COMPARISON OF BIOMATERIALS IN ABILITY FOR GENE EXPRESSION LEVELS OF *ALS1* AND *HWP1* IN *CANDIDA ALBICANS* AND *ICA1* IN *STAPHYLOCOCCUS EPIDERMIDIS**

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SUMMARY

This study describes a comparison of latex-silicone coated, polyvinyl chloride, polyvinylpyrolidone coated polyurethane and polyurethane catheters for the delivery of adhesion genes in an in vitro biofilm model. Aglutinin-like sequence (ALS1), hypha-specific surface protein (HWP1) genes of Candida albicans and intracellular adhesion (ICA1) gene for Staphylococcus epidermidis were targeted. Four different biofilm models were constructed on four different bio-materials. Biofilm mass was separeted from the surface of the catheter and genomic mRNA was isolated. It was quantified in a real time PCR assay. ALS1 and HWP1 gene copy numbers were found to be $2.50x10^5$, $1.30x10^4$ copy/ml, respectively, and ICA1 gene copy number was found to be $1.80x10^5$ copy/ml on the biofilm of the urinary catheters latex-silicone coated material. ALS1 and HWP1 gene copy numbers were found to be $7.56x10^6$, $6.88x10^5$ copy/ml, respectively, and ICA gene copy number was found to be $4.20x10^5$ copy/ml on the biofilm removed from endotracheal tube, made from polyvinyl chloride. Biofilm formation was not seen and biofilm associated C.albicans ALS1, HWP1 and S.epidermidis ICA genes were not expressed on the surface of nasogastric tube made from polyvinylpyrolidone coated polyurethane and the aspiration catheter made from polyurethane. In the PVP slime model, mean copy number was $2.70x10^2$, $2.27x10^1$ and $2.30x10^1$ for ALS1, HWP1 and ICA, respectively. Mean copy number of adhesion genes on PUC were $1.50x10^1$, $1.20x10^1$ and $3.10x10^1$ for ALS1, HWP1 and ICA, respectively. Adhesion genes were found as expressed in higher levels on the polyvinyl chloride catheter than those in siliconized one. Minimum gene expression levels were detected in polyvinylpyrolidone coated polyurethane and polyurethane catheters. It may result in the preferation of one material from other in the clinical use because of the different gene expression levels on the surface of these biomaterials.

Keywords: *ALS1, biofilm, Candida albicans, HWP1, ICA1, real time PCR, Staphylococcus epidermidis*

ÖZET

Biyomaddelerin *Candida albicans ALS1* ve *HWP1* ve *Stapylococcus epidemidis ICA1* Gen İfadesi Üzerine Etkilerinin Karşılaştırılması

Bu çalışma lateks-silikon kaplı, polivinil klorid, polivinilprolidon kaplı poliüretan ve poliüretan kateterlerin in-vitro biyofilm modelinde adezyon genlerinin ifadelenmesine etkilerini karşılaştırmak için düzenlenmiştir. Candida albicans'ın "aglutinin-like sequence" (ALS1), "hypha-specific surface protein" (HWP1) genleri ve Staphylococcus epidermidis'in "intracellular adhesion" (ICA1) geni hedeflenmiştir. Dört farklı biyolojik madde ile dört farklı biyofilm modeli oluşturulmuştur. Biyofilm katmanı kateter yüzeyinden sıyrılmış ve bu kitleden genomik mRNA izole edilerek gerçek zamanlı polimeraz zincir reaksiyonu (PCR) cihazında ölçülmüştür. Silikon kaplı idrar sondası yüzeyinde oluşan biyofilmde ALS1 ve HWP1 genlerinin kopya sayıları ortalaması, sırasıyla $2.50x10^5$ ve $1.30x10^4$ olarak, ICA geni kopya sayısı ortalama $1.80x10^5$ kopya/ml olarak bulunmuştur. Yapısı polivinil klorid olan trakeal tüp yüzeyinde oluşan biyofilmde ALS1 ve HWP1 genlerinin kopya sayıları ortalaması, sırasıyla $7.56x10^6$ ve $6.88x10^5$ kopya/ml olarak, ICA gen kopya sayısı ortalaması ise $4.20x10^5$ kopya/ml bulunmuştur. Polivinilprolidin kaplı poliüretan malzemeden yapılan nazogastrik tüp ve poliüretan malzemeden yapılan aspirasyon sondası yüzeyinde biyofilm oluşmadığı ve bu malzeme ile karşılaştırılan C.albicans örneğinde ALS1 ve HWP1 ile S.epidermidis örneğinde ICA gen ifedelerinin bulunmadığı gösterilmiştir. Polivinilprolidin kaplı poliüretan yüzeyde ALS $2.70x10^2$, HWP1 $2.27x10^1$ ve ICA1 $2.30x10^1$ düzeyinde iken, poliüretan yüzeyinde ALS1, HWP1 ve ICA genleri sırası ile $1.50x10^1$, $1.20x10^1$ ve $3.10x10^1$ bulunmuştur. Adezyon genleri polivinil klorid sonda yüzeyinde silikon olandan daha yüksek oranda gösterilmiştir. En düşük gen ifadelenmesi polivinilprolidon kaplı poliüretan kateter ve tek başına poliüretan yapılı kateterlerde gösterilmiştir. Kullanılan malzemelere bağlı olarak adezyon genlerinin arasında fark bulunması, klinik kullanımda bazı malzemelerin tercih edilmesini ve bazılarından sakınılmasını sağlayabilir.

Anahtar sözcükler: *ALS, biyofilm, Candida albicans, gerçek zamanlı PCR, HWP, ICA, Staphylococcus epidermidis*

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INTRODUCTION

The use of synthetic materials for temporary or permanent implantation has been accompanied by the emergence of a new challenging entity, namely, implant-associated infection. Different materials such as silicone, polyurethane, polyvinyl and polyethylene are used for the production of medical catheters. Catheter material is thought to affect the incidence of late catheter-related infection[13]. This effect may be related to the adherence of microorganisms and gene expression profiles.

Staphylococcus epidermidis and *Candida* spp. strains with the ability to form biofilms are the predominant pathogens. Biofilm production by *S.epidermidis* and *C.albicans* can occur on almost any kind of catheter and in a variety of other medical devices and implants[18].

Adherence of microorganisms to an implanted device is a crucial step in the development of biofilm and biomaterial-entered infection. Adhesion of *C.albicans* to host cells, has been studied most extensively, including research on adherence molecules, such as the agglutinin-like sequence *(ALS)* family, hyphal wall protein *(HWP)* and cell wall glycoproteins[15]. The intercellular adhesin *(ICA)* operon is a virulence factor identified in *S.epidermidis* related to biofilm production[16]. Different studies have already described changes in gene expression levels during biofilm development[1].

In the present study, we investigated the expression of *ALS1* and *HWP1* in *C.albicans* and ICA in *S.epidermidis* as housekeeping genes in biofilms by quantitative PCR analysis. We performed a comparison study conducting the influence of the biomaterials on the gene expression levels.

MATERIALS AND METHODS

Synthetic materials used for biofilm modeling

Disposable, latex-silicone coated catheter-SC (Well Lead Silicone Coated Foley Catheter, Well Lead Medical Ins., China), disposable, polyvinyl chloride catheter-PVC (Tracheal tube, Medimark Europe, France), disposable, polyvinylpyrolidone-PVP-coated polyurethane catheter (Nasogastric catheter, Medimark Europe, France) and disposable, polyurethane catheter-PUC (Neumann aspiration tube, Fujian Kanglite Group, Fuzhou, China) were used.

In vitro biofilm model

Slime positive clinical strains of *C.albicans* and *S.epidermidis* were used[4,6]. Catheter samples were cutted in 0.5 x 0.5 cm diameters and put in plastic conical tubes (Falcon Plastics, Oxnard, Calif.) containing 10 ml 0.9 % NaCl. A suspension of 500 µl broth medium including *C.albicans* (10^6/ml) and *S.epidermidis* (10^8/ml) were added onto seperate tubes. They were incubated in shaking bath at 37°C for 24 h at 150 rpm agitation for initial adhesion of cells. The squares were washed with 2 ml phosphate-buffered saline and moved to a fresh tubes containing 2 ml of fresh phosphate-buffered saline. These tubes were incubated at 37°C for an additional 48 h at 150 rpm agitation to allow biofilm formation.

Extraction of mRNA and conventional reverse transcription-polymerase chain reaction (RT-PCR)

Biofilm layer were removed from the surface of the catheter by scrubing with a cell-scrubber. Total RNA was isolated from biofilm layer using a commercially available kit (Heliosis, Metis Biyoteknoloji, Turkey) following the manufacturer's instructions. To avoid amplification of contaminating genomic DNA, all samples were treated with ribonuclease-free deoxyribonuclease (Qiagen, Hilden, Germany) for 15 min.

Real-time PCR assay conditions for ALS1 and HWP1 genes

Real time RT-PCR amplification mixtures (25 ml) contained 10 ng template cDNA, LightCycler Hybridization Probes Master Mix kit (Roche diagnostics, Tenay, Turkey), and SYBR Green I master mix buffer with fluorescein. LightCycler (Roche diagnostics, Tenay, Turkey) and LightCycler 3.5 software were used. Primers and probes were listed in Table 1. Fluorescence was collected at each polymerization step. Melting curve analysis was done.

Table 1. *Primer list for the targets.*

Name	Primer sequence
C. albicans ALS1 Forward	5′-GAC TAG TGA ACC AAC AAA TAC CAG A-3′
C. albicans ALS1 Reverse	5′-CCA GAA GAA ACA GCA GGT GA-3′
C. albicans HWP1 Forward	5′-ATG ACT CCA GCT GGT TC-3′
C. albicans HWP1 Reverse	5′-TAG ATC AAG AAT GCA GC-3′
S.epidermidis ICA Forward	5′-TAG TAA TCA CAG CCA ACA TCT T-3′
S.epidermidis ICA Reverse	5′-AAA CAA ACT CAT CCA TCC GAA T -3′
ICA Probe	5′-FAM-TGG ATA CCA ACT TAA AAA TAT CAG GCC AAC-3′- TAMRA

Taqman quantitative Real-time PCR for *ICA1* gene

Taqman primers and probes are summarized in Table 1. Probes were labeled with the reporter dye 6-carboxyfluorescein (6′-FAM) at the 5′end and with the quencher dye 6-carboxytetramethylrodamine (TAMRA) at the 3′end. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 45 repeats of 15 s at 95°C, and 1 min at 60°C. Data collection was performed during each annealing phase.

The standards for *ALS1, HWP1* and *ICA* consisted of six-fold dilution serials of the cDNA mixture (from 50 ng to 5 pg) were used as the standards. All standard curves showed correlation coefficients of greater than 0.99, indicating a precise log-linear relationship. The mRNA copy numbers were then calculated for each catheter sample using the standard curve to convert the obtained crossing threshold value into mRNA copy numbers.

Statistical methods:

The results were analysed using the one-way analysis of variance (ANOVA). The Bonferroni test was used as Post Hoc analysis. $p<0.05$ was considered as istatistically significant.

RESULTS

Isolates grown planktonically for 48 h show *ALS1, HWP1* and *ICA* mean copy number of 10^1 copies (data not shown). Each catheter was tested ten times and the mean copy numbers of the genes were calculated. Biofilm on the SC was augmented the gene expression level such as mean copies of $2.50x10^5$, $1.30x10^4$, $1.80x10^5$ for *ALS1, HWP1* and *ICA*, respectively. When the same strain was grown as a biofilm on PVC, mean copy number of *ALS1, HWP1* and ICA were $7.56x10^6$, $6.88x10^5$, and $4.20x10^5$ copies, respectively. In the PVP slime model, mean copy number was $2.70x10^2$, $2.27x10^1$ and $2.30x10^1$ for *ALS1, HWP1* and *ICA*, respectively. Mean copy number of adhesion genes on PUC were $1.50x10^1$, $1.20x10^1$ and $3.10x10^1$ for *ALS1, HWP1* and *ICA*, respectively. Real time PCR was more susceptible for the detection of expression of adhesion genes than that of conventional RT-PCR.

There was a statistically significant difference between the catheters in terms of expression levels of the adhesion genes ($p<0.05$). The highest levels of adhesion genes were found in PVC surfaces, while it was less in SC surface. PVP and PUC surfaces did not affect the gene expression levels when compared with planktonic cells.

Primer list for the targets was given on Table 1, the mean expression ratios of the *ALS1, HWP1* and *ICA1* genes in biofilm samples compared to the biomaterials and planktonic cells were given on Table 2, amplification curves of *ALS1* gene were shown on Figure 1, the com-

Table 2. *The mean expression ratios of the ALS1, HWP1 and ICA genes in biofilm samples compared to the biomaterials and planktonic cells.*

		Biomaterials coated with biofilm			
Gene	Planktonic cells	SC	PVC	PVP	PUC
C. albicans ALS1	10^1	$2.50x10^5$	$7.56x10^6$	$2.70x10^2$	$1.50x10^1$
C. albicans HWP1	10^1	$1.30x10^4$	$6.88x10^5$	$2.27x10^1$	$1.20x10^1$
S. epidermidis ICA	10^1	$1.80x10^5$	$4.20x10^5$	$2.30x10^1$	$3.10x10^1$

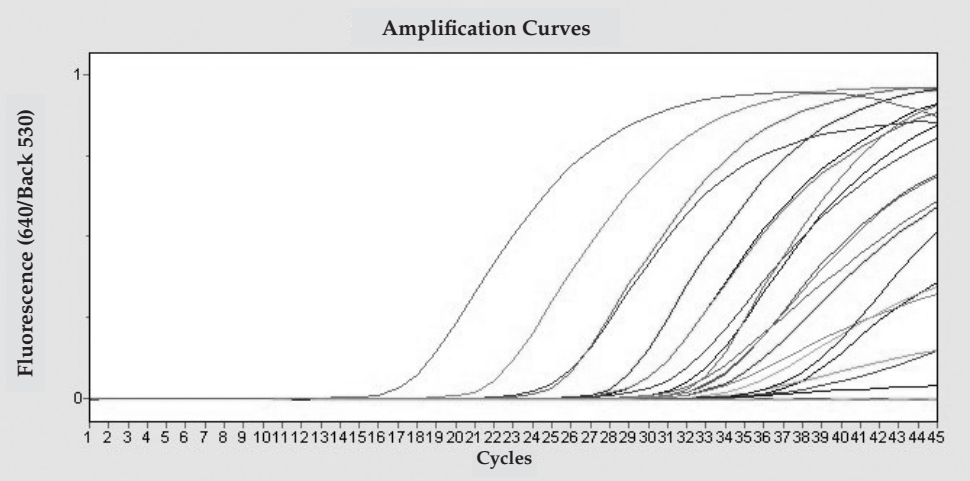


Figure 1. *Amplification curve of ALS1 gene.*

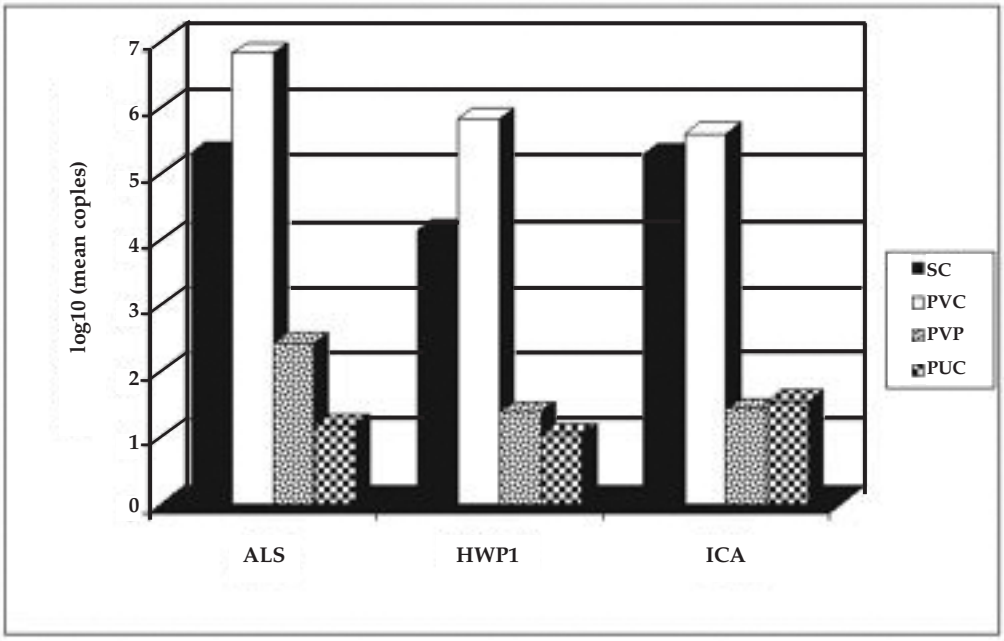


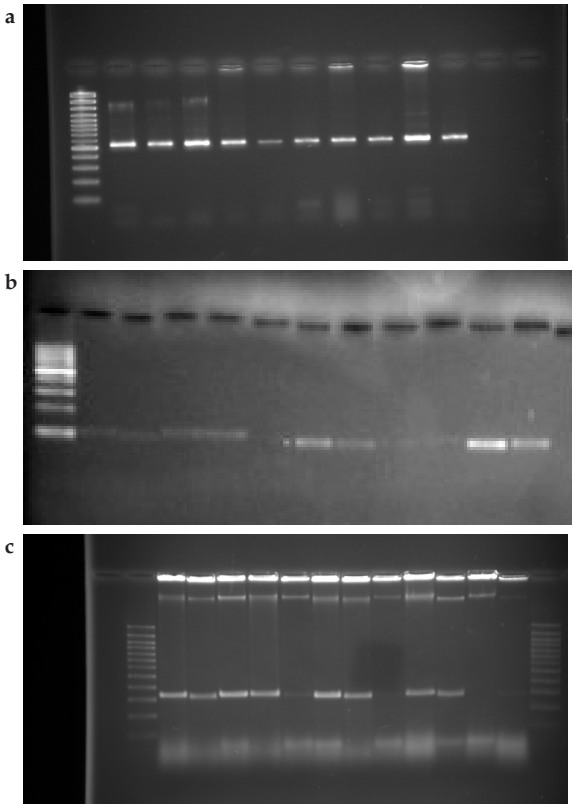
Figure 2. *The comparison of the catheters for the ability of gene expression in biofilms.*

parison of the catheters for the ability of gene expression in biofilms was shown on Figure 2 and *ALS1* cDNA amplicons (a:290 bp), *HWP1* cDNA amplicons (b:110 bp), *ICA* cDNA amplicons (c:350 bp) were shown on Picture 1.

DISCUSSION

In this study we compared latex-silicone coated (SC), polyvinyl chloride (PVC), poly (vinylpyrolidone) (PVP)-coated polyurethane and polyurethane catheter (PUC) for the delivery of adhesion genes in an in vitro biofilm model. Catheter material does affect the incidence of catheter-related infection when catheters are coated with a fibrin sheath[14] speculated that, this effect may be related to the expression of adhesion genes in the microorganisms which forms biofilms on different catheter materials through interactions with adhesive proteins.

Several studies have highlighted the differences in gene expression levels between biofilms and planktonic cells[6,19]. However, there



Picture 1. *a; ALS1 cDNA amplicons (290 bp), b; HWP1 cDNA amplicons (110 bp), c; ICA cDNA amplicons (350 bp).*

are large discrepancies between the studies, and the small number of differentially expressed genes that all (or most) of them have in common has caused several workers to question the existence of a "universal biofilm phenotype"[7,9]. We have chosen to analyze the expression of *ALS1, HWP1* in *C.albicans* and ICA in *S.epidermidis* because they encode large glycoproteins implicated in adhesion[2,10]. Therefore, these genes could be affected from material used in catheters and may contribute to biofilm formation on medical devices.

We found a difference between gene expression levels in biofilm cells isolated from biomaterials. High level of expression of *ALS1, HWP1* and *ICA1* genes were detected in the biofilms removed from PVC surfaces. SC, was found to be less affected to the expression of adhesion genes. Mean copy numbers of the adhesion genes were not significantly different in PVP and PUC surfaces when compared to planktonic cells. The difference was statisticaly significant between mean copies of genes in biofilms of SC, PVC, PVP and PUC surfaces.

Medical grade silicone rubber, also known as silastic, is easily modified and soft materials which is widely used for long-term access in animals and humans. In our study, we found this material has an disadvantage for the biofilm formation. Polyvinyl chloride, commonly abbreviated PVC, is a widely used thermoplastic polymer. But, we found that, using this material as a biomedical device, may result in biofilm formation. Polyurethane and polyvinylpyrolidone (PVP)-coated polyurethane catheter surfaces are hygroscopic and rapidly attracts water. The PVP coating induces a hydrophilic surface in body fluids for several hours[5]. We detected no biofilm formation on the surfaces of PVP and PUC catheters. We found these biomaterials are more suitable than those of PVC and SC as catheters in the human body. There are some similar results about the adhesions of the microorganisms on the PVC and SC surfaces. Bacterial adherence to PVC and siliconised latex was found to be significantly higher than PVC and SC surfaces[12]. Polyurethane caused the least adhesion formation among the four materials evaluated[17]. But, the quantitation of the adhesion genes was not reported before.

There are some limitations of our study. First, it is an in vitro study that needs confirmative in vivo studies. Second, we analysed only three adhesion genes in *C.albicans* and *S.epidemidis* biofilm cells. Other genes related to biofilm formation should be evaluated in another detailed study. Third, we did not analyse the amount of the biofilm mass which may be affected from the surface. But, our preliminary results, may open a new opinion in the biomaterial research.

Microorganism in a biofilm display significant phenotypical and genotypical changes[11]. Because of this, the contact with the biomaterial may be respond to an augmentation of adhesion gene expression differ from planktonic cell growth. In a biofilm, cell densities are substantially higher than in planktonic culture[3]. Because of this, it is not surprising that gene expression in sessile cells is very different from that in their free-floating counterparts.

Control strategies that have been used to

inhibit biofilm formation on catheters include antimicrobial ointments and lubricants, bladder instillation or irrigation, antimicrobial agents in the collection bags, impregnating the catheter with antimicrobial agents (silver oxide), and using systemic antibiotics for prophylaxis in catheterized patients[8]. With our study, another strategy was added to above cited ones; choosing the material according to biofilm formation rates of the surface.

CONCLUSION

Our study compares the catheters made from latex-silicone, polyvinyl chloride, polyvinylpyrolidone and polyurethane for the delivery of *ALS1, HWP1, ICA* adhesion genes in an in vitro biofilm model. High level of expression of adhesion genes were detected in the biofilms removed from PVC and SC surfaces. But, adhesion genes were not significantly different in PVP and PUC surfaces when compared to planktonic cells. It showed us, catheters materials may be choosen according to their ability to expression in adhesion genes.

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