*Indian Journal of Medical Microbiology, (2013) 31(1): 29-33*

*Original Article*

# Multiple virulence factors regulated by quorum sensing may help in establishment and colonisation of urinary tract by *Pseudomonas aeruginosa* during experimental urinary tract infection

**P Gupta, RK Gupta, *K Harjai**

## Abstract

**Purpose:** Damage caused by an organism during infection is attributed to production of virulence factors. Different virulence factors produced by the organism contribute to its pathogenicity, individually. During infectious conditions, role of virulence factors produced by the pathogen is different, depending upon the site of involvement. *Pseudomonas aeruginosa* is an opportunistic nosocomial pathogen known to cause infections of the respiratory tract, burn wound, urinary tract and eye. Importance of virulence factors produced by *P. Aeruginosa* during infections such as keratitis, burn wound and respiratory tract is known. The present study was designed to understand the importance of different virulence factors of *P. aeruginosa* in urinary tract infection *in vivo*. **Materials and methods:** An ascending urinary tract infection model was established in mice using standard parent strain PAO1 and its isogenic mutant, JP2. Mice were sacrificed at different time intervals and renal tissue homogenates were used for estimation of renal bacterial load and virulence factors. **Results:** Both parent and mutant strains were able to reach the renal tissue. $PAO_1$ PAO1was isolated from renal tissue till day 5 post-infection. However, the mutant strain was unable to colonise the renal tissue. Failure of mutant strain to colonise was attributed to its inability to produce protease, elastase and rhamnolipid. **Conclusion:** This study suggests that protease, elastase and rhamnolipid contribute to pathogenesis and survival of *P. aeruginosa* during urinary tract infection.

**Key words:** *Pseudomonas aeruginosa, urinary tract infections, virulence factors*

## Introduction

Establishment and survival of pathogen in host are important pre-requisites in initiation of any successful infection. *Pseudomonas aeruginosa*, an emerging and opportunistic pathogen, also needs host colonisation to cause infection. This pathogen is responsible for increased morbidity and mortality especially in nosocomial settings.[1] This pathogen has become notorious due to its increased antibiotic resistance, and ability to form biofilms. *P. aeruginosa* expresses multiple virulence factors, which contribute to its complex pathogenicity in various infections individually. Array of virulence factors includes cellular and extracellular virulence factors like pili, flagella, lipopolysaccharide, elastase, alkaline proteases, pyocyanin, pyoverdin, haemolysins, phospholipase C, rhamnolipids and biofilm formation that are under regulation of quorum sensing.[2]

Urinary tract infection (UTI) is a common mucosal infection of catheterised patients in hospital settings. *P. aeruginosa* is the third most important organism associated with catheter associated UTIs and is responsible for approximately 40% of total nosocomial infections.[3] Pathogenesis of *P. aeruginosa* in various infections such as respiratory tract infection, cystic fibrosis, burn wound infection and microbial keratitis have been attributed to its ability to express multiple virulence factors.[4-6] However, how these virulence factors contribute to pathogenesis of different infections is still not clear. Previously, researchers have shown the importance of some particular virulence factor, associated specifically with particular infection. Elastase and protease have been shown to be important for the dissemination of *P. aeruginosa* through infected skin of mice in the experimental burn wound infection model.[5] In another study, Zhu *et al*.[6] have shown that production of elastase does not make any difference in severity of microbial keratitis in mice model. On the other hand, Azhgani *et al*.[4] reported that production of elastase plays important role in pathogenesis of *P. aeruginosa* during respiratory tract infections. All these studies have indicated that out of wide variety of virulence factors produced by *P. aeruginosa*, individual virulence factor may govern

*Corresponding author (email: < kusum_harjai@hotmail.com>)
Department of Microbiology, BMS Block, Panjab University, Chandigarh, India
Received: 13-07-2012
Accepted: 06-01-2013

| Access this article online | |
|---|---|
| Quick Response Code: | Website: www.ijmm.org |
| | |
| | DOI: 10.4103/0255-0857.108715 |

and contribute differentially in the outcome of different infection.

In case of UTI, most of the work in relation to role of virulence factor in outcome of infection has been carried out with respect to *Escherichia coli* only. Earlier, importance of iron acquiring molecules, haemolysins and siderophores has been shown in case of *E. coli* induced UTI.[7] To the best of our knowledge, no such study has been carried out in case of *P. aeruginosa*-induced UTI. Since, elastase, protease and rhamnolipid are considered to be important virulence factors of *P. aeruginosa* and their role has been established in other mucosal infections, it becomes imperative to study the contribution of these virulence factors along with other factors such as siderophore and haemolysin in the pathogenesis of experimental UTI caused by *P. aeruginosa*.

## Material and Methods

### *Bacterial strains*

*P. aeruginosa* standard strain PAO1and its isogenic mutant strain $JP_2$(Δ*LasI:* Δ*RhlI:* Tn 501 $Tc^r$ $Hg^r$) were obtained from Dr. Barbara H. Iglewski, University of Rochester, New York, USA. JP2 is a double mutant lacking *lasI* and *rhlI* gene whereas PAO1 is parent strain with both the functional QS systems. For the growth and maintenance of JP2 tetracycline and mercuric chloride (50 μg/mL and 7.5 μg/mL, respectively) were added to the growth media.

### *Induction of acute ascending pyelonephritis*

In-house laboratory standardised method was followed. For the use of animals, Institutional Animal Ethical Committee granted the approval. Female LACA (Swiss Webster) mice, 6-8 weeks old, free of bacteriuria were used. Fifty micro litre of $10^8$ colony forming unit per millilitre (cfu/mL) of bacterial inoculum was introduced into the bladder of mice using a soft non-radio-opaque polyethylene tubing (outer diameter 0.61 mm, Clay Adams, BD USA). The catheter was withdrawn slowly without any further manipulation.

### *Bacteriological examination and tissue homogenate*

Mice were sacrificed at different time intervals (12 h, day 1, day 3 and day 5). Kidneys were removed and weighed aseptically. Kidney tissues were homogenised in 1 mL sterile saline. Quantitative bacterial count was made after plating appropriate dilutions on MacConkey agar plates. Bacterial counts were expressed as Log cfu per gram of kidney tissue. Remaining tissue homogenates were centrifuged at 1500 × g and filtered with 0.45 μm membrane filter (Millipore, USA) and filtrate obtained was used for the estimation of different virulence factors as below.

### *Protease estimation*

Proteolytic activity was estimated according to the method of Visca *et al*.[8] Briefly, 200 μL of tissue homogenate was diluted in 10 mM Tris buffer (pH 7.5) supplemented with 1 mM $CaCl_2$ and incubated with 7 mg hide powder azure (Sigma Chemicals, USA) at 37°C for 1 h. Absorbance was measured at 595 nm and results were expressed in units per millilitre (U/mL).

### *Elastase estimation*

The elastase activity was measured following the method of Visca *et al*.[8] One milligram of elastin-congo red (Sigma Chemicals, USA) was suspended in 200 μL of tissue homogenate mixed with 200 μL of 100 mM Tris-HCl buffer (pH 7.0) supplemented with 1 mM $CaCl_2$. Tubes were kept at 37°C for 2 h under shaking conditions. Reaction was stopped by adding 300 μL of 0.7 M sodium phosphate buffer (pH 6.0) and was centrifuged at 1500 × g for 5 min at 4°C. Absorbance was read at 495 nm and results were expressed in units per millilitre (U/mL).

### *Rhamnolipid estimation*

Rhamnolipid was quantified by the orcinol method.[9] Tissue homogenates were extracted with diethyl ether. This extract measuring 100 μL was diluted with 1:10 in freshly prepared orcinol reagent (7.5 volume of 60% $H_2SO_4$ and 1 volume of 1.6% (w/v) orcinol in distilled water) and mixture was heated in water bath at 80°C for 30 min. Absorbance was measured at 421 nm. L-Rhamnose (Sigma chemicals, USA) was used to standardise the assay.

### *Pyochelin estimation*

Quantitation of pyochelin was done by the method of Yadav *et al*.[10] Tissue homogenate measuring 200 μL, 200 μL each of 0.5 N hydrochloric acid, nitrite molybdate reagent and 1N NaOH were mixed and the final volume was made to 1 mL with distilled water. Absorbance was read at 510 nm.

### *Haemolysin estimation*

Cell free and cell bound haemolysin was estimated following the method of Linkish and Voget.[11]

### *Cell bound haemolysin*

350 μL of 2% washed human RBC suspension was mixed with 350 μL of cells and incubated at 37°C for 2 h. Assay mixture was centrifuged at 5000 g for 15 min. Supernatant was collected and absorbance was read at 545 nm. Haemolysin (mg/mL) was calculated by using lyophilised haemoglobin (Hb) as reference.

### *Cell free haemolysin*

350 μL of 2% washed human RBC suspension was mixed with 350 μL of tissue homogenate and incubated at

37°C for 2 h. Assay mixture was centrifuged at 5000 g for 15 min and absorbance was taken at 545 nm.

### *Statistical analysis*

All experiments were carried out in triplicates to validate the reproducibility. Results were analysed statistically by Student's *t*-test using Graphpad prism software to calculate *P* values.

## Results

### *Kidney tissue bacteriology*

To determine the bacterial lodgement of renal tissue of infected mice, log CFU was calculated. It was observed that both standard and mutant strains were able to reach the kidney tissue within first 12 h of infection. Standard strain PAO1 was able to colonise kidney tissue till day 5 (log cfu 3.7 ± 0.27) with peak infection on day 3 post-infection (4.85 ± 0.48). On the other hand, mutant strain JP2 was cleared from renal tissue within first 24 h of infection (2.7 ± 0.15). Moreover, the viable count of mutant strain JP2 was significantly less than that of standard strain PAO1 ($P < 0.01$, Figure 1).

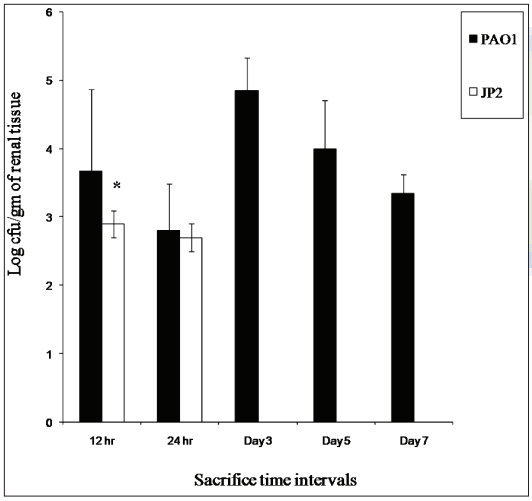


**Figure 1:** Comparative renal bacterial load (log cfu/g renal tissue) of mice after interuretheral challenge with standard strain PAO1 and its isogenic mutant JP2 (*$PAO_1$ *vs.* JP2 $P < 0.01$)

### *Virulence factor estimation*

#### Protease estimation

Protease levels were determined in renal tissue homogenate of mice infected with PAO1 and JP2. In tissue homogenate of mice infected with PAO1, protease levels were detectable immediately after 12 h post-infection with peak production on day 3 post-infection followed by decrease at day 5 post-infection [Table 1]. On the other hand, JP2 was unable to produce protease at any time interval.

#### Elastase estimation

Elastase levels were also detectable within 12 h post-infection in renal homogenate of mice infected with PAO1 with peak production on day 1 post-infection. Elastase levels declined on day 3 post-infection with slight increase on day 5 post-infection [Table 1]. While, JP2 was unable to produce elastase at any post-infection time interval.

#### Rhamnolipid estimation

Standard strain PAO1 showed subsequent increase in production of rhamnolipid from 12 h post-infection till day 5 post-infection. Peak level was observed on day 5 post-infection. However, mutant strain JP2 was unable to produce rhamnolipid at any time interval [Table 1].

#### Haemolysin and pyochelin estimation

Both PAO1 and JP2 produced haemolysin and pyochelin within 12 h post-infection. However, the level produced by mutant strain JP2 was significantly less as compared to PAO1 ($P < 0.01$). In the case of PAO1, maximum haemolysin production was observed at 12 h of infection. On the other hand, no detectable levels of haemolysin were found after day 1 post-infection in strain JP 2 [Table 1]. The pyochelin level was maximum on day 3 post-infection in the case of PAO 1and on day 1 post-infection in case of JP 2 [Table 1].

## Discussion

Colonisation of host tissue during the initial phase of infection is a prerequisite for establishment of any

**Table 1: Production of protease, elastase, rhamnolipid, phychelin and haemolysin (cell free and cell bound) in renal tissues of mouse under experimental Urinary tract infection induced by standard strain of *Pseudomonas aeruginosa* PAO1 and quorum sensing double mutant $JP_2$ (**P*<0.01; ***P*<0.001; ****P*<0.025)**

| Sacrifice Time intervals | Protease (U/mL) | | Elastase (U/mL) | | Rhamnolipid (mg/mL) | | Pyochelin (O.D. 510) | | Cell free haemolysin (Hb mg/mL) | | Cell bound haemolysin (Hb mg/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time interval | PAO1 | JP2 | PAO1 | JP2 | PAO1 | $JP_2$ | PAO1 | JP2 | PAO1 | JP2 | PAO1 | JP2 |
| 12 hr | 1±0.21 | -- | 2±0.28 | -- | 0.3±0.01 | -- | 1.4±0.21 | 1.23±0.02 | 1.3±0.14 | 1.1±0.07 | 4.25±0.14 | 0.5±0.03** |
| 24 hr | 1.4±0.28 | -- | 2.5±0.14 | -- | 0.25±0.02 | -- | 1.35±0.14 | 1.6±0.21 | 1.25±0.07 | 1.0±0.25 | 1.9±0.14 | 1.1±0.14* |
| Day 3 | 4.1±0.141 | -- | 1±0.28 | -- | 0.45±0.03 | -- | 1.76±0.14 | 1.35±0.16* | 0.25±0.02 | -- | 1.1±0.14 | -- |
| Day 5 | 1.4±0.35 | -- | 1.5±0.14 | -- | 0.49±0.01 | -- | 0.53±0.07 | 0.27±0.04*** | 0.9±0.14 | -- | 0.33±0.21 | -- |

UTI: Urinary tract infection

successful infection. Virulence factors (VFs) produced by the pathogen help in the host colonisation, which leads to development of infection. Like many other infections, establishment of UTI also requires pathogen colonisation. In the case of UTI, siderophores and haemolysins have been shown to be important for establishment of UTI caused by *E. coli*. However, VFs elaborated by *P. aeruginosa* to cause UTI is still under investigation. In *P. aeruginosa*, production of VFs is under control of QS. Therefore, in this study, the role of VFs was determined by employing $JP_2$ mutant strain, having mutations in the inducer regions of both QS systems responsible for the regulation of most of the VFs. Its lodgement in renal tissue was compared with the establishment of standard strain PAO1(elaborating both *las* and *rhl* QS system). Different groups of mice were infected with standard parent strain and mutant strain, and the mice were sacrificed on different time intervals post-infection. It was observed that mutant strain was able to reach the kidneys with in 12 h post-infection but was unable to survive in the renal tissue. While, standard strain persisted in kidneys for a time interval of 5 days with peak infection on day 3 post-infection. Survival of a pathogen in host depends on its ability to attach to host cells, produce VFs and alter/modulate host immune responses. Few workers have demonstrated that mutant strains, under stress conditions *in vivo*, can behave differently and can restore production of certain VFs.[12] Therefore, for real time evaluation of contribution of individual VFs in establishment of *P. aeruginosa in vivo*, level of protease, elastase, rhamnolipid, pyochelin and haemolysin were estimated in renal tissue homogenates of mice infected with standard strain PAO1 and mutant strain JP2.

In the case of *P. aeruginosa*, virulence factors elastase, protease and rhamnolipid are known to inhibit host immune responses and promote infiltration.[13-16] Proteases are known for their role in tissue damage during infection. Their ability to degrade laminin and other substrates suggests their role in tissue invasion and dissemination.[13] In this study, renal tissues of mice infected with PAO 1 showed detectable levels of protease within first 12 h post infection with peak levels on day 3 post-infection. On the other hand, renal tissue of mice infected with JP 2 showed no detectable level of protease, indicating that this enzyme has important role in survival and establishment of *P. aeruginosa* in kidney tissue of mice. Elastase alone or in conjunction with protease inactivates several biologically important molecules including connective tissue and immune system component such as immunoglobulins. It plays a major role in degrading complex elastin and collagen matrix present in lungs during respiratory tract infection caused by *P. aeruginosa*. It also inhibits the action of protease inhibitors.[14] The role of these enzymes in the pathogenesis of respiratory tract infections, burn wound infections and microbial keratitis has been reported, previously.[4,5] Expression of these enzymes has shown to be important depending upon the type of infection involved. In this study, mutant strain JP 2 was cleared from renal tissue at early infection hour. While standard strain PAO 1remained colonised till day 5 indicating that production of elastase and protease is essential and critical for the pathogen establishment. JP 2 was unable to produce these factors, and hence was cleared early from the kidney suggesting that elastase and protease help *P. aeruginosa* by down regulating host immune factors and by helping it to invade the host tissue. Moreover, the absence of elastase enables host immune factors to be strong enough to clear the pathogen from renal tissue. Lack of or diminished production of these enzymes might have strong impact on the outcome of infection. Link between the level of protease production and severity of infection has also been shown earlier,[6] where strong protease producers were shown to be more virulent than low producer strains.

Rhamnolipid is a biosurfactant and a heat stable extracellular haemolysin. It inhibits the phagocytic action of macrophages and hence helps pathogen in evading phagocytosis. Rhamnolipid is a known virulence factor, found in higher concentrations in sputa of cystic fibrosis patients. The production of rhamnolipid and persistence of *P. aeruginosa* in kidney tissue indicate the importance of rhamnolipid in UTI caused by *P. aeruginosa.* Rhamnolipid being a biosurfactant, also promote biofilm formation and dispersion,[16] which leads to persistence and chronic infections. Previously, it has been reported that strains lacking rhamnolipid production were less virulent and were cleared from the lungs at early stages.[17] The results of this study corroborated with previous findings where rhamnolipid non-producer strain (mutant strain JP2) was cleared from renal tissue at a very early stage in the course of UTI.

Iron is unavailable for bacteria in the urinary tract but at the same time; it is also essential for the survival. For most pathogens, including *P. aeruginosa,* there is intense competition for iron within the host.[17] *P. aeruginosa* produces pyochelin and haemolysin to sense and sequester iron from its environment.[18] Haemolysin is not only known for acquiring iron from the surroundings, but it also helps in survival of pathogen by inhibiting host factors.[19] In this study, it was observed that both PAO1 and JP2 elaborated haemolysin and pyochelin within 12 h post-infection [Table 1]. Although, mutant strain JP2 also produced haemolysin and pyochelin at all-time intervals but their level was significantly less as compared to PAO1 ($P < 0.01$). It has been found that expression of some genes may be up regulated by environmental or nutritional factors.[20] Hence, the variable level of pyochelin production may attribute to such unknown factors, as observed in case of JP2. Results of this study showed that although mutant strain JP2 was able to produce iron-sequestering molecules comparable to that of standard strain PAO1, it did not help JP2 in colonisation of renal tissue. These findings indicate

that production of siderophores alone is not sufficient for successful colonisation, as it did not offer any advantage to mutant strain JP2, which was unable to establish in renal tissue 24 h post-infection.

## Conclusion

The results of this study highlights that production of virulence factors is essential and critical for survival and establishment of *P. aeruginosa* during UTI. These virulence factors play a major role in host tissue damage and invasion and hence lead to colonisation of pathogen. Protease, elastase and rhamnolipid significantly contribute to establishment of *P. aeruginosa* during UTI. Although, production of siderophores and haemolysins have been shown to be important for the establishment of *E. coli* during UTI,[19] in this present study siderophore and haemolysin production did not help the mutant strain JP2 in establishment. Mutant strain JP2 producing these factors and not producing protease, elastase and rhamnolipid, was not able to colonise the urinary tract of mice ascending model of UTI. On the other hand, strain producing these factors (PAO1) survived till day 5. Results suggest that not only siderophore and haemolysin, but also production of protease, elastase and rhamnolipid are essential for the establishment of *P. aeruginosa* in the urinary tract, hence indicating their role in pathogenesis of *P. aeruginosa*. These results provide the first evidence of involvement of these VFs of *P. aeruginosa* in UTI.

## Acknowledgment

Authors gratefully acknowledge Prof. Barbara Iglewski for providing the standard and mutant strains of *Pseudomonas aeruginosa*.

## References

1. Leone M, Albanèse J, Garnier F, Sapin C, Barrau K, Bimar MC, *et al*. Risk factors of nosocomial catheter associated urinary tract infection in polyvalent intensive care unit. Intensive Care Med 2003;29:1077-80.
2. Gupta RK, Setia S, Harjai K. Expression of quorum sensing and virulence factors are interlinked in *Pseudomonas aeruginosa*: An *in vitro* approach. Am J Biomed Sci 2011;3:116-25.
3. Wagenlehnera FM, Weidnerb W, Naber KG. Emergence of antibiotic resistance amongst hospital-acquired urinary tract infections and pharmacokinetic pharmacodynamic considerations. J Hosp Infect 2005;60:191-200.
4. Azghani O, Bedinghaus T, Klein R. Detection of Elastase from *Pseudomonas aeruginosa* in Sputum and its Potential Role in Epithelial Cell Permeability. Lung 2000;178:181-9.
5. Rumbaugh KP, Iglewisky BH, Hamood AN. Contribution of Quorum Sensing to the Virulence of *Pseudomonas aeruginosa* in Burn Wound Infections. Infect Immun 2009;67:5854-62.
6. Zhu H, Thuruthyil SJ, Willcox MD. Determination of quorum-sensing signal molecules and virulence factors of *Pseudomonas aeruginosa* isolates from contact lens-induced microbial keratitis. J Med Microbiol 2002;51:1063-70.
7. Johnson JR. Virulence factors in *Escherichia coli* urinary tract infection. Clin Microbiol Rev 1991;4:80-128.
8. Visca P, Chiarini F, Mansi A. Virulence determinants in *Pseudomonas aeruginosa* strains from urinary tract infections. Epidemiol Infect 1992;10:323-36.
9. Koch AK, Kappeli O, Fiechter A, Reiser J. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J Bacteriol 1989;173:4212-9.
10. Yadav V, Harjai K, Kaur R. Urovirulence of *Pseudononas aeruginosa*: Planktonic cells vs. biofim cells. Folia Microbiol 2004;49:465-70.
11. Linkish VG, Voget W. Direct haemolysin activity of phospholipase- A. Biochem Biophys Acta 1972;270:241-50.
12. Van Delden C, Iglewski BH. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis 1998;4:551-60.
13. Morihara K, Homma JY. *Pseudomonas* proteases. In: Holder JA, editor. Bacterial enzymes and virulence. Boca Raton: FL, CRC Press; 1985.
14. Schmidtchena A, Holstb E, Tapperc H. Elastase-producing *Pseudomonas aeruginosa* degrade plasma proteins and extracellular products of human skin and fibroblasts, and inhibit fibroblast growth. Microb Pathogen 2003;34:47-55.
15. Zulianello L, Canard C, Köhler T, Caille D, Lacroix JS, Meda P. Rhamnolipids are virulence factors that promote early infiltration of primary human airway epithelia by *Pseudomonas aeruginosa*. Infect Immun 2006;74:3134-47.
16. Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1. J Bacteriol 2003;185:1027-36.
17. Meyer JM, Neely A, Stintzi A. Pyoverdin is essential for virulence of *Pseudomonas aeruginosa.* Infect Immun 1996;64:518-23.
18. Poole K, Zhao Q, Neshat S, Heinrichs DE, Dean CR. The *Pseudomonas aeruginosa tonB* gene encodes a novel TonB protein. Microbiology 1996;142:1449-58.
19. Johnson MK, Allen JH. The role of haemolysin in corneal infections with *Pseudomonas aeruginosa*. Invest Opthalmol Vis Sci 1978;17:480-3.
20. Bollinger N, Hassett DJ, Iglewski BH, Costerton JW, McDermott TR. Gene expression in *Pseudomonas aeruginosa*: Evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. J Bacteriol 1996;183:1990-6.

**How to cite this article:** Gupta P, Gupta RK, Harjai K. Multiple virulence factors regulated by quorum sensing may help in establishment and colonisation of urinary tract by *Pseudomonas aeruginosa* during experimental urinary tract infection. Indian J Med Microbiol 2013;31:29-33.

**Source of Support:** Nil, **Conflict of Interest:** None declared.