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Comparing the Detection of Various Microorganisms on Metal and Glass Surfaces Using Incubation Methods on Selective Agars, Modified PCR Methods and Simultaneous Detection by A Unified Parallel PCR Method

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Although person-to-person transmission is the most likely route for health-care associated infections, the role of the environment should not be ignored and may also contribute to the spread of nosocomial infections. It has been proven that microorganisms are able to survive on environmental surfaces up to several weeks, therefore providing a potential for biotransfer. Assessment of environmental cleanliness is mainly conducted by surface sampling and swabbing followed by classical incubation methods based on phenotypic detection of microorganisms which take between 2 to 4 days. Novel quick methods based on DNA detection using PCR methods is a possibility for quick detection of the presence of DNA. In the present study, we investigated the presence of the following challenge microorganisms: *Enterococcus faecium*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* on metal and glass surface after 24 hour drying using classical incubation methods and PCR methods. We detected all challenge microorganisms at initial concentrations as low as 10^2 cfu/mL and final concentration after drying at 30 cfu/mL. PCR methods proved to be equal or more sensitive compared to classical incubation methods for all challenge microorganisms. A new PCR run was conducted where all challenge microorganisms were simultaneously detected in one run under the following conditions: initial denaturation at 94°C for 15 min; followed by 38 cycles of amplification (1 min denaturation at 94°C; 1 min annealing at 53°C; 1 min extension at 72°C) and final extension at 72°C for 10 min. Significance and impact: In this study we developed a unified parallel PCR method that enables simultaneous detection of all challenge microorganisms within a few hours at concentrations as low as 30 cfu/mL and can be used as a tool for quick evaluation of the cleanliness of surfaces.

Key words: PCR, detection limit, *Enterococcus faecium*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, parallel PCR program.

The main source of nosocomial or health-care associated infections are the individual patient, medical equipment or devices, the hospital environment, the healthcare personnel, contaminated drugs, contaminated food, and contaminated patient care equipment (Collins, 2008; Gastmeier *et al*., 2005). Although the person-to-person transmission route is the most likely, the role of the environment should not be ignored and may also contribute to the spread of nosocomial infections (Bureau-Chalot *et al*., 2004; Dancer, 2004). Therefore preventing diseases transmitted from inanimate surfaces common in hospital areas, such as: metal, glass, plastic, ceramics, and textiles etc., is also important. It has been proven that microorganisms are able to survive on environmental surfaces for periods up to several

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weeks (Wilks, 2006; Friedman *et al*, 2012), providing a significant biotransfer / cross contamination / cross-infection potential (Verran, 2002) that should not be overlooked (Sexton *et al*., 2011).

Survival on glass and steel surfaces has been investigated for several types of microorganisms. In the research by Oomaki and co-workers on the contamination of surfaces of working tables with *Staphylococcus aureus* in ward staff centers (2006) it was found that *S. aureus* was detected on 51.8 % of the working tables that were not regularly disinfected or washed. They also found that regular disinfection by wiping the surfaces with 80 % ethyl alcohol is necessary to reduce microbial counts. Robine and co-workers (2000) found that *Pseudomonas fluorescens* and *Enterococcus faecalis* aerosols survived on glass, polyvinyl chloride and stainless steel surfaces at various humidity levels and are linked to the colonization ability of the microorganisms onto the chosen surfaces as well as environmental conditions such as temperature and humidity. Kusumaningrum and co-workers (2003) also found that *Salmonella enteridis*, *Staphylococcus aureus* and *Campylobacter jejuni* survived on stainless steel surfaces at various concentrations for several days and can lead to transfer of these pathogens from kitchen sponges to stainless steel surfaces and from these surfaces to foods, thus highlighting the fact that pathogens remain viable on dry stainless steel surfaces and present a contamination hazard dependant on the contamination level and type of pathogen. In the study by Young-Min and co-workers (2012) it was found that various food-borne pathogens form biofilms on stainless steel surfaces at certain environmental conditions (temperature, humidity, availability of nutrient components etc.) thus enabling the transmission of pathogens to foods in the food-processing industry. Fuster-Valls and co-workers (2008) also found that the survival of *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* on stainless steel surfaces was influenced by different environmental conditions and that microorganisms on wet surfaces produced biofilms within 24 hours, whilst adverse conditions such as slow air-drying caused a significant number of injured cells that did not grow under conditions provided in plate counting methods. In the study by Shiomori and co-workers (2001) of the existence of airborne methicillin-resistant Staphylococcus aureus (MRSA) in a hospital environment it was found that MRSA particles were isolated within respirable range and were also found on inanimate environments, such as sinks, floors, and bedsheets, in the rooms of the patients with MRSA infections as well as from the patients' hands. The epidemiological study in this research demonstrated that clinical isolates of MRSA in the investigated ward were of one origin and that the isolates from the air and from inanimate environments were identical to the MRSA strains that caused infection or colonization in the inpatients. The authors concluded that measures should be taken to prevent the spread of airborne MRSA to control nosocomial MRSA infection in hospitals. A similar conclusion was noted in a review of intensive care unit admissions (Dress *et al*, 2008), where it was shown that previous room occupation by a patient carrying vancomycin-resistant enterococci (VRE) increased the odds that the next room occupant will contact a VRE infection. The authors concluded that increased attention to environmental disinfection is warranted. Important measures in preventing infections from inanimate surfaces besides hand hygiene of healthcare workers is regular cleaning and disinfection of surfaces as this reduces the risk of transmitting pathogens between patients and thus reduces the risk of exogenously acquired infections (Beggs *et al*. 2004).

Evaluating the disinfection efficiency of various surfaces by surface hygiene sampling as an indicator of health hazards in premises such as health-care establishments, kitchens, food-processing industry etc. is the next step. Although routine sampling of environmental sites is not likely a cost-effective intervention (Harris, 2008), it should be conducted when the cause of outbreaks are not at hand. Until now the most commons methods used for surface sampling include: RODAC surface sampling and swabbing followed by classical incubation methods based on phenotypic detection of microorganisms which can take between 2 to 4 days to be completed. Another possibility is the use of Petrifilms (Fijan *et al*., 2008) and other similar commercial contact methods, however they do not report actual bacterial counts but measure some characteristics related to bacterial mass etc. Much of the increase in surface

sapling has been brought by the introduction of hazard analysis and critical control points (HACCP) (Fijan *et al*, 2006). High-through put sequencing is of course very reliable method of the future, however at present multiple bacterial sequencing is usually optimized to bacterial phyla, families, or to a limited very amount of species (Flores *et al*, 2011). A fast and reliable possibility compared to incubation methods and commercial methods is to use PCR detection of microorganisms from swabs, shortening the detection process to a few hours. PCR methods detect the DNA of present microorganisms and multiplex PCR are assays where two or more targets are simultaneously amplified in one reaction thus giving potential for greater reliability, flexibility and cost reduction (Xu *et al*, 2012). This cost-saving technique is applicable for large-scale scientific, clinical and commercial applications such as infectious microorganism detection (Pinar *et al*., 2004), diagnosis of infectious disease (Elnifro *et al*., 2000), gene expression (Hess *et al*., 2004) etc. However, there are several disadvantages of using one reaction tube multiplex PCR, such as complex manipulation, lower sensitivity, self-inhibition, amplification disparity resulting from different primers etc. (Xu *et al*, 2012). One possibility is to use PCR strip tubes and search for eight or twelve most common pathogens in one simultaneous parallel PCR run by using eight or twelve separate reaction tubes for each oligonucleotide primer pair, thus conducting a parallel PCR assay. Such an assay could be done routinely by various laboratories to quickly ascertain the disinfection efficiency of cleaning at a reasonable price.

In the first part of this paper PCR methods are compared to the classical incubation methods. The challenge microorganisms chosen in this research are representatives of Gram positive bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, and *Bacillus subtilis*; Gram negative bacilli: *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and yeast representative: *Candida albicans*. In the second part of the research a parallel PCR protocol is developed enabling the possibility of simultaneous detection of all challenge DNA in one PCR run, thus providing an example of a parallel PCR assay that could be used for regular detection of surface disinfection.

MATERIALS AND METHODS

Challenge microorganisms

48 hour cultures of *Enterococcus faecium* (ATCC 6057), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (DSM 618), *Escherichia coli* (DSM 1562), *K. pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 2091) grown in tryptic soy nutrient broth (Fluka) were used. Different concentrations of microorganisms were prepared in descending order (noted as 1 to 6) by diluting with saline solution. Before each experiment viable counts of all concentrations of microorganisms were made, to enable the calculation of the number of cells inoculated onto the surfaces by the method noted below.

Application of microorganisms on chosen surfaces

Two surfaces were investigated in this research: stainless steel and glass. Glass Petri dishes with a diameter 15 cm (area 176 cm^2) were used and stainless steel dishes (13.3 x 13.3 cm) with the same area as the glass Petri dishes and a 1 cm edge were custom-made. All surfaces were sterilized before use. For each experiment 1 mL of the prepared suspension of microorganisms was applied onto the surface and evenly distributed by carefully manually rotating the glass and steel containers. All work was repeated for each concentration and each microorganism in duplicate. The experiments were conducted in a laminar flow cabinet and samples were left in the laminar flow cabinet without air at 22°C for 24 hours to allow the cell suspensions to dry on the surfaces under environment conditions (slow air-drying surfaces).

Swabbing

Cotton swabs were pre-treated by dipping them into a prepared 0.9 % NaCl and 0.2 % Tween 80 solution. The tested surfaces were swabbed firmly and evenly with one side of the swab in a horizontal direction, and with the other side in a vertical direction back and forth to cover the entire area. The swab was transferred into 5 mL NaCl and Tween 80 solution, vortexed for 30s, and then left for 10 minutes and revortexed for 30s.

Incubation and colony counting

Viable cell counting was determined by making 10-fold serial dilutions (in duplicate) in 0.85 % NaCl of all original samples and samples retrieved from swabs followed by spreading 100 µL onto

selected media for each microorganism. At low concentrations 1000 µL was spread onto appropriate media. The following selective agars were used as a medium for incubating (at 37 °C) the microorganisms:

- *E. faecium*: kanamycin esculinazide agar base (incubation 48 hours).
- *S. aureus:* Baird-Parker agar base with added egg-yolk tellurite emulsion (incubation 48 hours).
- *B. subtilis*: MYP agar base with added egg yolk emulsion supplement and polymyxin supplement (incubation 48 hours).
- *E. coli*: violet red bile dextrose *Agar base* (incubation 48 hours).
- *K. pneumoniae:* Hi Crome *Klebsiella* selective agar base with *Klebsiella* selective agar base (incubation 24 hours).
- *P. aeruginosa:* cetrimide agar base with added glycerol (incubation 48 hours).
- *C. albicans:* HiCrome Ogye agar base with oxytetra selective supplement (incubation 72 hours).

After incubation, colonies were counted and the cfu was calculated. All tests were done in duplicate, the counts were converted into decimal logarithmic values and the mean log cfu/mL as well as the standard deviation was calculated. Counts obtained from glass and steel surfaces were compared using the paired T-test with IBM SPSS 20.0 software.

DNA detection

Isolation of DNA

Bacterial and fungal genomic DNA was extracted from the suspension of microorganisms retrieved from swabs. PrepMan Ultra Sample Preparation Reagent (Applied Biosystems) was used in accordance with manufacturer's instructions. Bacterial and fungal genomic DNA extracted from an overnight culture in liquid broth was used as a positive control. Extracted DNA was stored at -20° C until use.

Oligonucleotide primer selection

The target genes for the seven challenge microorganisms are shown in Table 1. The following oligonucleotide primer pairs were used: EM1 for *E. faecium* (Cheng *et al*, 1997); spa for *S. aureus* (Matussek *et al*. 2007); Bsub for *B. subtilis* (Wattiau *et al*., 2001), *GADA* for *E. coli* (McDaniels *et al*., 1996), ITS for *K. pneumoniae* (Liu *et al*., 2008), *gyrB* for *P. aeruginosa* (Motoshima *et al*., 2007) and ALB for *C. albicans* (Jordan and Durso, 1996).

Individual DNA amplification

PCR was performed using HotStarTaq DNA polymerase from Qiagen following the manufacturers' instructions where. The reaction mix with a final volume of 50 µL was separately prepared for each challenge microorganism and consisted of 1.5 mM10x PCR buffer with 15 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each oligonucleotide primer and 2.5 units of HotStarTaq polymerase per reaction (Rabuza *et al*., 2012). 5 µL of DNA template was added for all challenge bacteria and 1 µL of DNA template was added for the fungi *C. albicans*. Sensoquest S lab cycler was used for amplification under conditions noted in table II. $MgCl_2$ was also added to the PCR reactions in different concentrations as follows: 1 mM for *E. faecium, E. coli, P. aeruginosa* and *C. albicans*; 3.5 mM $MgCl_2$ for *B. subtilis* and 0.5 mM *S. aureus*. Each PCR reaction included positive controls directly from nutrient broths and negative controls containing sterile water. DNA amplification procedures noted in the selected literature (Cheng *et al*, 1997; Matussek *et al*. 2007*;* Wattiau *et al*., 2001; McDaniels *et al*., 1996; Liu *et al*., 2008; Motoshima *et al*., 2007; Jordan and Durso, 1996) were slightly modified and are noted in table II.

Simultaneous parallel DNA amplification

In the final set of experiments a simultaneous parallel DNA amplification was conducted with initial denaturation at 94°C for 15 min followed by 38 cycles (denaturation at 94°C for 1 min; annealing at 53°C for 1 min and extension at 72°C for 1 min) and final extension for 10 min at 72°C.

Detection of PCR amplicons

Agarose gel electrophoresis was performed to visualize amplified products with 1.2 % agarose gel (Sigma) in 0,5 TBE buffer (89 mMTris base, 89 mM Boric acid, 2 mM EDTA) stained with SYBR Green I nucleic acid gel stain (Sigma Aldrich) with a 100 bp ladder (Promega). Gels were visualized under UV illuminator Transilluminator Super-Bright (VilberLourmat) at 312 nm using a gel images system Doc Print VX2 (VilberLourmat) to confirm the presence of the amplified DNA. Images were processed by the Photo-Capt software.

RESULTS

The results of swabbing metal and glass surfaces inoculated with different concentrations

Table 1. Oligonucleotides used for PCR amplification

Target	Primer	Primer 5'———3'	Product size (bp)
Enterococcus faecium	EM1[1]	f (5'-TTG AGG CAG ACC AGA TTG ACG-3') r (5'-TAT GAC AGC GAC TCC GAT TCC-3')	658
Staphylococcus aureus	Spa[2]	f (5'-TAA AGA CGA TCC TTC GGT GAC C-3') r (5'- CAG CAG TAG TGC CGT TTG CTT-3')	380
Bacillus subtilis	Bsub[3]	f (5'-AAG TCG AGC GGA CAG ATG G-3') r (5'-CCA GTT TCC AAT GAC CCT CCC C-3')	595
Escherichia coli	gadB[4]	f (5'-ACC TGC GTT GCG TAA ATA-3') r (5'-GGG CGG GAG AAG TTG ATG-3')	670
Klebsiella pneumoniae	ITS[5]	f (5'-ATT TGA AGA GGT TGC AAA CGA T-3') r (5'-TTC ACT CTG AAG TTT TCT TGT GTT C-3')	130
Pseudomonas aeruginosa	*gyrB*[6]	f (5'-CCT GAC CAT CCG TCG CCA CAA C-3') r (5'-CGC AGC AGG ATG CCG ACG CC-3')	222
Candida albicans	ALB[7]	f (5'-CGC CTC TTG ATG GTG ATG AT-3') r (5'-TCC GGT ATC ACC TGG CTC-3')	122

Notes: Cheng *et al*, 1997[1]; Matussek *et al*. 2007[2]; Wattiau *et al*., 2001[3];McDaniels *et al*., 1996[4]; Liu *et al*., 2008[5]; Motoshima *et al*., 2007[6]; Jordan and Durso, 1996[7]

Table 2. Conditions for individual PCR amplification for each microorganism and unified parallel PCR amplification for all challenge microorganisms

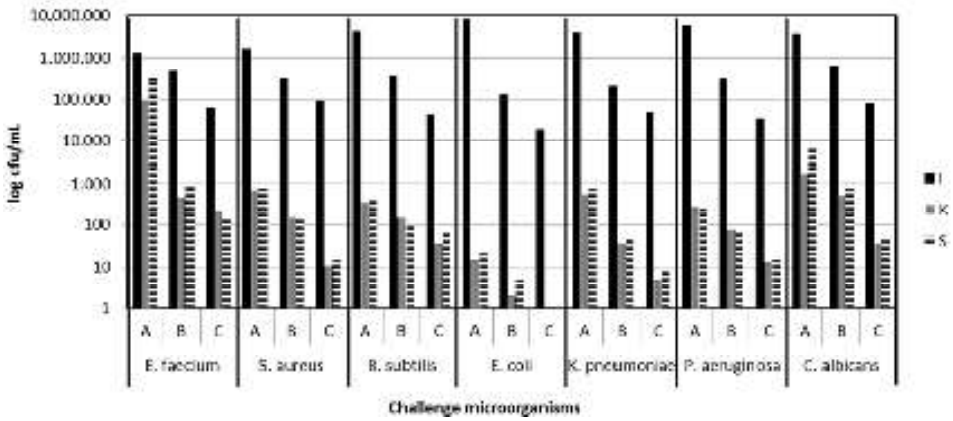
Microorganism	Initial denaturation	PCR amplification: - Denaturation - Annealing - extension	Number of cycles	Final extension
Enterococcus faecium	94°C/15 min	94°C / 1 min 54°C / 1 min 72°C / 1 min	40	72°C/7 min
Staphylococcus aureus	94°C/15 min	94°C / 1 min 58°C / 1 min 72°C / 1 min	40	72°C/7 min
Bacillus subtilis	94°C/15 min	94°C / 30 s 65°C / 2 min 72°C / 2 min	30	72°C/7 min
Escherichia coli	94°C/15 min	94°C / 30 s 52°C / 30 s 72°C / 1 min	35	72°C/7 min
Klebsiella pneumoniae	94°C/15 min	94°C / 1 min 56°C / 1 min 72°C / 1 min	42	72°C/7 min
Pseudomonas aeruginosa	94°C/15 min	94°C / 1 min 55°C / 1 min 72°C / 1 min	40	72°C/7 min
Candida albicans	94°C/15 min	94°C / 1 min 54°C / 2 min 72°C / 1 min	30	72°C/7 min
Unified parallel PCR	94°C/15 min	94°C / 1 min 53°C / 1 min 72°C / 1 min	38	72°C/10 min

of challenge microorganisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans*) after 24 hour drying at room temperature are noted in table III for all concentrations and in figure 1 for three initial inoculation concentrations (A = 10^6 cfu/mL, B = 10^5 cfu/mL and C = 10^4 cfu/mL). It was found that *Escherichia coli* proved to be the least tolerant challenge microorganism as a positive result for both PCR and incubation on selective agar was found at the original concentration of inoculation at 5.11 log cfu/mL. All microorganisms exhibited very similar results after 24 hour drying on glass and steel surfaces, with survival on glass surfaces being only slightly higher (based on slightly higher mean for glass), and insignificantly different according to the paired T-test (p>0,5).

Table 3. Results of swabbing metal and glass surfaces after 24 hour drying

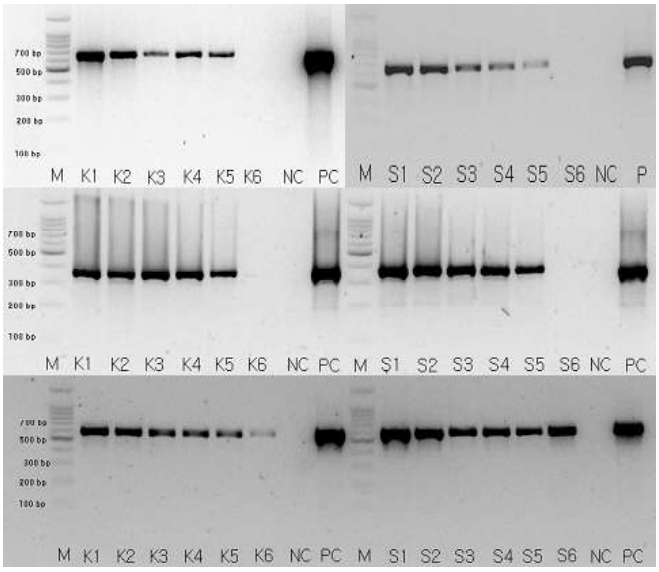
Challenge microorganism		Mean numbers (log cfu/mL) ± SD					
		1	2	3	4	5	6
E. faecium	I	6.11 ± 0.33	5.67 ± 0.30	4.82 ± 0.24	4.08 ± 0.16	3.20 ± 0.21	2.54 ± 0.22
	K	4.95 ± 0.57	2.65 ± 0.48	2.33 ± 0.47	1.83 ± 0.24	0.30 ± 0.14	0.00*
	S	5.18 ± 0.84	2.93 ± 0.57	2.10 ± 0.28	1.61 ± 0.41	0.48 ± 0.24	0.00
	P	+/+	+/+	+/+	+/+	+/+	–/–
S. aureus	I	7.99 ± 0.33	7.08 ± 0.21	6.20 ± 0.21	5.48 ± 0.24	4.96 ± 0.20	3.31 ± 0.28
	K	4.59 ± 0.55	3.45 ± 0.57	2.82 ± 0.34	2.15 ± 0.35	1.04 ± 0.23	0.00
	S	4.62 ± 0.52	3.11 ± 0.78	2.86 ± 0.47	2.18 ± 0.31	1.18 ± 0.16	0.00
	P	+/+	+/+	+/+	+/+	+/+	–/–
B. subtilis	I	7.54 ± 0.23	6.63 ± 0.24	5.94 ± 0.23	5.54 ± 0.23	4.64 ± 0.20	3.39 ± 0.21
	K	3.95 ± 0.68	2.54 ± 0.33	2.42 ± 0.54	2.14 ± 0.51	1.52 ± 0.18	1.11 ±0.32
	S	3.28 ± 0.85	2.64 ± 0.49	2.18 ± 0,45	1.99 ± 0.30	1.81 ± 0.25	1.04 ±0.37
	P	+/+	+/+	+/+	+/+	+/+	+/+
E. coli	I	8.78 ± 0.23	7.64 ± 0.23	6.92 ±0.11	5.11 ± 0.27	4.28 ± 0.16	3.52 ± 0.32
	K	4.38 ± 0.69	3.36 ± 0.42	1.18 ± 0.45	0.30 ± 0.23	0.00	0.00
	S	4.23 ± 0.62	2.89 ± 0.86	1.34 ± 0.51	0.70 ± 0.41	0.00	0.00
	P	+/+	+/+	+/+	+/+	–/–	–/–
K. pneumoniae	I	8.18 ± 0.37	7.69 ± 0.16	6.59 ±0.23	5.32 ± 0.25	4.70 ± 0.21	2.85 ± 0.24
	K	3.95 ± 0.74	2.88 ± 0.44	2.73 ± 0.37	1.53 ± 0.20	0.69 ± 0.23	0.00
	S	4.30 ± 0.42	3.13 ± 0.81	2.89 ± 0.30	1.68 ± 0.27	0.95 ± 0.25	0.00
	P	+/+	+/+	+/+	+/+	+/+	+/+
P. aeruginosa	I	8.11 ± 0.27	7.10 ± 0.28	6.77 ± 0.18	5.46 ± 0.28	4.54 ± 0.25	3.63 ± 0.31
	K	3.99 ± 0.44	3.18 ± 0.59	2.42 ± 0.34	1.87 ± 0.17	1.11 ± 0.24	0.00
	S	3.92 ± 0.54	3.04 ± 0.44	2.38 ± 0.31	1.80 ± 0.42	1.15 ± 0.40	0.00
	P	+/+	+/+	+/+	+/+	+/+	+/+
C. albicans	I	7.88 ± 0.17	6.57 ± 0.28	5.77 ± 0.16	4.92 ± 0.25	3.11 ± 0.27	2.99 ± 0.22
	K	4.76 ± 0.66	3.23 ± 0.45	2.71 ± 0.27	1.52 ± 0.34	0.30 ± 0.32	0.00
	S	4.51 ± 0.41	3.83 ± 0.38	2.88 ± 0.17	1.68 ± 0.30	0.78 ± 0.45	0.00
	P	+/+	+/+	+/+	+/+	+/+	–/–

Notes: counts below the detection limit were given a log value of 0.00*; Where: log cfu: calculated average number of colony forming units per mL and standard deviance from twofold experiments converted into decimal logarithmic values; I: initial log cfu of challenge microorganism in nutrient broth; numbers 1 to 6: different dilutions in descending order; K: log cfu of challenge microorganism on metal surface after 24 hour drying; S: log cfu of challenge microorganism on glass surface after 24 hour drying; P: detection of DNA using PCR for K and S samples respectively



Where I: initial concentration of microorganisms on surfaces, K: concentration of microorganisms on metal surfaces; S: concentration of microorganisms on glass surfaces

Fig. 1. Comparison of cfu of challenge microorganisms after 24 hour drying on metal and glass surface at analogue initial concentrations ($A=10^6$, $B=10^5$ and $C=10^4$ cfu/mL)



where M: molecular weight marker; NC: negative control, PC: positive control; K1-6: samples from metal surfaces; S1-6: samples from glass surfaces

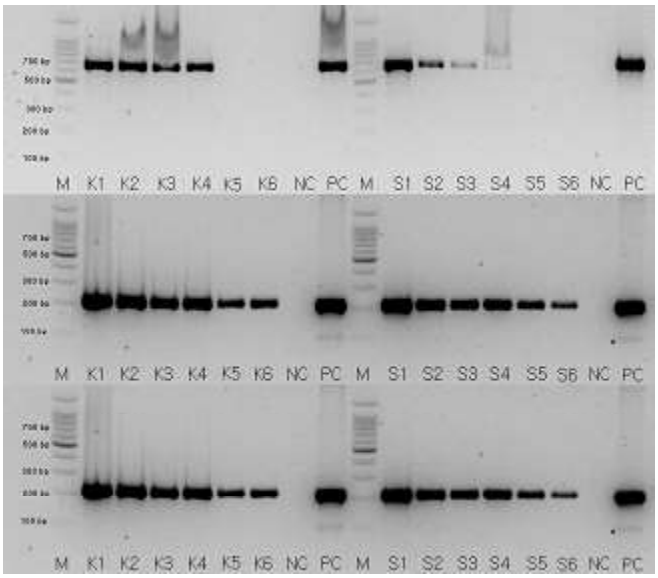
Fig. 2. Gel electrophoresis of PCR products for challenge Gram positive bacteria of swabbing metal and glass surfaces after 24 hour drying Upper part: *Enterococcus faecium* (658 bp); middle part: *Staphylococcus aureus* (380 bp); lower part: *Bacillus subtilis* (595 bp)

Perhaps greater differences occur after 24 hour initial drying. This finding corresponds to the finding of Robine *et al* (2000) where the highest mortality was measured on PVC surfaces and then decreasingly on glass and steel. *Enterococcus faecium* and *Candida albicans* showed on Fig. 1 the lowest log reduction at all initial concentration of inoculations thus proving to be the most tolerant challenge microorganisms.

Figures 2, 3 and 4 show results of gel electrophoresis of PCR products of swabbing metal and glass surfaces after 24 hour drying for challenge Gram positive bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Bacillus*

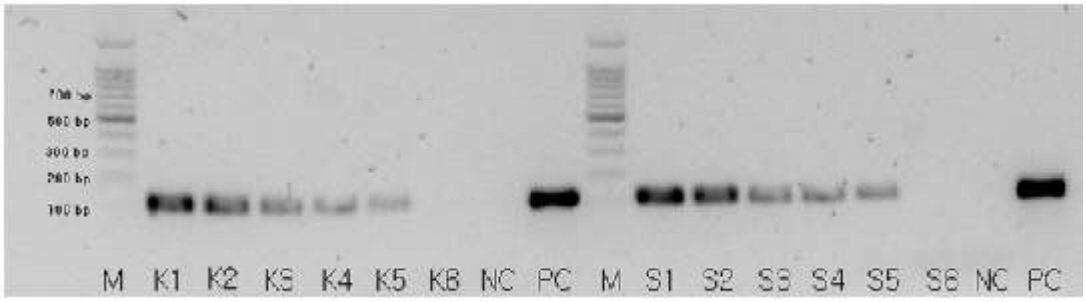
subtilis); Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and challenge fungi (*Candida albicans*) respectively. We found positive results for samples with an original concentration of inoculation of at least 3 log cfu/mL for *Enterococcus faecium*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans*. On the other hand we found that *Staphylococcus aureus* and *Escherichia coli* were not found on the steel and glass surfaces at the original concentration of inoculation of 3.31 log cfu/mL and 3.52 log cfu/mL respectively. *Klebsiella pneumoniae* proved to be the only challenge microorganism that, in the same sample, yielded a positive PCR result and negative result from incubation on selective agar, however, this result was at the lowest initial inoculated concentration of 2.85 log cfu/mL.

Figure 5 upper part corresponds to the results of the optimized unified parallel PCR program that yielded a positive band for all challenge microorganisms at lowest and highest concentrations except for *Enterococcus faecium*



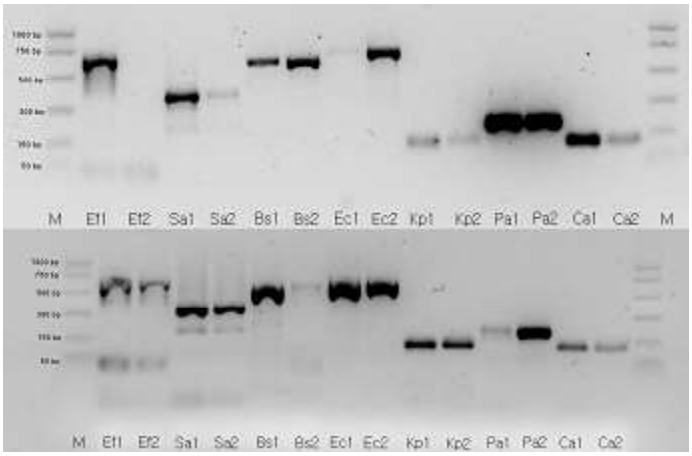
where M: molecular weight marker; NC: negative control, PC: positive control; K1-6: samples from metal surfaces; S1-6: samples from glass surfaces

Fig. 3. Gel electrophoresis of PCR products for challenge Gram negative bacilli of swabbing metal and glass surfaces after 24 hour drying Upper part: *Escherichia coli* (670 bp); middle part: *Klebsiella pneumoniae* (130 bp); lower part: *Pseudomonas aeruginosa* (222 bp);



where M: molecular weight marker; NC: negative control, PC: positive control; K1-6: samples from metal surfaces; S1-6: samples from glass surfaces

Fig. 4. Gel electrophoresis of PCR products for challenge fungi *C. albicans* (122 bp) of swabbing metal and glass surfaces after 24 hour drying



Where Ef: *E. faecium* (658 bp); Sa: *S. aureus* (380 bp); Bs: *B. subtilis* (595 bp); Ec: *E. coli* (670 bp); Kp: *K. pneumoniae* (130 bp); Pa: *P. aeruginosa* (222 bp); Ca: *C. albicans* (122 bp); M: molecular weight marker; NC: negative control, PC: positive control; 1: highest concentration; 2: lowest detected concentration.

Fig. 5. Gel electrophoresis of PCR products after unified parallel PCR amplification of swabbing metal surfaces after 24 hour drying Upper part: isolated DNA of each challenge microorganism was added separately; lower part: samples were taken from a mixture of isolated DNA;

at the lowest concentration. In figure 5 lower part the results of the optimized PCR program run on a mixture all isolated DNA is noted. It is obvious that all challenge microorganisms were detected at both low and high concentrations.

DISCUSSION

The limit of detection for incubation methods in our study was 1 cfu per mL (1 mLis the amount that was inoculated onto the selective media) based on the theory that one colony forming unit corresponds to one or more initial viable cells thus the estimation of microbial numbers by cfu will, in most cases, slightly undercount the number of living cells present in the original sample. The initial volume of saline solution with Tween 80 into which the swabs were dipped was 5 mL; therefore, if the whole solution contained only 1 viable cell, this meant that there was only a 20 % probability of capturing this cell and inoculating it onto the selective media. In theory; PCR methods also have a limit of 1 single DNA copy, however if 1 cell is in a 5 mL volume and 5 µL is used for one PCR reaction, then the probability of capturing this cell is very low (1 positive reaction out of 200). Therefore, in actual experiments, the situation at very low concentrations is a result of chance. According to Mata and co-workers (2004) the detection limit per PCR was between 30 to 60 cells for various streptococci species and around 100 cells for *Lactococcus* species. In our research we found similar results as samples with an original concentration of inoculation of at least 3 log cfu/mL yielded positive results above 1 cfu/mL (detection limit of experiments) by incubation methods on selective agars and positive PCR bands (see figures 2 to 4 and table III) for *Enterococcus faecium*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans*. However, since plate counts below 30 cfu/mL are not statistically reliable, we can thus conclude that incubation methods and PCR methods produced positive results at a concentration of at least 30 cells per mL. On the other hand we found that *Staphylococcus aureus* and *Escherichia coli* were not found on the steel and glass surfaces at the original concentration of inoculation of 3 log cfu/mL. However, this was connected to the fact that the drying process decreased the number of cells in a slightly higher concentration that for the other challenge microorganisms and was not connected to a different detection limit. *Klebsiella pneumoniae* proved to be the only challenge microorganism that, in the same sample, yielded a positive PCR result and negative result from incubation on

selective agar, however, this result was at the lowest initial inoculated concentration (< 3 log cfu/mL) thus being below the above mentioned threshold for detection of inoculated concentration.

Fuster-Valls and co-workers (2008) in the research on the survival of *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* on stainless steel surfaces found that adverse conditions such as slow air-drying caused a significant number of injured cells that did not grow under conditions provided in plate counting methods, but were present in direct epifluorescence microscopy, thus still presenting a danger. However, according to the authors, this method technique has two important limitations: a detection limit of 2-3 logarithmic reductions (Grand *et al*., 2011) and equipment and qualified personnel requirements. These findings also suggest that cultivation methods are not always an exact indicator of the presence of microorganisms on surfaces. It is for this reason that in our research PCR was used.

In the second part of the research we conducted a series of experiments using eight PCR strip tubes, where we added into seven reaction tubes (final volume of 50mL) 1.5 mM 10x PCR buffer with 15 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 units of HotStarTaq polymerase and nuclease free water. Then, we added into individual reaction tubes 5 µL of only one type of isolated bacterial DNA and 1 µL of isolated fungal DNA (samples from metal surfaces) together with the corresponding oligonucleotide primer pair, thus creating seven different reaction mixes. We conducted several PCR amplifications at various conditions and found that the optimal annealing temperature was at 53°C. The optimized unified parallelPCR program was as follows: initial denaturation at 94°C (15 min) followed by 38 cycles: denaturation at 94°C (1 min); annealing at 53°C (1 min); extension at 72°C (1 min); and final extension for 10 min at 72°C. This simultaneously run program yielded a positive band for all challenge microorganisms at lowest and highest concentrations except for *Enterococcus faecium* at the lowest concentration. Thus proving that a unified PCR protocol where all samples can be processed simultaneously in one run is possible. Of course, to simulate real conditions we created a mix of individual DNA templates of the challenge microorganisms and repeated the unified PCR program to see if various DNA templates inhibit each other. We added 10 µL of each bacterial DNA template and 5 µL of the challenge fungal DNA template thus creating a diverse sample with a volume of 65 µL. 5 µL of this diverse sample was then added to seven different reaction mixes prepared analog to the above mentioned protocol. This yielded positive results for all challenge microorganisms at low and high concentrations (figure 5, lower part). It is obvious from this figure that no inhibition occurred due to the presence of various DNA templates, thus proving that this method could be applied routinely for the detection of most common pathogens on various surfaces. Although this system is based on the detection DNA and not based on incubation methods of viable cells, it has been shown in our research that when we detected DNA, we almost always detected viable cells through incubation methods. Although it is not necessary in general that detection of DNA means the presence of infectious microorganisms, it is also important to detect the presence of DNA of pathogenic microorganisms on inanimate surfaces since extracellular DNA may also be important for transferring genetic information between individuals and species (Nielsen *et al*., 2007) as accumulating nucleotide sequence data suggest that acquisition of foreign DNA by horizontal gene transfer (HGT) is of considerable importance in bacterial evolution. The uptake of extracellular DNA by natural transformation is one of several ways bacteria can acquire new genetic information given sufficient size, concentration and integrity of the DNA.

Future work would include extending list of chosen microorganisms to twelve by finding oligonucleotide primer pairs for these pathogens that work at the conditions of the unified parallel PCR program and do not interfere with other DNA templates as well as checking the reliability of the protocol for different strains of the challenge species and testing the system on other surfaces and environments thus becoming a quick, efficient and cost-effective method for detecting hygiene of surfaces. Cleaning audits as well as education and reinforcement of requirements of cleaning and disinfection mostly take place in institutions (Friedman *et al*, 2012) such as health-care

associated facilities, pharmaceutical industry, food-processing industry, however more frequent audits using quick, efficient and cost-effective detection methods may become the overall approach to reducing environmental contamination of frequently touched surfaces in these settings and thus limit the transfer of environmentally persistent pathogens that can cause serious illness to patients, healthcare workers, workers in the food-processing industry and pharmaceutical industry etc.

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REFERENCES

1. Beggs, C.B., Shepherd, S.J.andKerr, K.G., How does health care worker hand hygiene behaviour impact up on the transmission of MRSA between patients?: ananalysis using a Monte Carlo model. *BMC Infect Dis* 2009; **9**: 64.
2. Bureau-Chalot, F.,Piednoir, E., Camus,J. and Bajolet.O., Microbiologic quality of linen and linen rooms in short-term care units. *J Hosp Infect* 2004; **56**: 329-331.
3. Cheng, S.,McCleskey, F.K.,Gress, M.J., Petroziello, J.M., Liu, R., Namdari, H., Beninga, K., Salmen, A.and DelVecchio, V.G., A PCR assay for identification of *Enterococcus faecium*. *J Clin Microbiol* 1997; **35**: 1248-1250.
4. Collins, A.S., Preventing Healthcare—Associated Infections. In Patient Safety and Quality: An Evidence-Based Handbook for Nurses ed.Hughes, R.G. RockvilleMD: Agency for Healthcare Research and Quality 2008 pp. 547–575.
5. Dancer, S.J., How do we assess hospital cleaning? A proposal for microbiological standards for surface hygiene in hospitals. *J Hosp Infect* 2004; **56***:* 10-15.
6. Drees, M., Snydman, D.R., Schmid, C.H., Barefoot, L., Hansjosten, K., VueP.M., Cronin, M., Nasraway, S.A. andGolan, Y., Prior environmental contamination increases the risk of acquisition of vancomycin-resistant enterococci. *ClinInfectDis* 2008; **46**: 678–685.
7. Elnifro, E., Ashshi, A., Cooper, R. and Klapper, P., Multiplex PCR: optimization and application in diagnostic virology. *ClinMicrobiol Rev* 2000; **13**: 559–570.
8. Fijan, S., Cenciè, A. andŠostar-Turk, S., Hygiene monitoring of textiles used in the food industry. *Braz j microbiol* 2006; **37**: 356-361.
9. Fijan, S., Gunnarsen, J.T.H., Weinreich, J.and Šostar-Turk, S., Determining the hygiene of laundering industrial textiles in Slovenia, Norway and Denmark. *Tekstil* 2008; **57**: 73-95.
10. Flores, G.E., Bates, S.T., Knights, D., Lauber, C.L., Stombaugh, J., Knight, R. andFierer, N., Microbial biogeography of public restroom surfaces. *PLoS One* 2011; **6**: e28132.
11. Friedman, N.D., Walton, A.L., Boyd, S., Tremonti, C.,Low, J. Infection Control GD, Styles, K., Harris, O., Alfredson, D. and Athan, E., The effectiveness of a single-stage versus traditional three-staged protocol of hospital disinfection at eradicating vancomycin-resistant Enterococci from frequently touched surfaces. *Am J Infect Control,* 2012. **6553**(12): 780-8.
12. Fuster-Valls, N., Hernandez Herrero, M., Marin-de-Mateo, M. and Rodriguez Jerez, J., Effect of different environmental conditions on the bacteria survival on stainless steel surfaces. *Food control* 2008; **19**: 308-314.
13. Gastmeier, P., Stamm-Balderjahn, S., Hansen, S., Nitzschke-Tiemann, F.,Zuschneid, I., Groneberg, K. and Rüden, H., How outbreaks can contribute to prevention of nosocomial infection: Analysis of 1,022 outbreaks. *Infect Control Hosp Epidem.* 2005; **26**: 357–361.
14. Grand, I., Bellon-Fontaine, M.N, Herry, J.M., Hilaire, D., Moriconi, F.X.andNaitali, M., Possibleoverestimation of surface dis infection efficiency by assessment methods mased on liquid sampling procedures as demonstrated by In situ quantification of spore viability. *Appl Environ Microbiol* **77**: 6208-6214.
15. Harris, A.D., How important is the environment in the emergence of nosocomial antimicrobial-resistant bacteria? *ClinInfectDi*s 2008; **46**: 686-8.
16. Hess, C.J., Denkers, F., Ossenkoppele, G.J., Waisfisz, Q., Mc Elgunn, C.J., Eldering, E., Schouten, J.P. and Schuurhuis, G.J., Gene expression profiling of minimal residual disease in acute myeloid leukaemia by novel multiplex-PCR-based method. *Leukemia* 2004; **18**: 1981–1988.
17. Jordan, J.A. and Durso, M.B., Rapid Speciation of the Five Most Medically Relevant *Candida* species using PCR Amplification and a Microtiter Plate-Based Detection system. *Mol Diagn* 1996; **1**: 51-58.

18. Kusumaningrum, H.D., Riboldi, G., Hazeleger, W.C. and Beumer, R.R., Survival of foodbornepathogens on stainless steel surfaces andcross-contamination to foods. *Int J Food Microbiol* 2003; **85**: 227-236.
19. Liu, Y., Liu, C., Zheng, W., Zhang, X., Yu, J., Gao, Q., Hou, Y. and Huang, X., PCR detection of *Klebsiella pneumoniae* in infant formula based on 16S-23S internaltranscribedspacer. *Int J FoodMicrobiol* 2008; **31**: 230-5.
20. Matussek, A., Taipalensuu, J., Einemo, I.M., Tiefenthal, M. and Löfgren, S., Transmission of *Staphylococcus aureus* from maternity unit staff members to new borns disclosed throughs patyping. *Am J Infect Control* 2007; **35**: 122-5.
21. Mata, A.I., Gibello, A., Casamayor, A.,Blanco, M.M., Domíngue, L.Z. and Fernández-Garayzábal, J.F., Multiplex PCR Assay for Detection of Bacterial Pathogens Associated with Warm-Water Streptococcosis in Fish. *Appl Environ Microbiol* **70**: 3183–3187.
22. McDaniels, A.E., Rice, E.W., Reyes, A.L., Johnson, C.H., Haugland, R.A. and Stelma G.N. Confirmational identification of *Escherichia coli,* a comparison of genotypic and phenotypic assays for glutamate decarboxylase and beta-D-glucuronidase. *Appl Environ Microbiol* 1996; **62**: 3350.
23. Motoshima, M., Yanagihara, K., Fukushima, K., Matsuda, J., Sugahara, K., Hirakata, Y., Yamada, Y., Kohno, S. and Kamihira, S. (2007) Rapid and accurate detection of *Pseudomonas aeruginos* abyreal-time polymera secha in reaction with melting curve analysis target in ggyrB gene. *Diagn Microbiol Infect Dis* 1996; **58**: 53-58.
24. Nielsen, K.M., Johnsen, P.J., Bensasson, D. and Daffonchio, D., Release and persistence of extracellular DNA in the environment. *Environ Biosafety Res* 2007; **6**: 37-53.
25. Oomaki, M., Yorioka, K., Oie, S. and Kamiya, A., *Staphylococcus aureus* contamination on the surface of working tables in ward staff centers and its preventive methods. *Biol Pharmac Bull* 2006; **29**: 1508-1510.
26. Pinar, A., Bozdemir, N., Kocagöz, T. and Alaçam, R., Rapid detection of bacterial a typical pneumoniae agents by multiplex PCR. *Cent Eur J Public Health* 2004; **12**: 3-5.
27. Rabuza, U., Šostar-Turk, S. and Fijan S., Efficiency of four sampling methods used to detect two common nosocomial pathogens on textiles. *Tex Res J*, 2012; **82**: 2099-2105.
28. Robine, E., Derangere, D., and Robin, D., Survival of a *Pseudomonas fluorescens* and *Enterococcus faecalis* aerosol on inert surfaces. *Int J Food Microbiol* 2000; **55**: 229-234.
29. Sexton, J.D., Tanner, B.D., Maxwell, S.L.andGerba C.P., Reduction in the microbial load on high-touch surfaces in hospital rooms by treatment with a portable saturated steam vapor disinfection system. *Amer Jour Inf Contr* 2011; **39**: 655-662.
30. Shiomori, T., Miyamoto, H. and Makishima, K., Significance of airborne transmission of methicillin-resistant *Staphylococcus aureus* in anotolaryngology-head and necksurgery unit. *Arch Otolaryngol Head Neck Surg* 2001; **127:** 644-648.
31. Verran, J., Biofouling in food processing: biofilm or biotransfer potential? *Inst Chem Eng Trans C: Food Bioprod Process* 2002; **80**: 292-298.
32. Wattiau, P., Renard, M.-E., Ledent, P., Debois, V., Blackman, G.and Agathos. S.N., A PCR test to identify *Bacillus subtilis* and closely related species and its application to the monitoring of waste water biotreatment. *Appl Microbiol Biotechnol* 2001; **56**: 816-819.
33. Wilks, S.A., The survival of *Escherichia coli* O157 on a range of metal surfaces. *Int J Food Microbiol* 2005; **105**: 445-454.
34. Xu, W., Zhai, Z., Huang, K., Zhang, N., Yuan, Y., Shang, Y. and Luo, Y., A novel universal primer-multiplex-PCR method with sequencingg elelectrophoresis analysis. *PLoS One*7, 2012; 22900.
35. Young-Min, B., Seung-Youb, B., and Sun-Young, L., Resistance of pathogenic bacteria on the surface of stainless steel depending on attachment form and efficacy of chemical sanitizers. *Int J Food Microbiol* 2012; **153**, 465-473.