface and provides a tool to study altered adhesin expression. This protocol is not suitable for the study of microbes that cannot be selectively isolated on differential medium. The methodology is also dependent on the availability of monoclonal antibody to virus-specific proteins. Although analysis in this study was enhanced by the size differential between yeast and bacteria, use of differentially fluorescently tagged, e.g. Texas red, microbe specific monoclonal antibody would be an effective work-around should the microbes in question be similar in morphology and size.

Disclosures

The authors have nothing to disclose.

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