Diagnostic multiplex polymerase chain reaction assay for the identification of *Pseudomonas aeruginosa* from the skin biopsy specimens in burn wound infections and detection of antibiotic susceptibility

Rasoul Y. Mashouf, MSc, PhD, *Alireza Zamani,* MSc, PhD, *Hadi S. Farahani,* BSc, MSc.

ABSTRACT

الأهداف: الكشف عن المكورات الزيفانية الهوائية من عينات أخذت من البشرة في التهابات الحروق بواسطة التفاعل التسلسلي المناعي المركب (PCR) وكشف استجابة البكتيريا المعزولة بالزراعة للعلاج بالمضادات الحيوية.

الطريقة: أجرينا هذه الدراسة المقطعية العرضية على 140 مريضاً يعانون من التهابات الجروح، وأدخلوا لمركز الحروق التخصصي في مستشفى موتاهاري بطهران – إيران، خلال 12 شهرا من عام 2005م وحتى عام 2006م. تم أخذ عينات من الجلد من كل مريض، أجري على أحدها اختبار التفاعل التسلسلي المناعي (PCR)، وأجري على الأخرى الزراعة. وقد تم استخدام اختبار التفاعل التسلسلي المناعي (PCR) القائم على التكثير المتزامن لصبغين من البروتينات الدهنية وهما الصبغ (oprI) والصبغ (oprL) للكشف المباشر عن المكورات الزيفانية والمكورات الزيفانية الهوائية في عينات الجلد. تحددت استجابة المكورات الزيفانية الهوائية لستة عشر مضادا حيويا باستخدام طريقة الانتشار القرصي.

النتائج: من بين المائة وأربعين عينة، كشف اختبار التفاعل التسلسلي المناعي المركب عن 66 بكتيريا (47.2%)، بينما كشفت الزراعة عن 57 بكتيريا (40.7%) على أنها مكورات زيفانية هوائية. تحققت النتائج الموجبة لكل من الصبغين، والتي كشفت عن المكورات الزيفانية الهوائية بينما تم تكثير الصبغ آي من المكورات الزيفانية الأخرى (العدد 12)، وجميع أنواع البكتيريا الأخرى التي تم اختبارها (العدد 62) كانت سالبة نتيجة لاختبار التكثير. كان أكثر أنواع المضادات الحيوية فعالية ضد المكورات الزيفانية الهواء هو عقار سيفيبايم (79%)، وأزيتريونام (76%)، وحامض تريارسيلين–كلافولانيك (68%)، وتوبرامايسين (62%)، واميكاسين (61%).

خاتمة: تعتبر مجموعة اختبار التفاعل التسلسلي المناعي (PCR) واعدة من أجل الكشف السريع والحساس عن المكورات الزيفانية الهوائية من العينات التي أخذت من حروق الجلد. كما أن التكثير المتزامن لصبغين من صبغيات البروتينات الدهنية وهما الصبغ (oprI) والصبغ (oprL) يمكن أن يكشف عن المكورات الزيفانية الهوائية، ويمكن أن يكشف الصبغ (oprI) فقط عن المكورات الزيفانية الأخرى.

Objectives: To identify *Pseudomonas aeruginosa (P. aeruginosa)* from the skin biopsy specimens in burn wound infections by multiplex polymerase chain reaction (M-PCR) and detection of antimicrobial susceptibility of isolates from culture.

Methods: We conducted this cross-sectional study in 140 patients with wound infections who admitted to the referral burn center of Motahari, Tehran, Iran, during a 12-month period from 2005-2006. Skin biopsy specimens were aseptically taken from each patient, one for PCR and one for bacterial culture. A M-PCR test based on the simultaneous amplification of 2 lipoprotein genes: *oprI* and *oprL*, was used to directly detect fluorescent pseudomonades and *P. aeruginosa* in skin biopsy specimens. The susceptibility of *P. aeruginosa* isolates to 16 antibiotics was determined using the disc diffusion method.

Results: Out of 140 biopsy specimens, M-PCR detected 66 (47.2%) isolates, while culture detected 57 (40.7%) isolates as *P. aeruginosa*. Positive results for both genes which observed only for *P. aeruginosa*, while only one gene, *oprI*, was amplified from other fluorescent pseudomonades (n=12) and all other bacterial tested (n=62) were negative by the amplification test. The most effective antibiotics against isolate of *P. aeruginosa* were cefepime (79%), azetreonam (76%), ticarcillin-clavulanic acid (68%), tobramycin (62%), and amikacin (61%).

Conclusion: Multiplex PCR assay appears promising for the rapid and sensitive detection of *P. aeruginosa* from the burned skin biopsy specimens. Simultaneous amplification of 2 lipoprotein genes: *oprI* and *oprL*, could detect *P. aeruginosa*, and *oprI* gene only for other fluorescent pseudomonades.

Saudi Med J 2008; Vol. 29 (8): 1109-1114

From the Departments of Medical Microbiology (Mashouf, Farahani), and Immunology (Zamani), School of Medicine, Hamadan University of Medical Sciences. Hamadan, Iran.

Received 7th April 2008. Accepted 1st July 2008.

Address correspondence and reprint request to: Dr. Alireza Zamani, Department of Immunology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran. Tel. +98 (811) 82762958 Ext. 338. Fax. +98 (811) 8276299. E-mail: zamanialireza@yahoo.com

Pseudomonas aeruginosa (*P. aeruginosa)* is a key opportunistic pathogen causing severe acute and chronic nosocomial infections in hospitalized patients. This organism is responsible for a wide range of hospital-acquired infections such as burn wound infections, pneumonia, urinary tract infections, or bacteremia.[1-4] The so-called fluorescent Pseudomonads are characterized by the production of pigments and fluoresce brightly under UV light. These are including *P. aeruginosa, Pseudomonas putida (P. putida), Pseudomonas alcaligenes (P. alcaligenes), Pseudomonas asplenii (P. asplenii), Pseudomonas aureofaciens (P. aureofaciens), Pseudomonas chlororaphis (P. chlororaphis), Pseudomonas cichorii (P. cichorii), Pseudomonas corrugata (P. corrugata), Pseudomonas fluorescens (P. fluorescens), Pseudomonas Syringae (P. Syringae),* and *Pseudomonas flavescens (P. flavescens).*[5] The burn wound represents a site susceptible to opportunistic colonization. *Pseudomonas aeruginosa* is prevalent in burn wound infections and it is generally multi-drug resistant. Cross-transmission from patient to patient may occur via the hand of the health care staff or through contaminated materials or reagents. Thus, a number of outbreaks of nosocomial infection due to *P. aeruginosa* have been reported in burn wound units.[6-8] The experience accumulated over the past 3 decades, in the early interventional treatment of burns patients have dramatically changed the cause of death; it is now estimated that approximately 75% of the mortality following burn injuries is related to infections, instead of osmotic shock and hypovolemia.[9-11] Therefore, knowledge of the responsible organisms of burn wounds, its prevalence and bacterial resistance, is of crucial importance for fast and reliable therapeutic decisions. Early diagnosis of wound colonization or prediction of burn wound infection with *P. aeruginosa* provides an opportunity for suitable therapeutic intervention.[12,13] Understanding the genetic programs underlying infection is essential to develop highly needed new strategies for prevention and therapy. Alterations of drug targets or enzymatic inactivation of antimicrobial agents are the well-known mechanisms of antimicrobial drug resistance. Besides, these well-known mechanisms, recent studies have shown that a further resistance mechanism, active drug efflux, has become increasingly important in the current threat of multidrug resistance.[14-19] The outer membrane proteins of *P. aeruginosa,* which play important roles in the interaction of the bacterium with the environment seem as a good candidate for molecular detection and differentiation from other bacteria.[20,21] The gene of *oprI* code a polypeptide contain 82 amino acids and gene of *oprL* also code a polypeptide contains 168 amino acids.[22,23] The purpose of present study was to identify *P. aeruginosa* and other fluorescent pseudomonades from the skin biopsy specimens in burn wound infections by multiplex polymerase chain reaction (M-PCR) test based on the simultaneous amplification of 2 lipoprotein genes, *oprI* and *oprL* and detection of levels of antimicrobial susceptibility of isolated from culture.

Methods. ***Patients and sample collection.*** *Pseudomonas aeruginosa* (ATCC 17933), *P. putida* (ATCC 12633) and *Staphylococcus aureus* (*S. aureus*) (ATCC 29213) as control organisms and a total of 140 burned patients with wound infections who admitted to the referral burn center of Motahari, Tehran, Iran, during a 12-month period from 2005-2006 were included. Skin biopsy specimens (10 mg) were aseptically taken by a surgeon and inoculated in sterile microcentrifuge tubes. One specimen of each infected patient was transferred to bacteriology laboratory to culture and other used for PCR. None of the patients included in the study had antibiotics therapy before sampling and informed consent was obtained from the patients.

All samples were submitted to M-PCR reactions, using primer pairs targeting specific genomic sequences of each species. An M-PCR test based on the simultaneous amplification of 2 lipoprotein genes, *oprI* and *oprL*, was used to directly detect fluorescent pseudomonades (by amplification of only *oprI* open reading frame, 249 bp) and *P. aeruginosa* (by amplification of *oprI* open reading frame, 249 bp and *oprL* open reading frame, 504 bp simultaneously) in skin biopsy specimens. Detection of amplification of 2 mentioned lipoprotein genes, 500 µl of the following buffer was added to the microcentrifuge tubes containing the biopsy specimens: 50 mM Tris-hydrochloric acid (HCl) (pH 8.5), one mM EDTA, and 0.5% Tween 20. The samples were first boiled in a water bath for 10 minutes, and then proteinase K (Boehringer Mannheim) at 200 µg/ml was added. The tubes were further incubated in a water bath at 65°C for 40 minutes. After deactivation of the proteinase K by heat treatment (10 minutes in a boiling water bath), the tubes were centrifuged at 13,000 rate per minute, and 10 ml of the supernatant was taken as DNA template for the M-PCR.

Multiplex PCR. The M-PCR mixture (20 µl final volume) contained 7.6 µl of distilled, filter-sterilized, and autoclave water, 2 µl of 10x PCR buffer [500 mM-potassium chloride, 100 mM Tris-HCl [pH 8.4], 15 mM magnesium chloride (MgCl2)], 2 µl of a

Disclosure. This study was funded by the research deputy of Hamadan University of Medical Sciences, Hamadan, Iran.

deoxynucleotide mixture [deoxyguanosine triphosphate (dGTP), deoxythymidine TP (dTTP), deoxyadenosine TP (dATP), and deoxycytidine TP (dCTP); 2 mM each], one µl of template DNA, one µl of each primer (10 mM), one µl of Taq polymerase (0.5 unit/reaction), and 2.4 µl of MgCl2 (17 mM). Two sets of primers were used in this M-PCR; one set (primers PS1 and PS2) consisted of primers corresponding to the beginning and the end of the open reading frame of the *oprI* gene, while the other set of primers (primers PAL1 and PAL2) was designed to amplify the open reading frame of the *oprL* gene. The primers had the following sequences: PS1, 5-ATGAACAACGTTCTGAAATTCTCTGCT-3 (a 27-mer corresponding to the beginning of the open reading frame of *oprI*); PS2, 5-CTTGCGGCTGGCTTTTTCCAG-3 (a 21-mer corresponding to the end of the open reading frame of *oprI*); PAL1, 5-ATGGAAATGCTGAAATTCGGC-3 (a 21-mer corresponding to the beginning of the open reading frame of *oprL*); and PAL2, 5-CTTCTTCAGCTCGA CGCGACG-3 (a 21-mer corresponding to the end of the open reading frame of *oprL*). The amplification program was set at 35 cycles of denaturation at 94°C for 40s (Eppendorf, Thermocycler). The annealing was carried out at 57°C with the same time settings mentioned above, while the extension was carried out at 72°C for 50s. Five µl of the reaction mixture was mixed with one µl of 6x loading buffer and was put on an agarose gel of 1.5% (wt/vol) for electrophoresis and visualization of the product after staining with ethidium bromide on a UV transilluminator.[21,23,24]

Bacterial culture. Cultivation and isolation of organisms were performed according to the methods outlined in the Bailey and Scott's diagnostic microbiology.[25] Specimens were aseptically smashed and cultured on appropriated culture media including 5% sheep blood agar and MacConkey agar (Merck, Germany), and Eosin methylen blue (EMB) agar (ANTEC, ATD, Germany). The cultures were incubated in 37°C for 24-48 hours, and then the colonies were removed for further studying. Biochemical and antigenic properties of isolates were verified for identification purposes.[25]

Antibiogram: In order to detect the susceptibility of isolates to routine antimicrobial drugs, all isolates of *P. aeruginosa* in vitro were assessed by an agar disk diffusion method recommended by the Clinical and Laboratory Standard Institute (CLSI).[26] A suspension of each isolate was made so that the turbidity was equal to 0.5 McFarland standard and then plated onto Muller-Hinton agar (Difcos) plate. Antibiotic disk (Oxoid) was applied to each plate. After incubation at 35°C for 24 hours, zone size was measured. Reference strains included were ATCC 25923 (*S. aureus*) and ATCC 35218 [*Escherichia coli (E. coli)*]. Sixteen antibiotics including imipenem (10 µg), meropenem (10 µg), cefepime (30 µg), ciprofloxacin (5 µg), and azetreonam (30 µg), cefotaxime (30 µg), gentamicin (10 µg), tazobactam-piperacillin (30 µg), cefalotine (30 µg), cefatazidime (30 µg), carbenicillin (100 µg), tetracycline (30 µg), tobramycin (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), and ticarcillin-clavulanic acid (30 µg) were used for antibiogram. The required data of the burn patients including age, gender, causes of burns and types of microorganisms isolated from skin biopsy were recorded in a special questionnaire and then analyzed using the SPSS version 10.0 (SPSS inc, Chicago, IL) software package.

Results. Of 140 burned patients, 82 were males, and 58 were females. The most frequent patients 43 (30.7%) were belonged to the 20-29 year old and the

Table 1 - The frequency distribution of the causes of burns in patients

Causes of burns	Male	Female	Total
Fire flame	42 (30)	38 (27.1)	80 (57.1)
Boiled water	11 (7.8)	13 (9.3)	24 (17.1)
Chemical agents	15 (10.7)	4 (2.9)	19 (13.6)
Explosive agents	9 (6.5)	3 (2.1)	12 (8.6)
Electrical shock	5 (3.6)	0 (0)	5 (3.6)
Total	**82 (58.6)**	**58 (41.4)**	**140 (100)**

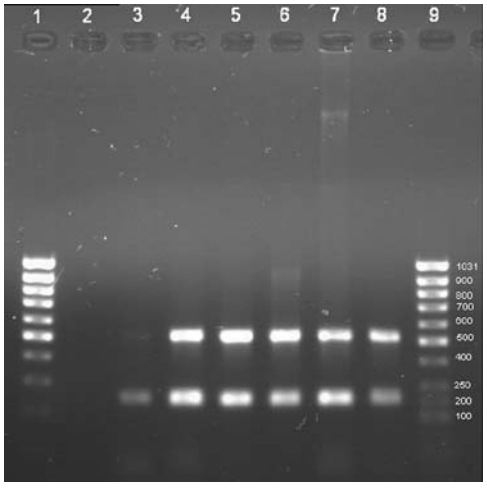

Figure 1 - Multiplex polymerase chain reaction (M-PCR) after agarose gel electrophoresis. Lane 1 and 9, molecular size marker, expressed in base pairs; Lane 2, *Staphylococcus aureus* (ATCC 29213) as a negative control; Lane 3, *Pseudomonas putida* (ATCC 12633) as a fluorescence pseudomonas; Lane 4, DNA of *Pseudomonas aeruginosa* (ATCC 17933) as a positive control; Lanes 5, 6, 7, and 8 skin biopsy specimen M-PCR.

Table 2 - Results of multiplex polymerase chain reaction (M-PCR) assay and culture for the skin biopsy specimens from burn patients.

Bacterial species	No. of isolates (M-PCR) n (%)	PCR results for oprI (249 bp)	PCR results for oprL (504 bp)	No. of isolates (Culture) n (%)
Pseudomonas aeruginosa	66 (47.2)	+	+	57 (40.7)
Non-*Pseudomonas aeruginosa*	12 (8.5)	+	-	12 (8.5)
Staphylococcus aureus	-	-	-	20 (14.3)
Staphylococcus epidermdis	-	-	-	17 (12.1)
Klebsiella pneumoniae	-	-	-	14 (9.9)
Escherichia coli	-	-	-	7 (4.9)
Proteus mirabli	-	-	-	2 (1.4)
Entrobacter agglomerans	-	-	-	2 (1.4)

lowest frequency belonged to the >60 years old. The distributions of the causes of burns are shown in **Table 1**. As it is shown, fire flame and boiled water were the most frequency agents of burns.

Figure 1 shows that the M-PCR, as visualized after agarose gel electrophoresis, is specific for *P. aeruginosa* (lane 4), since 2 amplification bands of 249 bp (*oprI*) and 504 bp (*oprL*) were observed when a *P. aeruginosa* colony lysate was used as the template for the PCR. Other fluorescent pseudomonades such as *Pseudomonas putida* (lane 3) are detected by the presence of only one band corresponding to the 249-bp open reading frame of *oprI*. Other bacteria such as *S. aureus* (lane 2), had no reaction in the M-PCR. As shown in lanes 5, 6, 7, and 8 a positive amplification after M-PCR was obtained for skin biopsy specimens from 4 burn patients infected with *P. aeruginosa*.

Table 2 presents the results of the PCR amplifications for *P. aeruginosa* and non-*P. aeruginosa* as well as for other bacteria. The data in **Table 2** confirm that *oprI* can be amplified from all members of the Pseudomonas, while the *oprL* band can only be amplified from *P. aeruginosa*, but it can amplify consistently. Indeed, among the 66 isolates of *P. aeruginosa* tested, none were found to be negative for *oprL* amplification. The results of the susceptibility of 57 isolates of *P. aeruginosa* to 16 routine antibiotics were as follows: Cefepime (79%), azetreonam (76%), ticarcillin-clavulanic acid (68%), tobramycin (62%), amikacin (61%), ciprofloxacin (58%), meropenem (52%), tazobactam-piperacillin (51%), imipenem (49%), cefotaxime (42%), gentamicin (40%), cefatazidime (38%), cefalotine (27%), carbenicillin (16%), and tetracycline (7%).

Discusion. Several studies demonstrated that *P. aeruginosa*, *S. aureus*, and *E. coli* are the most prevalent isolates from burn wound infections.[3,6,27,28] We found that *P. aeruginosa* was the most common isolates coincides with previous reports.[3,6,27,29] However, it was in contrast to other studies which report *S. aureus* as predominant organism.[28,30-33] The purpose of the present study was to perform rapid detection of *P. aeruginosa* and other fluorescent pseudomonades (such as *P. putida*) in tissue biopsy specimens of burned patients by M-PCR test based on the simultaneous amplification of 2 lipoprotein genes: *oprI* and *oprL*. Reliability of the most widely used PCR screenings for the human opportunistic pathogen *P. aeruginosa* was evaluated. Although the conventional bacteriological methods currently in use for *P. aeruginosa* identification are satisfactory, however, more rapid techniques may be useful in some specific situations.[20,21,34,35] Immunological screening of a *P. aeruginosa* cosmid library led to the identification of clones producing an 18 kDa outer-membrane protein. This protein reacted in Western blots with a polyclonal antiserum against outer-membrane proteins of *P. aeruginosa* and with a monoclonal antibody (MA1-6) specific for *oprL*, the peptidoglycan-associated outer-membrane lipoprotein (PAL). Sequencing of pOML7, a subclone expressing *oprL*, showed an open reading frame (ORF) of 504 bp encoding a polypeptide with a typical lipoprotein signal recognition sequence.[23]

In our study, both target genes used in this M-PCR (*oprI* and *oprL*) were detected in all *P. aeruginosa* strains tested. Results of this project show 66 patients of 140 total burn wound infections (47.2%) developed an infection which *P. aeruginosa* was involved. The data presented here indicate that the M-PCR technique based on the detection of *oprI* and *oprL* is very sensitive and provides results within few hours. The simultaneous detection of other fluorescent pseudomonads can be important since fluorescent pseudomonads other than *P. aeruginosa*, such as *P. stutzeri, P. fluorescens, P. mendocina,* and *P.*

putida, have sometimes been claimed to be involved in opportunistic infections.[5]

The specificity of the *oprI* and *oprL* PCR assay was also high in comparison with routine culture when it was based on testing genomic DNA from a collection of bacterial strains but appeared much lower in comparison with routine culture when it was based on a preliminary clinical evaluation. There may be some explanations for these discrepancies. These include false-positive M-PCR results due to sample or amplicon carry over contamination or to the presence of nonspecific homologous target sequences in other bacteria. Alternatively, the discrepancies can reflect true *P. aeruginosa* infection with a false-negative culture result due to sample overgrowth by other bacteria or to the presence of noncultivable organisms or auxotrophic mutations in the organism.[36]

Therapy for *P. aeruginosa*, a major cause of life-threatening nosocomial infection, is problematic because of the propensity for multiple-drug resistance.[37] Mechanisms of resistance of *P. aeruginosa* are dependent mainly on impermeability and multidrug efflux pumping.[21] The importance of periodically studying susceptibility and resistance patterns of *P. aeruginosa* in each setting in order to evaluate different therapeutic guidelines, as it is not always advisable to extrapolate data from different regions. These differences can be explained by the different use of antibiotics in each center and the geographic variations of the resistance mechanisms of *P. aeruginosa*. Some *P. aeruginosa* strains are only susceptible to the polymyxins.[15,38]

The survey of antibiotic susceptibility of *P. aeruginosa* showed high percentages of resistance to the different antibiotics. In this study resistance to imipenem (51%), and meropenem (48%) were relatively high in contrast to other studies.[39,40] Carbapenem (imipenem and meropenem) resistance is associated with metallo-beta-lactamases.[41] In our study, cefepime was most active against strains of *P. aeruginosa* followed by azetreonam that coincide with results of Rodriguez et al.[42] In comparison with third-generation cephalosporins, cefepime may be less likely to induce resistance. The third active antibiotic was ticarcillin-clavulanic acid.

In conclusion, M-PCR assay used in this study which can be performed in few hours appears promising for the rapid and sensitive detection of *P. aeruginosa* in biopsy specimens of burned wounds infections and prevent patients complication due to infection. Since a high proportion of samples had positive cultures, infection control is recommended as a strategy to minimize spread of resistant organisms. It is necessary to implement urgent measures to prevent the spreading of this multiresistant strain.

Acknowledgment. *Authors gratefully acknowledge T. Ghiasvan and N. Shabab for their technical assistance.*

References

1. Fonseca AP, Correia P, Sousa JC, Tenreiro R. Association patterns of *Pseudomonas aeruginosa* clinical isolates as revealed by virulence traits, antibiotic resistance, serotype and genotype. ***FEMS Immunol Med Microbiol*** 2007; 51: 505-516.
2. Gad GF, El-Domany RA, Zaki S, Ashour HM. Characterization of *Pseudomonas aeruginosa* isolated from clinical and environmental samples in Minia, Egypt: prevalence, antibiogram and resistance mechanisms. ***J Antimicrob Chemother*** 2007; 60: 1010-1017.
3. Ekrami A, Kalantar E. Bacterial infections in burn patients at a burn hospital in Iran. ***Indian J Med Res*** 2007; 126: 541-544.
4. Oudhuis GJ, Verbon A, Hoogkamp-Korstanje JA, Stobberingh EE; Susceptibility Surveillance Study Group. Antimicrobial resistance in *Escherichia coli* and *Pseudomonas aeruginosa* from Intensive Care Units in The Netherlands, 1998-2005. ***Int J Antimicrob Agents*** 2008; 31: 58-63.
5. Palleroni NJ. Pseudomonas. In: Borriello SP, Murray PR, Funke G, editors. Topley and Wilson's Microbiology and Microbial Infections, Bacteriology. 10th edition. London: Hodder Arnold, ASM Press; 2005. p. 1591-1598.
6. Agnihotri N, Gupta V, Joshi RM. Aerobic bacterial isolates from burn wound infections and their antibiograms-a five-year study. ***Burns*** 2004; 30: 241-243.
7. Lamia T, Bousselmi K, Saida BR, Allah MA. Epidemiological profile and antibiotic susceptibility of *Pseudomonas aeruginosa* isolates within the burned patient hospitalized in the intensive care burn unit. ***Tunis Med*** 2007; 85: 124-127.
8. Orrett FA. Antimicrobial susceptibility survey of *Pseudomonas aeruginosa* strains isolated from clinical sources. ***J Natl Med Assoc*** 2004; 96: 1065-1069.
9. Lawrence JC. Burn bacteriology during the last 50 years. ***Burn*** 1992; 18: S23-S29.
10. Vindenes H, Bjerknes R. Microbial colonization of large wounds. ***Burns*** 1995; 21: 575-579.
11. De Macedo JL, Santos JB. Bacterial and fungal colonization of burn wounds. ***Mem Inst Oswaldo Cruz*** 2005; 100: 535-359.
12. Vindenes H, Bjerknes R. Microbial colonization of large wounds. ***Burns*** 1995; 21: 575-579.
13. Harris A, Torres-Viera C, Venkataraman L, DeGirolami L, Samore M, Carmeli Y. Epidemiology and clinical outcomes of patients with multiresistant *Pseudomonas aeruginosa*. ***Clin Infect Dis*** 1999; 28: 1128-1133.
14. Wareham DW, Curtis MA. A genotypic and phenotypic comparison of type III secretion profiles of *Pseudomonas aeruginosa* cystic fibrosis and bacteremia isolates. ***Int J Med Microbiol*** 2007; 297: 227-234.
15. Lee YC, Ahn BJ, Jin JS, Kim JU, Lee SH, Song do Y, et al. Molecular characterization of *Pseudomonas aeruginosa* isolates resistant to all antimicrobial agents, but susceptible to colistin, in Daegu, Korea. ***J Microbiol*** 2007; 45: 358-363.
16. Mendes RE, Kiyota KA, Monteiro J, Castanheira M, Andrade SS, Gales AC, et al. Rapid detection and identification of metallo-beta-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. ***J Clin Microbiol*** 2007; 45: 544-547.
17. Lavenir R, Jocktane D, Laurent F, Nazaret S, Cournoyer B. Improved reliability of *Pseudomonas aeruginosa* PCR detection by the use of the species-specific ecfX gene target. ***J Microbiol Methods*** 2007; 70: 20-29.

18. Nikaido H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. ***Science*** 1994; 264: 382-388.
19. Livermore DM. Of Pseudomonas, porins, pumps and carbapenems. ***J Antimicrob Chemother*** 2001; 47: 247-250.
20. Cornelis P, Bouia A, Belarbi A, Guyonvarch A, Kammerer B, Hannaert V, et al. Cloning and analysis of the gene for the major outer membrane lipoprotein from *Pseudomonas aeruginosa*. ***Mol Microbiol*** 1989; 3: 421-428.
21. De Vos D, Lim A Jr, De Vos P, Sarniguet A, Kersters K, Cornelis P. Detection of the outer membrane lipoprotein I and its gene in fluorescent and non fluorescent pseudomonads: implications for taxonomy and diagnosis. ***J Gen Microbiol*** 1993; 139: 2215-2223.
22. Hancock RE, Siehnel R, Martin N. Outer membrane proteins of Pseudomonas. ***Mol Microbiol*** 1990; 4: 1069-1075.
23. Lim A Jr, De Vos D, Brauns M, Mossialos D, Gaballa A, Qing D, et al. Molecular and immunological characterization of OprL, the 18 kDa outer-membrane peptidoglycan-associated lipoprotein (PAL) of *Pseudomonas aeruginosa*. ***Microbiology*** 1997; 143: 1709-1716.
24. Sambrook J, Russell D. Molecular cloning: a laboratory manual. 3rd edition. New York (NY): Cold Spring Harbor Laboratory press, USA; 2001. p. 8.107.
25. Forbes BA, Sahm DF, Weissfeld AS. Bailey & Scott's Diagnostic microbiology. 11th Ed. Philadelphia (PA): Mosby Co; 2002. p. 368-375 and 204-213.
26. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial disk susceptibility tests. NCCLS documents M 100 S15. Wayne, PA: Clinical and Laboratory Standard Institute, USA; 2005.
27. Nasser S, Mabrouk A, Maher A. Colonization of burn wounds in Ain Shams University burn unit. ***Burns*** 2003; 29: 229-233.
28. Komolafe OO, James J, Kalongolera L, Makoka M. Bacteriology of burns at the Queen Elizabeth Central Hospital, Blantyre, Malawi. ***Burns*** 2003; 29: 235-238.
29. Sharma BR, Harish D, Singh VP, Bangar S. Septicemia as a cause of death in burns: an autopsy study. ***Burns*** 2007; 33: 538-539.
30. Vindenes H, Bjerknes R. Microbial colonization of large wounds. ***Burns*** 1995; 21: 575-579.
31. Lesseva M, Hadjiiski OG. Staphylococcal infections in the Sofia Burn Centre, Bulgaria. ***Burns*** 1996; 22: 279-282.
32. Vostrugina K, Gudaviciene D, Vitkauskiene A. Bacteremias in patients with severe burn trauma. ***Medicina (Kaunas)*** 2006; 42: 576-579.
33. Messadi AA, Thabet L, Bouselmi K, Ben Redjeb S. A study of sensitivity to antibiotics for *Staphylococcus aureus* strains isolated in a department of resuscitation of badly burnt patients. ***Tunis Med*** 2006; 84: 74-77.
34. Abd-El-Haleem D, Kheiralla ZH, Zaki S, Rushdy AA, Abd-El-Rahiem W. Multiplex-PCR and PCR-RFLP assays to monitor water quality against pathogenic bacteria. ***J Environ Monit*** 2003; 5: 865-870.
35. Dabrowski W, Czekajlo-Kolodziej U, Medrala D, Giedrys-Kalemba S. Optimisation of AP-PCR fingerprinting discriminatory power for clinical isolates of *Pseudomonas aeruginosa*. ***FEMS Microbiol Lett*** 2003; 218: 51-57.
36. Taylor RF, Hodson ME, Pitt TL. Adult cystic fibrosis: association of acute pulmonary exacerbation and increasing severity of lung disease with auxotrophic mutants of *Pseudomonas aeruginosa*. ***Thorax*** 1993; 48: 1002-1005.
37 Paramythiotou E, Lucet JC, Timsit JF, Vanjak D, Paugam-Burtz C, Trouillet JL, et al. Acquisition of multidrug-resistant *Pseudomonas aeruginosa* in patients in intensive care units: role of antibiotics with antipseudomonal activity. ***Clin Infect Dis*** 2004; 38: 670-677.
38 Ouderkirk JP, Nord JA, Turett GS, Kislak JW. Polymyxin B nephrotoxicity and efficacy against nosocomial infections caused by multiresistant gram-negative bacteria. ***Antimicrob Agents Chemother*** 2003; 47: 2659-2662.
39. Ivanov DV, Krapivina IV. Etiology of nosocomial surgical infections caused by gram-negative bacteria, and profile of their antibiotic resistance. ***Zh Mikrobiol Epidemiol Immunobiol*** 2007; 5: 90-93.
40. Lockhart SR, Abramson MA, Beekmann SE, Gallagher G, Riedel S, Diekema DJ, et al. Antimicrobial resistance among Gram-negative bacilli causing infections in intensive care unit patients in the United States between 1993 and 2004. ***J Clin Microbiol*** 2007; 45: 3352-3359.
41. Rasmussen BA, Bush K. Carbapenem-hydrolyzing ß-lactamases. Antimicrob. ***Agents Chemother*** 1997; 41: 223-232.
42. Rodriguez CN, Rodriguez-Morales AJ, Garcia A, Pastran B, Meijomil P. Antimicrobial resistance of *Pseudomonas aeruginosa* strains isolated from surgical infections in a 7-year period at a general hospital in Venezuela. ***Surg Infect (Larchmt)*** 2006; 7: 269-273.