Serotyping and Cross-Reactivity’s Between Different *Pseudomonas aeruginosa* Isolates Prevalent in Iran

Ahmadi H¹, Maleknia S², Taharae B³, Norouzian D³, Poormirza-gholi F³, Nejati M¹, Hedayati MH¹, Beik Mohammadi MR¹, Behnoodi A¹, Izadpanahi M¹

¹Department of Bacterial Vaccines and Antigen Production, Pasteur Institute of Iran, Tehran, Iran.  
²Department of Microbiology, Islamic Azad University, Zanjan Branch, Iran.

Received: January 2010, Accepted: April 2010.

**ABSTRACT**

**Background and Objectives:** 300 *Pseudomonas aeruginosa* strains were isolated from hospitalized patients in Iran. Using international antigenic typing system (IATS) antibodies, all strains were classified into 16 serotypes while serotype 14 was not identified among the 17 known serotypes. To evaluate the rate of cross-reactivity between O-antigenic determinants, monospecific polyclonal antibodies were made against whole-killed-cells and live cells of each serotype.

**Material and Methods:** Each antiserum was challenged against homologous and heterologous antigens using slide agglutination test. The degree of agglutination reaction is shown by -ve, 1+ve, 2+ve, 3+ve and 4+ve for 0, 25%, 50%, 75% and 100% agglutination respectively. Then, the results were tabulated for further study.

**Results:** The rate of cross-reactivity between O-antigenic determinants demonstrated that strains 10.55 and 15.14 had the highest agglutination reaction with serum of all the homologous and heterologous serotypes.

**Conclusion:** Evaluation of the results obtained from the present study can be applied in production of reliable vaccines and antisera as therapeutic agents or as diagnostic kits.

**Keywords:** *Pseudomonas aeruginosa*, agglutination test, cross-reactivity, antigenic schema.

**INTRODUCTION**

*Pseudomonas aeruginosa* is one of the most common opportunistic pathogen of nosocomial infections (1-9). Being amphibiotic in nature (parasite & saprophyte), *P. aeruginosa* causes a high epidemic spread in wound and burn infections and in patients with immune deficiency syndrome, neoplasia, cystic fibrosis, ones who have undergone surgery, organ transplantation or have received artificial organs (2, 3, 5, 6, 9, 10).

Based on the antigenic specification of the oligosaccharide side chain of LPS (O-Antigen), many serological classification systems for *P. aeruginosa* were proposed (11-14), but the most reliable typing system was the one suggested by the International Committee of Microbiology (ICM) in 1970 which adapted 17 heat stable O-antigenic typing system abbreviated by arabic numbers 1 through 17 (1, 7).

In the present study, we have performed the slide agglutination test according to the panel of the ICM typing system, using 300 pathogenic *P. aeruginosa* isolates collected from different hospitals in Iran to suggest a new model of antigenic schema for *P. aeruginosa* serotyping so that, to control or prevent the infection.

**MATERIALS AND METHODS**

**Bacterial strains.** Applying biochemical tests and using standard somatic typing antisera from Difco (Franklin Lakes, NJ USA) & Denka Seiken (Tokyo, Japan) Companies, all 300 clinical isolates...
of *P. aeruginosa* were classified among the Iranian isolates. Each serotype was lyophilized, encoded and kept as stock culture in the Collection of Standard Bacteria of the Pasteur Institute of Iran (CSBPI) till use. Standard strain of *P. aeruginosa* PTCC-1074 was used as a positive control.

**Antiserum preparation.** Each *P. aeruginosa* serotype was grown on Heart Infusion Agar (HIA) (MERK, Darmstadt, Germany) for 18 hours at 37°C. Cells were harvested by PBS (Phosphate Buffer Saline, pH = 7.2) containing 0.5% phenol and 2% (V/V) of 20% glucose solution. Each suspension was heated at 90°C for one hour in shaking water bath and then washed three times with the same buffer. A portion of each cell suspension was adjusted to 9×10⁸ cells/ml in sterile PBS (pH = 7.2) and then used as immunizing antigen.

A group of 2 white New Zealand rabbits, weighing 1.5 to 2 Kg, were immunized intravenously with each serotype suspension in increasing doses of 0.25, 0.5, 1, 1.5 and 2 ml at 4 day intervals 7 days after the last injection. The sera were collected from each group and pooled. After addition of 1:10000 (W/V) thioumeral, all sera were kept at 4°C until use.

**Rapid slide agglutination test.** This test was applied for both the live and heat killed cells of all 16 of *P. aeruginosa*. Each serotype was grown on Heart Infusion Agar (HIA) for 18 hours at 37°C. Cells were harvested by PBS (Phosphate Buffer Saline, pH = 7.2) containing 0.5% phenol and 2% (V/V) of 20% glucose solution. Each suspension was heated at 90°C for one hour in shaking water bath and then washed three times with the same buffer. A portion of each cell suspension was adjusted to 9×10⁸ cells/ml in sterile PBS (pH = 7.2) and then used as immunizing antigen.

A group of 2 white New Zealand rabbits, weighing 1.5 to 2 Kg, were immunized intravenously with each serotype suspension in increasing doses of 0.25, 0.5, 1, 1.5 and 2 ml at 4 day intervals 7 days after the last injection. The sera were collected from each group and pooled. After addition of 1:10000 (W/V) thioumeral, all sera were kept at 4°C until use.

**Rapid slide agglutination test.** This test was applied for both the live and heat killed cells of all 16 of *P. aeruginosa*. Each serotype was grown on Heart Infusion Agar (HIA) for 18 hours at 37°C. Cells were harvested by PBS (Phosphate Buffer Saline, pH = 7.2) containing 0.5% phenol and 2% (V/V) of 20% glucose solution. Each suspension was heated at 90°C for one hour in shaking water bath and then washed three times with the same buffer. A portion of each cell suspension was adjusted to 9×10⁸ cells/ml in sterile PBS (pH = 7.2) and then used as immunizing antigen.

A group of 2 white New Zealand rabbits, weighing 1.5 to 2 Kg, were immunized intravenously with each serotype suspension in increasing doses of 0.25, 0.5, 1, 1.5 and 2 ml at 4 day intervals 7 days after the last injection. The sera were collected from each group and pooled. After addition of 1:10000 (W/V) thioumeral, all sera were kept at 4°C until use.

**Rapid slide agglutination test.** This test was applied for both the live and heat killed cells of all 16 of *P. aeruginosa*. Each serotype was grown on Heart Infusion Agar (HIA) for 18 hours at 37°C. Cells were harvested by PBS (Phosphate Buffer Saline, pH = 7.2) containing 0.5% phenol and 2% (V/V) of 20% glucose solution. Each suspension was heated at 90°C for one hour in shaking water bath and then washed three times with the same buffer. A portion of each cell suspension was adjusted to 9×10⁸ cells/ml in sterile PBS (pH = 7.2) and then used as immunizing antigen.

A group of 2 white New Zealand rabbits, weighing 1.5 to 2 Kg, were immunized intravenously with each serotype suspension in increasing doses of 0.25, 0.5, 1, 1.5 and 2 ml at 4 day intervals 7 days after the last injection. The sera were collected from each group and pooled. After addition of 1:10000 (W/V) thioumeral, all sera were kept at 4°C until use.

**Rapid slide agglutination test.** This test was applied for both the live and heat killed cells of all 16 of *P. aeruginosa*. Each serotype was grown on Heart Infusion Agar (HIA) for 18 hours at 37°C. Cells were harvested by PBS (Phosphate Buffer Saline, pH = 7.2) containing 0.5% phenol and 2% (V/V) of 20% glucose solution. Each suspension was heated at 90°C for one hour in shaking water bath and then washed three times with the same buffer. A portion of each cell suspension was adjusted to 9×10⁸ cells/ml in sterile PBS (pH = 7.2) and then used as immunizing antigen.

A group of 2 white New Zealand rabbits, weighing 1.5 to 2 Kg, were immunized intravenously with each serotype suspension in increasing doses of 0.25, 0.5, 1, 1.5 and 2 ml at 4 day intervals 7 days after the last injection. The sera were collected from each group and pooled. After addition of 1:10000 (W/V) thioumeral, all sera were kept at 4°C until use.

**Rapid slide agglutination test.** This test was applied for both the live and heat killed cells of all 16 of *P. aeruginosa*. Each serotype was grown on Heart Infusion Agar (HIA) for 18 hours at 37°C. Cells were harvested by PBS (Phosphate Buffer Saline, pH = 7.2) containing 0.5% phenol and 2% (V/V) of 20% glucose solution. Each suspension was heated at 90°C for one hour in shaking water bath and then washed three times with the same buffer. A portion of each cell suspension was adjusted to 9×10⁸ cells/ml in sterile PBS (pH = 7.2) and then used as immunizing antigen.

A group of 2 white New Zealand rabbits, weighing 1.5 to 2 Kg, were immunized intravenously with each serotype suspension in increasing doses of 0.25, 0.5, 1, 1.5 and 2 ml at 4 day intervals 7 days after the last injection. The sera were collected from each group and pooled. After addition of 1:10000 (W/V) thioumeral, all sera were kept at 4°C until use.

**Rapid slide agglutination test.** This test was applied for both the live and heat killed cells of all 16 of *P. aeruginosa*. Each serotype was grown on Heart Infusion Agar (HIA) for 18 hours at 37°C. Cells were harvested by PBS (Phosphate Buffer Saline, pH = 7.2) containing 0.5% phenol and 2% (V/V) of 20% glucose solution. Each suspension was heated at 90°C for one hour in shaking water bath and then washed three times with the same buffer. A portion of each cell suspension was adjusted to 9×10⁸ cells/ml in sterile PBS (pH = 7.2) and then used as immunizing antigen.

A group of 2 white New Zealand rabbits, weighing 1.5 to 2 Kg, were immunized intravenously with each serotype suspension in increasing doses of 0.25, 0.5, 1, 1.5 and 2 ml at 4 day intervals 7 days after the last injection. The sera were collected from each group and pooled. After addition of 1:10000 (W/V) thioumeral, all sera were kept at 4°C until use.

**Rapid slide agglutination test.** This test was applied for both the live and heat killed cells of all 16
isolates. PBS also was used as negative control. Two drops of each antiserum was placed on a clean glass slide. A loop full of 18 hours growth from each live serotype was mixed evenly with the first drop and one drop of a thick suspension of each heat-killed cells was mixed second drop, slides were tilted by hand and the rate of agglutination reaction was recorded from four positive (4+ means strong agglutination appearing in a few seconds and one positive means a weak agglutination reaction at the end of one minute) (19).

The results were tabulated so that the rate of agglutination of both live and killed bacteria from homologous and heterologous strains against each serum was recorded.

### RESULTS

Comparison of the results observed in Tables 1 & 2 show a minor antigenic difference between the live and killed antigens. It was also observed that strains 10.55 and 15.14 had the highest agglutination reaction with serum of all the homologous and heterologous serotypes except serotype 8 and 9. Strain 1.101 had weak agglutination reaction. The sera against strains 6.95, 6.109, 7.107, 17.110 had maximum agglutination reaction with homologous and heterologous strains. Strains 11.106 and 15.14 showed the minimum agglutination reaction.

#### Table 2. Antigenic schema with heat - stable somatic antigens invtro by the slide agglutination test.

| Antigen | PTCC-1074 | 12:159 | 15:14 | 16:190 | 17:110 | 10:55 | 15:108 | 12:159 | 15:108 |
|---------|-----------|--------|-------|--------|--------|-------|--------|--------|--------|
| 1:101   | 4+ 2+ 2+ 1+ 1+ 1+ 2+ 2+ 2+ |           |       |        |        |       |        |        |        |
| 2:160   | 4+ 1+ 4+ 2+ 2+ 1+ 4+ 4+ 4+ |           |       |        |        |       |        |        |        |
| 3:172   | 2+ 4+ 2+ 3+ 2+ 4+ 2+ 1+ 1+ |           |       |        |        |       |        |        |        |
| 4:89    |           | 4+     |       |        |        |       |        |        |        |
| 5:60    | 4+ 3+ 1+ 4+ 1+ 3+ 1+ 1+ 1+ 2+ 2+ 1+ | 1+ 2+ | 1+ 2+ | 1+ |        |        |       |        |        |
| 5:111   | 4+ 4+ 4+ 4+                       |        |       |        |        |       |        |        |        |
| 6:95    |           | 4+     |       |        |        |       |        |        |        |
| 6:109   |           | 4+     |       | 4+     | 4+     | 4+     |        |        |        |
| 7:107   |           | 4+     |       | 4+     | 4+     | 4+     |        |        |        |
| 7:197   |           | 4+     |       | 4+     | 4+     |        |        |        |        |
| 8:98    |           | 4+     |       | 4+     | 4+     |        |        |        |        |
| 9:105   |           | 1+     | 2+     | 2+     | 3+     | 4+     | 4+     | 1+     |        |
| 10:55   | 4+ 4+ 4+ 4+ 3+ 4+ 4+ 4+ 4+ 4+ 4+ 3+ 3+ 4+ 3+ 3+ 3+ 4+ 3+ | 4+ 4+ |        |        |        |        |        |
| 11:106  |           | 4+     |       | 4+     | 4+     |        |        |        |        |
| 12:80   |           | 4+     |       | 4+     | 4+     |       |        |        |        |
| 12:159  |           | 4+     |       | 4+     | 4+     |       |        |        |        |
| 13:108  | 4+ 4+ 4+ 3+ 4+ 4+ 4+ 4+ 4+ 1+ 4+ 3+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 3+ | 4+ 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| 15:14   | 4+ 4+ 2+ 4+ 3+ 4+ 4+ 4+ 4+ 1+ 4+ 3+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 3+ | 4+ 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| 16:190  | 4+ 1+ 4+ 4+ 1+ 1+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ | 4+ 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| 17:110  | 4+ 4+ 4+ 4+ 4+ 3+ 4+ 4+ 3+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 4+ 3+ | 4+ 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| PTCC-1074 | 4+     | 2+     | 2+     |       |        |        |        |        | 4+     |
1. Kusama H. Serological classification of Pseudomonas aeruginosa by a slide agglutination test. J Clin Microbiol 1978; 8: 181-188.
2. Brokopp CD, Gomez-Lus R, Farmer JJ 3rd. Serological typing of Pseudomonas aeruginosa: use of commercial antisera and live antigens. J Clin Microbiol 1977; 5: 640-649.
3. Bouza E, Garcia-Garrote F, Cercenado E, Marin M, Diaz MS. Pseudomonas aeruginosa: a Survey of Resistance in European Hospitals. J Antimicrob Chemother 1999; 43: 981-982.
4. Penner JL, Whiteley R. Cross-protection of mice provided by active and passive immunization against experimental infection with virulent Proteus retgeri and Providencia bacteria. Infect Immun 1978; 20: 347-351.
5. Faure K, Shimabukuro D, Ajayi T, Allmond LR, Sawa T, Wiener-Kronish JP. O-antigen serotype and type III secretory toxins in clinical isolates of Pseudomonas aeruginosa. J Clin Microbiol 2003; 41: 2158-2160.
6. Masoud H, Sadovskaya I, Kiviat T, Altman E, Richards JC, Lam JS. Structural elucidation of the lipopolysaccharide core region of the O-chain-deficient mutant strain A28 from Pseudomonas aeruginosa serotype 06 (International Antigenic Typing Scheme). J Bacteriol 1995; 177: 6718-6726.
7. Higeta S, Yasunaga Y, Ogata M. Type-specific indirect hemagglutinating antibody in patients with Pseudomonas aeruginosa infection. J Clin Microbiol 1978; 8: 489-495.
8. Janet AM, Harris G, Govan RWJ. Revised. Pyocin typing method for Pseudomonas aeruginosa. J Clin Microbiol 1984; 20: 47-50.
9. Head NE, Yu H. Cross-Sectional Analysis of clinical and environmental isolates of Pseudomonas aeruginosa: Biofilm formation, virulence, and genome diversity. Infect Immun 2004; 72: 133-144.
10. Pier GB. Cross-protection by Pseudomonas aeruginosa polysaccharides. Infect Immun 1982; 38: 1117-1122.
11. Lémy B. Serological properties of Pseudomonas aeruginosa. I. Group-specific somatic antigens. Acta Microbiol Acad Sci Hung 1966/67; 13: 295-318.
12. Meittin T. Contribution à l’étude de la structure antigénique des B. pyocyaniques (Pseudomonas aeruginosaa). II. Individualisation des groupes sérologiques au moyen des antigènes. O. Arch Pathol Exp Microbiol 1964; 23: 679-688.
13. Habs J. Untersuchungen über die O-antigene von Pseudomonas aeruginosa. Z Hyg 1957; 114: 218-227.
14. Homma JY. A new antigenic scheme and live-cell slide-agglutination protocol for the intra-sub-specific, serologic classification of Pseudomonas aeruginosa. Jpn J Exp Med 1976; 46: 329-336.
15. Oldak E, Tarfany E, A. Secretion of protease by Pseudomonas aeruginosa bio-films exposed to ciprofloxacin. J Antimicrob Chemother 2005; 49: 3281-3288.
16. Miyairi YA, Bystrova OV, Kocharova NA, Zahringer U, Miutler T. Contribution à l’étude de la structure antigénique des B. pyocyaniques (Pseudomonas aeruginosaa). II. Individualisation des groupes sérologiques au moyen des antigènes. O. Arch Pathol Exp Microbiol 1964; 23: 679-688.
17. Dorin G, Pier GB. Vaccines and immunotherapy against Pseudomonas aeruginosa. Vaccine 2006; 24: 1381-1387.
18. Kairel YA, Bystryova OV, Kochorava NA, Zahringer U, Pier GB. Conserved and variable structural features in the lipopolysaccharide of Pseudomonas aeruginosa. J Endotox Res 2006; 12: 324-336.