Asian Journal of Biological and Life Sciences | Original Research

# Transmission electron microscopy of *In Vitro* biofilms formed by *Candida albicans* and *Escherichia coli*

**Sally C. Suba, Frederick R. Masangkay***

Department of Medical Technology, Institute of Arts and Sciences, Far Eastern University, Nicanor Reyes Sr. St., Manila, Philippines 1008

E-mail : frederick_masangkay2002@yahoo.com Contact No : +63-2-7355621, +63-2-516-63-82

Submitted : 05.01.2013 Accepted :19.03.2013 Published : 30.04.2013

## Abstract

To evaluate *in vitro* interactions of *Escherichia coli* and *C. albicans* in pure and dual-species biofilm, and to assess biofilm formation. Clinical isolates were grown in thioglycollate broth. Sessile cells were cultured in Blood agar plate (BAP) and grown on copper grids for Transmission Electron Microscopy (TEM) negative staining. Sessile cells were also grown in Eppendorf tubes with thioglycollate, preserved ultra-thin sections of which were processed for positive staining. *C. albicans* in pure and co-culture with *E. coli* in thioglycollate exhibited blastospore morphotypes only. Biofilm towers predominated in *C. albicans* pure culture and demonstrated "blob" formations. Networking architecture was observed in pure colonies of *E. coli*. In vitro co-cultures demonstrated down-regulated biofilm formation, decreased cellularity and degraded yeast cells. Biofilm phenotypes were present in both bacterial and fungal sessile cells from the urine sample and pose as obstacles in effective drug therapy. Therefore it is necessary that methodologies for effective isolation of sessile cells be employed for the judicious application and management of drug susceptibility testing directed towards sessile cells in vitro as baseline for antimicrobial therapy in vivo.

## INTRODUCTION

A biofilm is a multicellular community composed of prokaryotic and/or eukaryotic cells embedded in a matrix composed, at least partially, of material synthesized by the sessile cells in the community [1]. The formation of biofilms by most microorganisms involves the regulation of genes that are essential for surface attachment and production of extracellular matrices [2].

Organisms most commonly isolated from catheter biofilms are *Staphylococcus epidermidis, S. aureus, Candida albicans, P. aeruginosa, K. pneumoniae,* and *Enterococcus faecalis* [3,4]. Fungi most commonly associated with such disease episodes are in the genus *Candida*, most notably *C. albicans*, which causes both superficial and systemic disease. Even with current antifungal therapy, mortality of patients with invasive candidiasis can be as high as 40 percent [5]. *Candida* organisms are dimorphic, and may be found in humans during different phenotypic phases. In general, blastospores represent the phenotype responsible for transmission or spread of *Candida*, and are associated with asymptomatic colonization of the vagina. In contrast, germinated yeast producing mycelia most commonly constitute a tissue-invasive form of *Candida*, usually identified by the presence of symptomatic disease along with larger numbers of blastospores. *Candida* spp. infections, caused by *Candida albicans*, are increasingly associated with the use of medical devices such as central venous catheters, urinary catheters, and trachea-esophageal voice prostheses [6]. *Candida albicans* forms biofilms on these materials which are extremely difficult to eradicate with conventional antifungal therapy [7]. *Candida albicans* has the capacity to interact with bacteria through a series of extracellular signals called quorum sensing molecules [8]. Farnesol, a quorum sensing molecule secreted by the *C. albicans* prevents the hyphal growth and allows the yeast to interact with bacteria [9].

*E. coli* is a bacterium that is part of the intestinal microbiota which is the most common cause of urinary tract infections. *E. coli* causes about 70% of cases; other bacteria from the intestinal tract such as *Proteus* and *Klebsiella* cause about 10% of UTIs. Nonenteric bacteria like *Pseudomonas* and *Staphylococcus* occasionally cause UTIs as well [10]. Both its frequent community lifestyle and the availability of a wide array of genetic tools contributed to establish *E. coli* as a relevant model organism for the study of surface colonization [11]. *E. coli* is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract, where it thrives in an environment with structural characteristics of a multispecies biofilm [1,12]. With over 250 serotypes, *E. coli* is a highly versatile bacterium ranging from harmless gut commensal to intra or extraintestinal pathogens, including common colonizers of medical devices and the primary causes of recurrent urogenital infections [13].

## MATERIALS AND METHODS

### Isolates

Bacterial and fungal cells were isolated from an 80 year old female out-patient at The Lung Center of the Philippines who complained of fever, fatigue, and back pain for two days prior to collection of her urine. Urine sample showed greater than 10 WBC per HPF and yeast cells. Growth was observed in BAP, CAP and MAC. MAC produced 100,000 CFU/ml of lactose fermenting colonies of *Escherichia coli* after 24 hours of incubation at $37^0$C (Complicated UTI). Conventional Biochemical testing was done using Citrate, Triple Sugar Iron (TSI), Lysine Iron Agar (LIA), Motility Indole Ornithine (MIO), Methyl Red (MR), Vogues-Proskauer (VP), Glucose, and Lactose fermentation. Further identification of colonies was confirmed using API 20 E for *E. coli*. White colonies were Gram stained and observed using light microscopy. Yeast cells were seen and had been demonstrated to be germ tube test positive after two hours of incubation at $37^0$ C. Identity of fungal isolate was confirmed using API 20 C AUX and was identified as *C. Albicans*.

### Isolation of biofilms

Plated pure cultures of *E. coli* and *C. albicans* were inoculated in thioglycollate to a density of 1 McFarland (%T = 55.6, Abs = 0.257, at 600 nm) and incubated for 48 hours at 37$^0$ C. Thioglycollate was decanted and test tubes were gently washed with sterile PBS to remove nonadherent cells. Sessile (adherent) cells were swabbed from the sides of the test tube and inoculated in BAP and incubated for 72 hours at 37$^0$ C. Growth from these plates were inoculated in thioglycollate broth adjusted to 1 McFarland, 1000 µL of which were placed in 25 mm sterile plastic plates containing 2ml of thioglycollate and copper grids for Transmission Electron Microscopy (Negative Staining) of biofilm morphology of sessile colonies and incubated for 72 hours at 37$^0$C. 100 µL of 1 McFarland of sessile cells were placed in sterile 1.0 ml capacity Eppendorf tubes with 1ml of thioglycollate and incubated for 72 hours at 37$^0$ C and will be processed for electron microscopy of cross sections of the sessile cell colonies.

## Transmission Electron Microscopy

### Negative Staining

Sessile cell colonies grown on formvar-coated copper grids were stained with 2% Phosphotungstic acid (PTA) with a pH of 7.0 for 30 seconds and air-dried. Stained copper grids were viewed under transmission electron microscope (JEOL JEM 1010, Japan Electron Optics).

### Positive Staining

Sessile cell colonies in Eppendorf tubes were preserved using glutaraldehyde; washed with 0.1 M phosphate buffered saline (PBS) and centrifuged (5,000 RPM). Concentrated cells were fixed in 1% osmium tetroxide for 1 hour and dehydrated using increasing grades of acetone (50%, 75%, 95%, and absolute alcohol). Epon Resin was used as an infiltrating media in a 1:1 concentration with acetone; final infiltration was done with a 1:3 concentration of acetone and infiltrating media for 24 hours. Infiltrated cells were embedded in absolute embed mixture and left to polymerize in an oven at 70$^0$ C for 15 hours. Ultra-thin sections were stained with 7% methanolic uranyl acetate solution as the primary contrast media for 15 minutes and lead citrate as the secondary contrast media for 15 minutes and viewed under transmission electron microscope (JEOL JEM 1010, Japan Electron Optics).

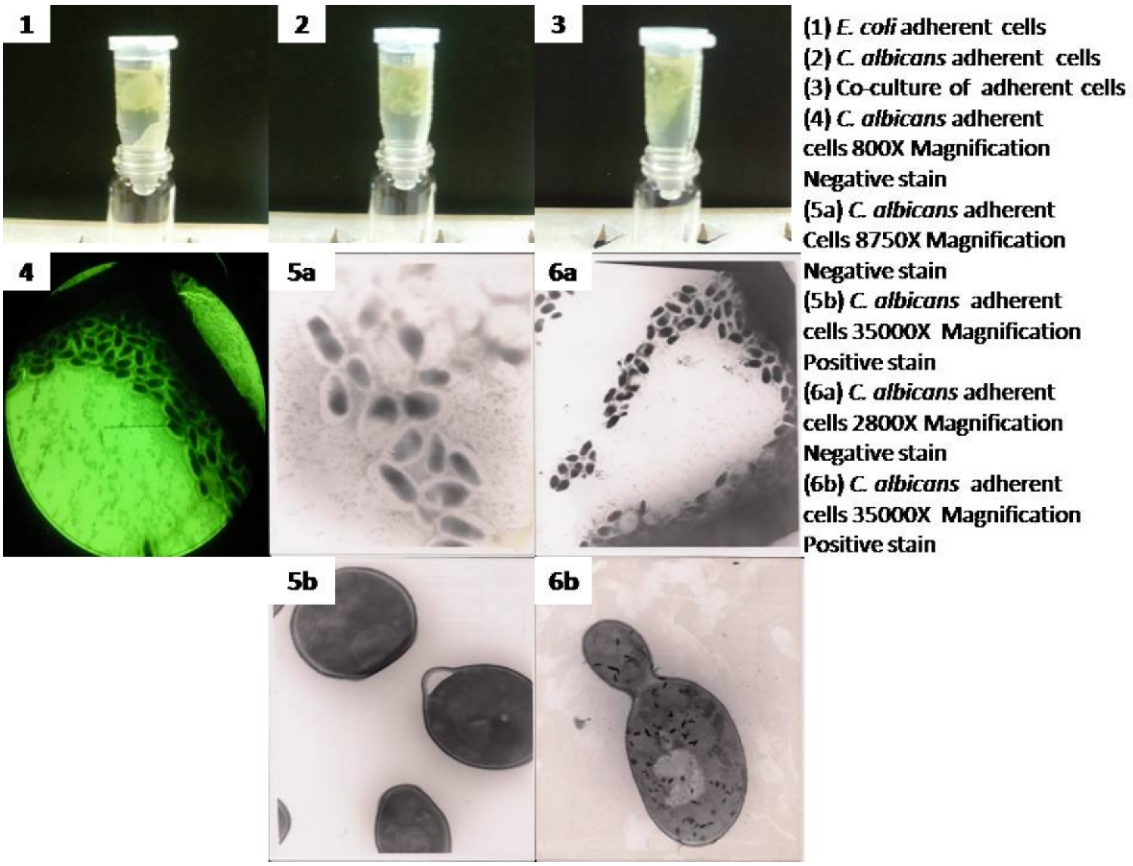
## RESULTS

### Isolated Biofilms of Sessile Cells

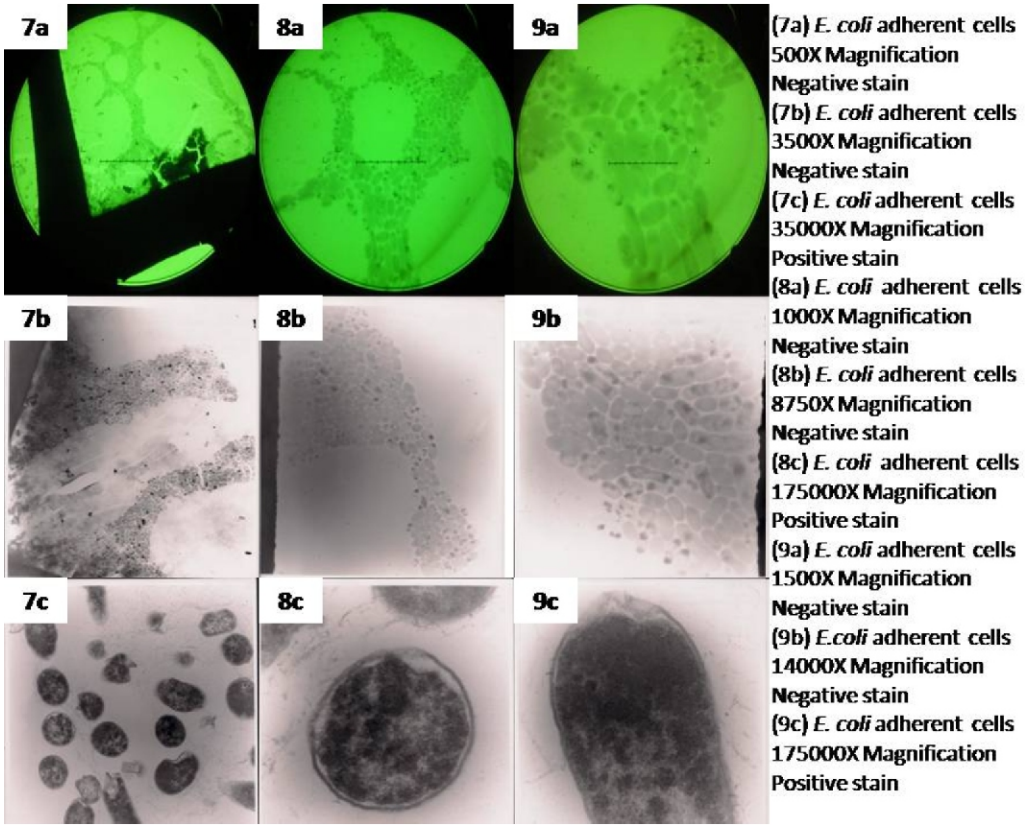
Structured networks that fill the entire test tube and can be removed as a single coherent mass were observed in the results of this study by the shroud-like mass formed by the adherent cell colonies in thioglycollate after 72 hours of incubation (Figures 1 to 3).

### *C. albicans* and *E. coli* Adherent Cells

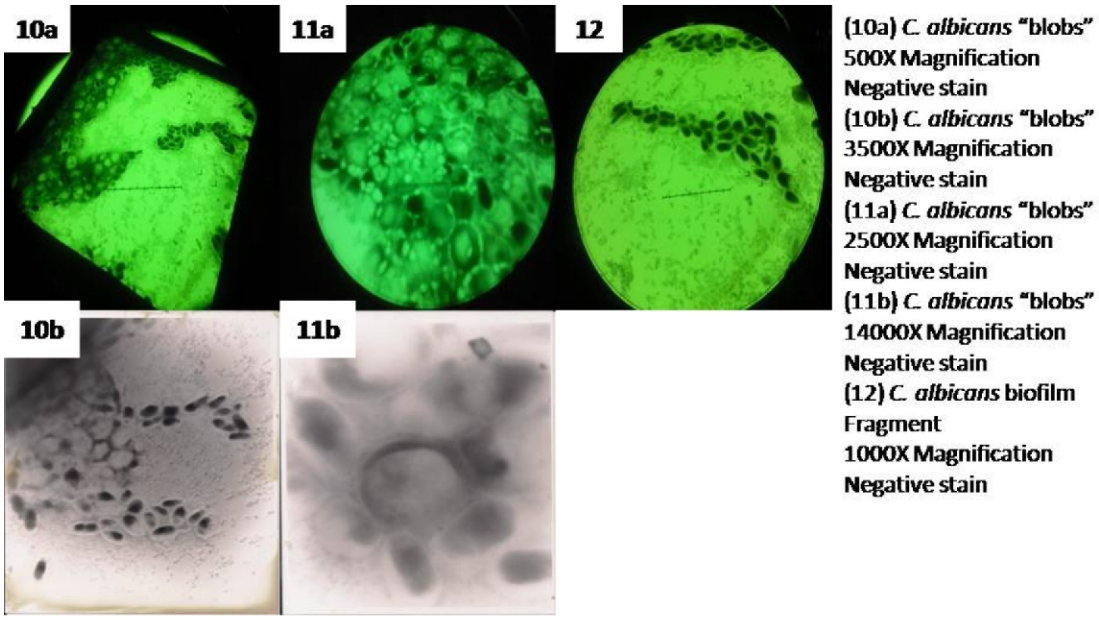
Sessile cells of *C. albicans* demonstrated only blastospore morphology; Hyphae or pseudohyphae were not seen in this study which may indicate that this fungal infection is not a tissue invasive one. Figures 4 to 9 show sessile cells of pure colonies of *C. albicans* and *E. coli* after 72 hours of incubation under TEM. The blastospore layer is in contact with the surface and anchors the final three-dimensional structure to the colonized substrate




Figures 1 to 6b



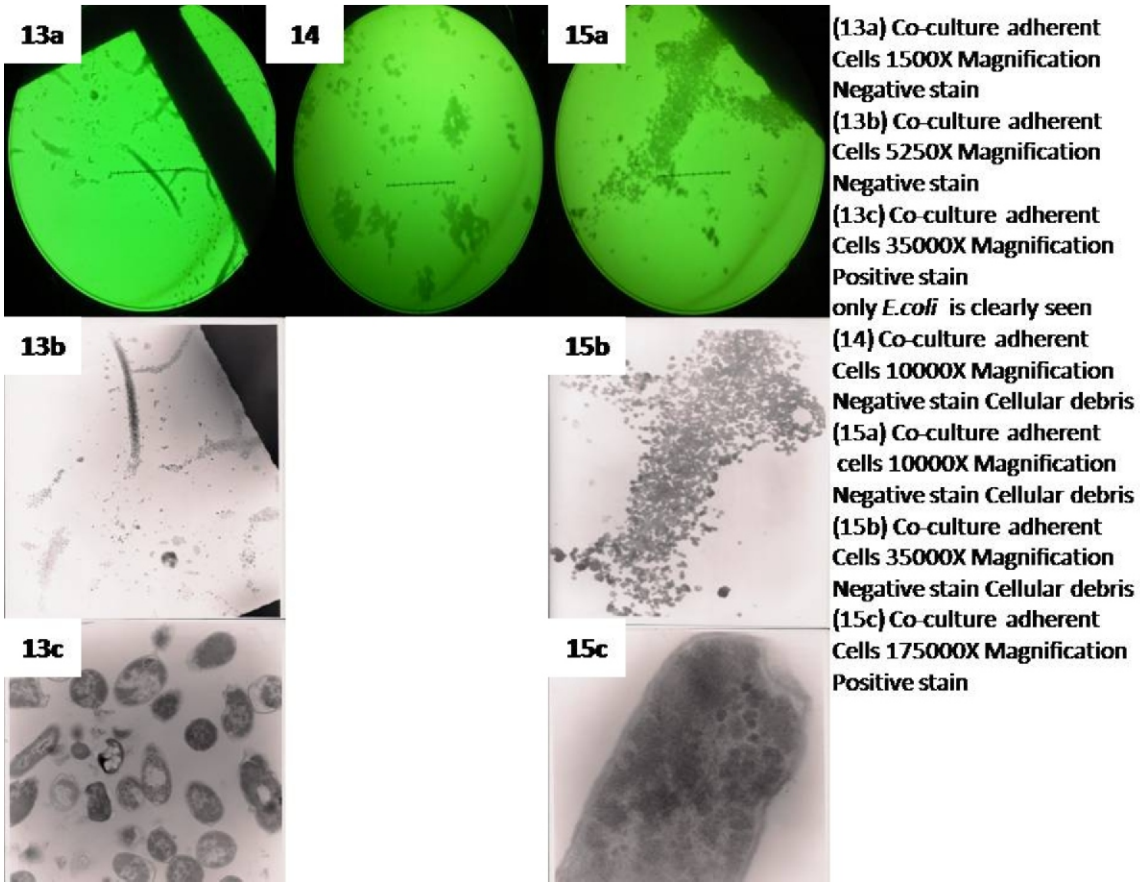

Figures 7 to 9c




Figures 10a to 11b

(Figure 4). Figures 5a and 6a show rich formation of extracellular matrix enclosing each fungal cell after 72 hours of incubation in thioglycollate broth. This abundance in extracellular matrices however was not clearly observable in *E. coli* adherent cells. Networking structures were observable in *E. coli* adherent cells as void areas surrounded by a network of adherent cell colonies anchored to the copper grid surface (Figure 7a, 7b, and 8a).

The microcolonies of sessile cells isolated in this study were seen to take the form of simple towers, or of mushrooms (Figure 6a), and the water channels were devoid of cells surrounded by




Figures 13a to 15c

colonies that are clearly seen to be spreading in all directions in a web-like pattern (Figure 7a, 7b, 8a, 8b, 9a, 9b).

Sessile cells of *C. albicans* measured an average of 3.3 µm to 5.1 µm in both negative and positive staining methodology indicating that the dehydration process in positive staining does not reduce the cell size; it does however dissolve the exopolysaccharide matrix that surrounds the adherent cells making positive staining an ineffective method for demonstrating exopolysaccharides. Positive staining however is an effective method of demonstrating cell walls which in this case cannot be demonstrated by negative staining.

"Blobs" (Figure 10a, 10b, 11a, 11b) were clearly demonstrated in this study and are seen to comprise mature *C. albicans* biofilm. This study demonstrates that this phenomenon can occur even in pure cultures (single-specie) of biofilms *in vitro* which in this case is made up of *C. albicans* sessile yeast cells. The biofilm blobs in single-specie culture of *C. albicans* adherent cells measured an average of 4.29 µm X 5.14 µm. Biofilm Fragments (Figure 12) were also seen as detached colonies seen in figure12; adherent cells in the biofilm fragment are all still enclosed in thick extracellular matrices.

Dual-species biofilms demonstrated scanty architecture. Co-cultures demonstrated reduction in the cellularity of the biofilm. Thus, in general, the dual-species biofilm had a reduced number of cell layers and degraded yeast cells (Figure 13a, 13b, 14, 15a, 15b).

## DISCUSSION

*Candida* spp. occurs as asporogenous yeast cells but can also form hyphae or pseudohyphae under certain conditions [14]. Hyphae or pseudohyphae were not seen in this study. In general, blastospores represent the phenotype responsible for transmission or spread of Candida and are associated with asymptomatic colonization of the vagina. Our study supports this by demonstrating an all blastospore population of *C. Albicans*. This means that the method of isolation of sessile cells were indeed highly selective for these cell types which are primarily responsible for colonization of medical devices and are difficult to eradicate by conventional antifungal therapy as results of study by Kojic 2004 and Bauters *et al.,* 2002 demostrated [6,7]. *Candida albicans* biofilms have the capacity to interact with bacteria through a series of extracellular signals called quorum sensing molecules [8]. Notably, Farnesol, a quorum sensing molecule secreted by *C. albicans* prevents the hyphal growth and allows the yeast to interact with bacteria [9] which accounts for the all-blastospore population of *C. albicans*. Blastospore layers observed were in close contact with the surface of the copper grid and anchored the final three-dimensional structure to the colonized substrate. The initial adherence is followed after 3 to 4 hours by the formation of microcolonies on the colonized surface. After 11 hours, a thick fungal *C. albicans* growth can be observed. The intermediate phase (12-24 hours) is mainly characterized by the synthesis of the extracellular matrix, covering the *C. albicans* cells of the premature biofilm and increasing with longer

incubation times [15-19].

Mature *Candida* biofilms show an extensive spatial heterogeneity, with typical microcolony/water channel architecture and cells embedded in an extracellular polymer matrix. The heterogeneous build-up of mature biofilms allows an influx of water and nutrients and efflux of metabolites/waste products [20-21] which accounts for the architecture observed in *E. coli* sessile cells as void spaces surrounded by a network of cell colonies anchored to the copper grid surface. *E. coli* exoploysaccharide is significantly thinner than those seen in *C. albicans* and aside from differences in cell wall thickness these can be explained because subcultures in liquid culture media lead to loss of exopolysaccharide structures, and in the case of *E. coli* some pili and flagella, so that the outer membrane of Gram-negative cells become their outermost component [1]; it would be very interesting then to observe *E. coli* biofilms formed in solid and semisolid media.

Adherent cells of *C. albicans* measured an average of 3.3 µm to 5.1 µm in both negative and positive staining methodology indicating that the dehydration process in positive staining does not reduce the cell size; it does however dissolve the exopolysaccharide matrix that surrounds the adherent cells making positive staining an ineffective method for demonstrating exopolysaccharides. Positive staining however is effective in demonstrating cell walls which in this case cannot be demonstrated by negative staining.

Recent studies of natural mixed-species biofilms by Lawrence *et al.,* 2003 [22] have shown large "blobs" of exopolysaccharide that don't always enclose bacterial cells but do comprise of a large part of the volume of these sessile communities [1]. This study demonstrated that this phenomenon can occur even in pure-cultures (single-specie) *in vitro* which in this case are made up of *C. albicans* sessile yeast cells.

Biofilm fragments observed in this study (Figure 6a and 12) constitute a special problem because (unlike detached planktonic cells) they still express the biofilm phenotype. Adherent cells in the biofilm fragment are all still enclosed in thick extracellular matrices, and are therefore inherently resistant to conventional antibiotics as they move and settle in new locations [1,23].

Biofilms of co-cultures demonstrated scanty architecture with reduced visible cell counts at all stages of biofilm development, despite profuse growth and dense colonization in their single-specie counterparts which parallels the result of Kojic and Darouiche [6]. Co-cultures of adherent cells in this study demonstrated a reduction in the cellularity of the biofilm. Thus, in general, the dual-species biofilm had reduced number of cell layers and degrading yeast cells.

Data indicate that *E. coli* and *C. albicans* species in co-culture mutually modulate biofilm development, both quantitatively and qualitatively, and that *E. coli* LPS appears to be a key component in mediating these outcomes [24]. A study by Thein *et al.,* 2006 [25] noted that there is significant inhibition of *C. albicans* yeast cells co-cultured with high concentrations of *E. coli* ($2.5\times10^6$, $5\times10^6$ and $1\times10^7$ c.f.u. $ml^{-1}$) at 24 hours. The results of this study were able to corroborate their results showing significant inhibition of *C. albicans* yeast cells co-cultured with *E. coli* up to 72 hours of incubation.

The results of studies mentioned above as well as the results of this study strongly suggest that there are molecular factors influencing modulation of colonial growth and biofilm formation in co-cultures of *E. coli* and *C. albicans* aside from specie to specie interaction. It is highly suspected that the nature of nutrients (artificial or biological) and the growth environment (*in vivo/in vitro*) plays a major role in the up-regulation or the down-regulation of signalling molecules responsible for the observed phenomenon. It is note-worthy to mention also that this study observed the behaviour demonstrated by actual clinical isolates compared to other studies which utilized ATCC strains.

## CONCLUSION

In conclusion, the present study demonstrated that the methodology employed in the isolation of both bacterial and fungal sessile cells was effective in harvesting high yields of sessile cell population which are the cells that should be of primary concern in the drug susceptibility testing of clinical isolates *in vitro* for the proper application and management of antibiotic therapy of the patients *in vivo*.

## ACKNOWLEDGEMENT

The authors would like to acknowledge The Lung Center of the Philippines for providing them with the clinical isolates; The Research Institute of Tropical Medicine for the Confirmatory Identification of the clinical isolates; The Research and Biotechnology Division, Electron Microscopy Department of St. Luke's Hospital, Quezon City for the Transmission Electron Microscopy procedure; Professor Romulo Villanueva the Program Coordinator of the English Department as our language consultant and The Far Eastern University, Manila University Research Council for this Research Grant.

## REFERENCES

1. Costerton JW. The Biofilm Primer. Springer-Verlag Berline Heidelberg. New York 2006: 3-5, 43, 56, 113-116, 120,148.

2. Danese PN, Pratt LA, Kolter R. Biofilm formation as a developmental process. Methods Enzymol. 2001; 336: 19-26.

3. Elliott TSJ, Moss HA, Tebbs SE, Wilson IC, Bonser RS, Graham TR, et al. Novel approach to investigate a source of microbial contamination of central venous catheters. Eur J Clin Microbiol Infect Dis. 1997; 16:210-3.

4. Raad II, Sabbagh MF, Rand KH, Sherertz RJ. Quantitative tip culture methods and the diagnosis of central venous catheter-related infections. Diag Microbiol Infect Dis. 1992;15:13-20.

5. Wey SB, Mori M, Pfaller MA, Woolson RF, Wenzel RP. Hospital-acquired candidemia. The attributable mortality and excess length of stay. Arch.Intern.Med. 1988; 148:2642-2645.

6. Kojic, EM, Darouiche RO. Candida infections of medical devices. Clin Microbiol Rev. 2004; 17(2):25567.

7. Bauters TGM, Moerman M, Vermeersch H, Nelis HJ. Colonization of voice prostheses by albicans and non-albicans Candida species. Laryngoscope. 2002; 112:70812.

8. Cugini C, Calfee MW, Farrow JM, Morales DK, Pesci EC. Farnesol, a common sesquiterpene, inhibits PQS production in Pseudomonas aeruginosa. Mol Microbiol. 2007; 65: 896906.

9. Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B. Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol. Appl Environ Microbiol. 2001; 67: 29822992.

10. Bauman R. Microbiology: Alternate Edition with Diseases

by Body System, Pearson Benjamin Cummings. 2006; 710-711.

11. Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, Haagensen J, Molin S, Prensier G, Arbeille B, Ghigo J. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Mol Microbiol. 2004*; 51:659674.

12. Probert HM., Gibson GR. Bacterial biofilms in the human gastrointestinal tract. *Curr Issues Intest Microbiol. 2002;* 3:2327.

13. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. *Nat Rev Microbiol. 2004;* 2:123140.

14. McCullough MJ, Ross BC, Reade P. C. *Candida albicans*: a review of its history, taxonomy, epidemiology, virulence attributes, and methods of strain differentiation. Int J Oral Maxillofac Surg. 1996; 25: 136-44.

15. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol. 2001;* 183:5385-5394.

16. Argimón S, Wishart JA, Leng R. Developmental regulation of an adhesion gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. Eukaryotic Cell. 2007; 6: 682-92.

17. Hoyer LL, Green CB, Oh S-H, Zhao X. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene familya sticky pursuit. Med Mycol. 2008; 46: 1-15.

18. Nobile CJ, Schneider HA, Nett. Complementary adhesion function in *C. albicans* biofilm formation. Curr Biol. 2008; 18: 1017-24.

19. Zhao X, Daniel KJ, Oh S-H. *Candida albicans* Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. Microbiology. 2006; 152: 2287-99.

20. Ramage G, Vande Walle K, Wickles BL, López-Ribot JL. Biofilm formation by *Candida dubliniensis*. J Clin Microbiol. 2001; 39: 3234-40.

21. Ramage G, Martínez JP, López-Ribot JL. *Candida* biofilms on implanted biomaterials: a clinically significant problem. FEMS Yeast Res. 2006; 6: 979-86.

22. Lawrence JR, Swerhone GDW, Leppard GG, Araki T, Zhang X, West MM, Hitchcock AP. Scanning transmission X-Ray, laser scanning, and transmission electron microscopy mapping of the exopolymeric matrix of microbial biofilms. Appl Environ Microbiol. 2003; 69:55435554.

23. Olson ME, Lam K, Bosey GP, King EG, Costerton JW. Evaluation of strategies for central venous catheter replacement. Crit Care Med. 1992; 20:797804.

24. Bandara HM, Yau JY, Watt RM, Jin LJ, Samaranayake LP. Escherichia coli and its lipopolysaccharide modulate in vitro Candida biofilm formation. J Med Microbiol. 2009; Dec;58 (Pt 12):1623-31.

*25.* Thein ZM, Samaranayake YH, Samaranayake LP. In vitro effect of dietary sugars, serum and antimicrobial resistance on dual species biofilm formation of C. albicans and E. coli Acta Pathologica. Microbiologica et Immunologica Scandinavica (APMIS). 2007; 115: 00-00.