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Original Article

# Rapid detection of Pseudomonad at species level by multiplex PCR in surgical units and ICU of Dhaka Medical College Hospital.

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**Abstract**
Pseudomonads are the most important gram negative organisms involved in various types of infection. This cross sectional study was conducted from January to December 2010 to isolate and identify Pseudomonad at species level in different clinical samples by culture and multiplex polymerase chain reaction (PCR) and to evaluate the efficacy of PCR in rapid detection of the bacteria at species level. Wound swab and tips of endotracheal tube were collected from hospitalized patients from different surgical units and intensive care unit (ICU) of Dhaka Medical College Hospital, Dhaka. Pseudomonads were isolated and identified at species level by culture, microscopy, different biochemical tests and PCR. Among 230 samples, 52.6% were surgical wound, 34.3% were burn wound and 9.6% were traumatic wound samples and 3.4% were tips of endotracheal tubes. Twenty six percent isolated organisms were *Pseudomonas* spp., 30.4% were *Escherichia coli*, and 13.5% were *Staphylococcus aureus*. Others were Proteus, *Klebsiella pneumoniae, Acinetobacter baumannii* and *Enterobacter spp*. In 19.67% samples mixed infections by other organism (*Esch coli, Staph aureus, Proteus* spp, *Klebsiella* spp) with *Pseudomonas* were detected and its distribution was highest in traumatic and burn wound. Multiplex PCR and different biochemical tests were used to identify 3 bacterial species of Pseudomonad. Among the species identified, 95.52% was *Pseudomonas aeruginosa*, 2.99% was *Stenotrophomonas maltophilia* and 1.49% was *Burkholderia cepacia*. The sensitivity of multiplex PCR was 95.08% and specificity 94.67%. PCR was the most rapid and more accurate method for detection of Pseudomonad at species level.

**Key words:** Pseudomonad, multiplex PCR, species.

## Introduction:

Wound infection is one of the commonest hospital acquired infections and is an important cause of morbidity and account for 70-80% mortality worldwide[1]. Major types of wound infection include- surgical, traumatic, burn etc. Regardless the types of wounds, both surgical and non-surgical, Pseudomonads are the important etiological agents and draw the attention of researchers because of its multi-drug resistant pattern[2].

Pseudomonad contributes substantially to wound-related morbidity and mortality worldwide[3]. *P aeruginosa* is ubiquitous in distribution and can be isolated from a number of sites in the hospital environment, such as respiratory care equipments, baths, sinks, cold water humidifier, bed pans and floors[4].

Among the Pseudomonad species, *P aeruginosa* is more frequently isolated which can colonize the healthy individual as harmless saprophyte and opportunistic pathogen of human[5]. It is the leading cause of nosocomial infection related to ICU, particularly ventilator associated

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pneumonia[4,6]. *Burkholderia pseudomallei* cause melioidosis in human, primarily in Southeast Asia and Northern Australia. Sepsis due to *B pseudomallei* has a mortality rate of 80%, if untreated[7]. *B cepacia* can be transmitted to cystic fibrosis patients causing necrotizing pneumonia and bacteremia[8]. *Stenotrophomonas maltophilia* is an increasingly important cause of hospital acquired infection in patients who are receiving antimicrobial therapy and in immunocompromised patients[9].

A more rapid test for detection of Pseudomonad species i.e. *P aeruginosa, P maltophilia* and *B cepacia* was demonstrated with a simple and rapid polymerase chain reaction (PCR) technique. Multiplex PCR can be performed with the set of three primer pairs based on 16s rRNA sequences[10].

Phenotype based identification is time consuming and may misidentify different Pseudomonad species but multiplex PCR overcomes the problem of variable phenotype to provide more accurate and rapid species identification. So the study was conducted to identify Pseudomonad at species level by using multiplex PCR more accurately and within possible shortest time.

**Methods:**

This cross sectional study was conducted in the department of Microbiology of Dhaka Medical College, Dhaka, for a period of one year.

A total of 230 specimens (wound swab and tips of endotracheal tube) were tested for the presence of Pseudomonad at species level. Pseudomonad species was isolated and identified by microscopy, culture, biochemical tests and multiplex polymerase chain reaction. Samples were collected from hospitalized patients in different units and intensive care unit of Dhaka Medical College Hospital, Dhaka.

The protocol was approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of Dhaka Medical College. Informed written consent was taken from each patient before collection of sample.

**Study population:** Specimens were collected from patients having clinical symptoms of microbial infection irrespective of age and sex of varying socio-economic status.

**Microbiological methods:**

**Culture of wound swab:** Wound swab was inoculated in blood agar and MacConkey's agar media and incubated at 370C aerobically for 48 hours.

**Culture of endotracheal tube tip:** Tip of the endotracheal tube was put into glucose broth and incubated for 4 hours at 37$^{o}$C aerobically. From broth culture, inoculation was done in blood agar and MacConkey's agar media and was incubated at 37$^{o}$C aerobically for 48 hours.

Isolation and identification of bacteria was done following standard procedure[11].

**Molecular detection:**

**DNA extraction:** DNA was extracted from samples following bead extraction method[12] and from the colony growth by simple boiling method[13].

**Amplification of DNA:** Multiplex PCR was performed using the following primers (Ist Base, Singapore):

| | | | |
|---|---|---|---|
| VIC1 | -TTC CCT CGC AGA GAA AAC ATC | 520 bp | P aeruginosa |
| VIC2 | -CCT GGT TGA TCA GGT CGA TCT | | |
| Malt1 | -TAC CAC CCG TAC CTG GAC TT | 149 bp | S maltophilia |
| Malt2 | -ATC GCA TCG TTG CTG TTG TA | | |
| Eub16-1 | -AGR GTT YGA TYM TGG CTC AG | 463 bp | B cepacia complex |
| CeMuVi16-2 | -CCG RCT GTA TTA GAG CCA | | |

PCR was performed in a final reaction volume of 25µl, containing 1µl of each three primers, 3µl of extracted DNA, 12.5µl Mastermix and 6.5µl of nuclease free water (Promega Corporation, USA).

**Interpretation of result:** Amplified PCR products showing presence of specific DNA band corresponding to 520 bp, 149 bp and 463 bp bands were considered positive for the presence of *P aeruginosa, S maltophilia* and *B cepacia* complex respectively.

**Data analysis:** The result was compiled and analyzed using Microsoft Office Excel 2007 program according to the objectives of the study. Sensitivity and specificity were calculated using standard statistical formula.

**RESULT**

Among 230 samples 61 (26.5%) yielded growth of *Pseudomonas* spp. in culture, 70 (30.4%) were *Esch* coli, and 31 (13.5%) were *Staphylococcus aureus*. Other isolated bacteria were *Proteus* spp. 6.5%, *Klebsiella pneumoniae* 3.9%, *Acinetobacter baumannii* 0.4% and *Enterobacter* 0.4% (Table-I).

Of the isolated *Pseudomonas* species, 19.67% samples had mixed infections with other organism (*Esch coli*-5, *Proteus*-4, *Staph aureus*-2, *Klebsiella*-1). Distribution of mixed infection was highest in traumatic wound and burn wound (Table-II).

Among the 67 identified Pseudomonad species by multiplex PCR, 64 (95.52%) were *P aeruginosa*, 1 (1.50%) was *B cepacia* and 2 (2.98%) were *S maltophilia* (Table-III).

Of 230 samples multiplex PCR identified 67 samples as Pseudomonad spp., 58 samples were positive by both culture and PCR whereas three samples were positive in culture was found negative by PCR. Considering culture as gold standard, the sensitivity of multiplex PCR was 95.08%. Nine of the culture negative samples were positive in PCR and the specificity of PCR was 94.67% (Table-IV).

**Table-I: Isolation of different organisms from different samples (n-230)**

| Organism | Surgical wound (n=121) | Burn wound (n=79) | Traumatic wound (n=22) | Endotracheal tube (n=8) | Total (n=230) |
|---|---|---|---|---|---|
| Pseudmonas spp. | 35(28.9) | 18(22.8) | 5(22.8) | 3(37.5) | 61(26.5) |
| Esch coli | 39(32.2) | 26(32.9) | 4(18.2) | 1(12.5) | 70(30.4) |
| Staph aureus | 15(12.4) | 12(15.2) | 3(13.7) | 1(12.5) | 31(13.5) |
| Proteus spp. | 9(7.4) | 4(5.1) | 2(9.1) | 0(0) | 15(6.5) |
| Klebsiella spp. | 4(3.3) | 2(2.5) | 2(9.1) | 1(12.5) | 9(3.9) |
| Others* | 1(0.8) | 1(1.3) | 0(0) | 0(0) | 2(18.3) |

Others*-Acinetobacter baumannii and Enterobacter spp.
Note: Figures in parentheses represent percentage.

**Table-II: Pattern of mixed organisms isolated from different samples.**

| Organisms | Burn (n=79) | Trauma (n=22) | Endotracheal Tube (n=8) | Surgery (n=121) |
|---|---|---|---|---|
| *P aeruginosa + Esch coli* | 3 | 1 | 0 | 1 |
| *P aeruginosa + Proteus spp.* | 3 | 1 | 0 | 0 |
| *P aeruginosa + Staph aureus* | 1 | 1 | 0 | 0 |
| *P aeruginosa +Klebsiella spp.* | 0 | 0 | 1 | 0 |
| Total (12) | 7(8.9%) | 3(13.6%) | 1(12.5%) | 1(0.8%) |

Note: Number of individual organism of mixed growth: *P aeruginosa*-12, *Esch coli*-5, *Proteus*-4, *Staph aureus*-2, *Klebsiella*-1.

**Table-III: Different Pseudomonad species identified by multiplex PCR or biochemical tests (n-67).**

| Name of species | Number n(%) |
|---|---|
| *Pseudomonas aeruginosa* | 64 (95.52) |
| *Burkholderia cepacia* | 1 (1.49) |
| *Stenotrophomonas maltophilia* | 2 (2.99) |

**Table-IV: Comparison between results of multiplex PCR with culture of isolated Pseudomonad (P aeruginosa, B cepacia, S maltophilia).**

| PCR | Culture | |
|---|---|---|
| | Positive n (%) | Negative n (%) |
| **Positive (n=67)* | 58 (95.08) | 9 (5.33) |
| *Negative (n=163)* | 1+2** (4.92) | 160 (94.67) |
| Total | 61 (100) | 169 (100) |

* positive for P aeruginosa, B cepacia and S maltophilia.
**positive for P alcaligenes, P Fluorescences

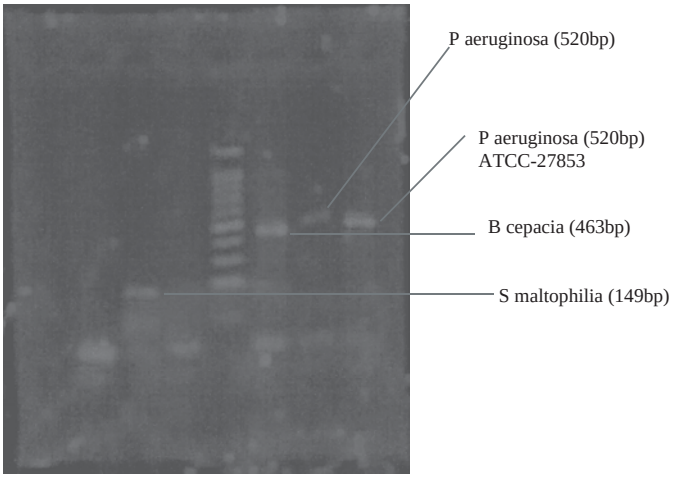


***Figure-1:*** Multiplex PCR of DNA samples obtained from: Lane 3-*S maltophilia*, Lane 4-negative control, Lane 5 -molecular size marker (100 bp), Lane 6-*B cepacia* complex, Lane 7-*P aeruginosa*, and Lane 8-Positive control for *P aeruginosa* (ATCC-27853).

## DISCUSSION

Wound infections remain an important source of concern in hospitals throughout the world resulting from the associated morbidity and mortality[3]. In the present study Pseudomonad spp. causing wound infections were isolated and identified.

Among 230 clinical specimens Pseudomonas spp. was isolated from 26.52% samples in culture and 127 organisms were other than Pseudomonas and 42 specimens yielded no growth. The frequency of Pseudomonas infection (26.52%) in the study was second to Esch coli (30.43%). In Bangladesh it was reported that in 28% cases the causative agent of wound infection was Pseudomonas spp. which is in agreement with this study14. Other studies in India showed a range of 22% to 32% of wound infection was caused by Pseudomonas spp[15,16,17]. Incidence of Pseudomonad infection varies from place to place, country to country due to different therapeutic and preventive policy[18].

Total 25 (13.29%) specimens yielded mixed infection in culture that is in accordance with a study in Bangladesh14. Among isolated Pseudomonas species, 19.67% samples had

mixed infection and the causative organisms (Esch coli, Staph aureus, Proteus spp, Klebsiella spp.) isolated in those mixed infection were same as reported in a study[14]. Maximum mixed infections were found from traumatic wound and burn wounds. It is probable that mixed infection occurs more often in burn and traumatic wound due to wide exposed area of the wound where opportunistic organisms easily colonize. Mixed infection in traumatic wound may also probably be attributed to the contamination of the wound with soil and other environmental microbes as majority of the cases were victims of road traffic accident.

Species of Pseudomonad were identified by culture on blood agar, MacConkey's agar media, gram staining, different biochemical tests and by multiplex PCR. By multiplex PCR 67 samples were identified as Pseudomonas and among them 95.52% were detected as Pseudomonas aeruginosa; 2.99% were S maltophilia and 1.49% was B cepacia. The results are consistent with a study in Bangladesh where more than 90% of the isolated species were Pseudomonas aeruginosa[19].

Multiplex PCR was done on all the 230 samples. Three sets of primer pair were used for identification of P aeruginosa, B cepacia and S maltophilia. By PCR 67 samples were identified as Pseudomonad spp., 58 samples were positive by both culture and PCR whereas three samples that were positive in culture were found negative by PCR. Considering culture as the gold standard, the sensitivity of PCR was 95.08% and the specificity was 94.67%. PCR was the most rapid method for identification of Pseudomonad with a detection time of less than 24 hours for DNA directly from sample whereas conventional methods required about 72 hours.

Three of the culture positive specimens were not positive for Pseudomonad DNA by PCR. Among them two organisms were identified as P fluorescence and P alcaligenes by biochemical tests but were negative by PCR as primers specific for them were not used. The remaining one was identified as Pseudomonas spp. biochemically but PCR showed negative result. This might be due to some other species of Pseudomonad which could not be differentiated biochemically from P aeruginosa. Alternatively the primers used in the study might have failed to detect P aeruginosa due to mutation in the annealing site of DNA.

In this study, nine culture negative specimens gave positive result by PCR. Strict procedures were employed during laboratory work to avoid contamination. Also the DNA-free negative controls that were used during each amplification cycle were all negative. Thus it is also possible that these 'culture-negative, PCR-positive' results represent the better effectiveness of PCR in detection of Pseudomonad in specimen or this may be due to failure of growth of the Pseudomonad in culture, probably due to insufficient or non-cultivable bacteria in sample. PCR requires as few as 10 organisms in a sample for detection whereas for culture the detection limit is higher[20]. As PCR had a very good sensitivity and specificity it may be an alternative for detection of organisms from clinical samples which will provide a more rapid result.

## CONCLUSION

Pseudomonads were the second most common organisms next to Esch coli isolated from infected wound in a tertiary level hospital in Bangladesh and 91.8% species was Pseudomonas aeruginosa. Multiplex PCR was a rapid and might be a reliable detection method for Pseudomonad spp. from samples.

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**Conflict of interest:**
We do not have any potential conflicts of interest.

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