DOI: 10.7860/JCDR/2016/18612.7892

Original Article

Microbiology Section

Microbiological Characterization of *Haemophilus influenzae* Isolated from Patients with Lower Respiratory Tract Infections in a Tertiary Care Hospital, South India

PADMAJA ANANTH SHENOY[1], KIRAN CHAWLA[2], SHASHIDHAR VISHWANATH[3], DIPIKA SHAW[4]

ABSTRACT

Introduction: *Haemophilus influenzae* is responsible for wide range of localized and invasive lower respiratory tract infections (LRTI) with the highest burden of disease in low and middle income countries.

Aim: The aim of the present study was to characterize the *H.influenzae* isolates from suspected LRTI.

Materials and Methods: A prospective study was conducted over a period of one and half years (December 2012 to May 2014) including patients with LRTI. *H.influenzae* was isolated from lower respiratory specimens following standard procedures. Complete characterization of the isolates was performed by bio typing, capsular serotyping, molecular genotyping and antibiotic susceptibility testing. The predisposing factors and clinical presentation were studied in the infected patients.

Results: A total of 8995 samples were received during the study period, out of which growth was significantly observed in 2848 (31.7%) samples. Among the various respiratory pathogens, *H.influenzae* was isolated from 175 (6.14%) patients. Majority (78.9%) of the patients presented with acute exacerbations of chronic obstructive pulmonary disease. The isolates most frequently were of Biotype II (35.42%). Only four of the 50 isolates subjected to capsular serotyping were typeable and were of type b, e and f. All the 50 isolates tested were found to be non-typeable by PCR for capsular genotyping. Maximum resistance was found against ampicillin (9.71%).

Conclusion: *H.influenzae* was found to be a significant cause of LRTI. Majority of the isolates were found to be non typeable strains. Non typeable *H. influenzae* isolates should not be neglected as they can colonize the respiratory tract in COPD patients and can lead to biofilm formation and treatment failure.

Keywords: Antibiotic susceptibility testing, Biotyping, Genotyping, Non-typeable strains

INTRODUCTION

Haemophilus influenzae is one of the common causes of community-acquired respiratory tract infection affecting mostly young children and the elderly with the highest burden of disease in low and middle income countries [1,2]. The organism is known to be carried in the nasopharynx of young children [3]. The encapsulated *H. influenzae* type b strains are the major bacterial pathogens of invasive infections, while non-typeable *H.influenzae* (NTHi) accounts for majority of respiratory infections. NTHi has been found commonly in association with lung disease such as recurrent exacerbations of COPD [4,5]. *H. influenzae* type b is still a major cause of concern in the developing countries, where incidence of invasive infection was much higher than that of the developed countries in pre vaccination era [3]. The implementation of *H. influenzae* type b vaccination has led to decline in the incidence of *H. influenzae* type b disease with relative upsurge in occurrence of the other capsular types (a, c-f) and non-capsulate (NC) strains [6].

Until the recent past, the penicillin group of drugs such as Ampicillin/Amoxicillin was commonly used for empirical treatment of *Haemophilus* disease. The third-generation cephalosporins (e.g., ceftriaxone) were used following the occurrence of ampicillin resistance in causative organisms [7]. In many parts of the world, resistance among *H. influenzae* isolates to β-lactam antibiotics is most frequent followed by trimethoprim-sulfamethoxazole [1].

It is very important to perform capsular typing of *H. influenzae*, as the capsule is a major virulence factor in the pathogenesis of the disease. The organism has been classified on the basis of capsular polysaccharide into six serotypes (a-f) as well as non-capsulated (NC) type. The identification of *H. influenzae* capsular type can be determined by the slide agglutination test (serotyping) using type specific antisera. Since serotyping is not a fully reliable method, Maaroufi Yet al., and LaClaire LL et al., incorporated PCR for capsular typing in their studies [8,9]. The amplification of genes involved in capsule expression was found to be rapid, specific, and highly sensitive for *H.influenzae* detection [8,9].

Considering the limited data on the burden of *H. influenzae* in developing countries [10], the present study was undertaken to determine the prevalence, biotype, serotype, genotype and the antimicrobial susceptibility pattern of *H. influenzae* in patients with lower respiratory tract infections in our tertiary care hospital.

MATERIALS AND METHODS

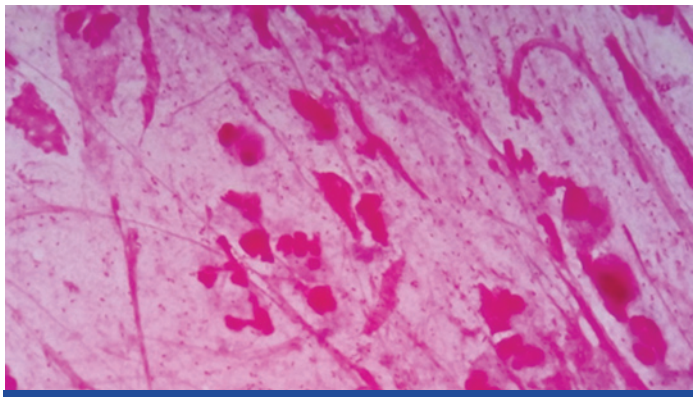
A prospective study was conducted in tertiary care centre of costal Karnataka over a period of 18 months from December 2012 to May 2014 following clearance from Institutional Ethics Committee. In suspected patients of Lower Respiratory Tract Infection (LRTI), history and demographics were collected from medical records. Respiratory specimens including, Bronchoalveolar Lavage (BAL), Endotracheal (ET) aspirate and sputum were collected from patients suffering from LRTI with history of cough and expectoration with or without dyspnea. The samples from patients below 15 years of age were excluded from the study. The collected samples after transporting immediately

to laboratory were processed by gram stain [Table/Fig-1] and culture. The samples whose gram stain was fulfilling the Barlett's grading with less than 5 squamous epithelial cells and more than 25 polymorphonuclear neutrophils per low power field [11] with pleomorphic gram negative coccobacilli, were cultured on sheep chocolate agar and 5% sheep blood agar [12]. The plates were incubated at 35-37°C in presence of 5-10% CO_2 for 18-24 hours. The *Haemophilus* isolates were identified on sheep chocolate agar with characteristic small (0.5-1mm) smooth, translucent, convex colonies with entire edge [Table/Fig-2]. Confirmation of *H. influenzae* isolates was done by gram stain from culture, positive catalase and oxidase, growth requirement of factor X (hemin) & V (Nicotinamide adenine dinucleotide) and positive satellitism test. In satellitism test, *S.aureus* was streaked across the surface of an inoculated 5% sheep blood agar plate, incubated at 35-37°C for 18-24 hours in presence of 5-10% CO_2. The colonies of *H.influenzae* nearer to the *S.aureus* appeared larger because of the release of V factor into the medium. These procedures were done according to standard techniques [12]. Antibiotic susceptibility testing of the *H. influenzae* isolates to ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg) and ceftriaxone (30 µg) was performed on *Haemophilus* test medium (HTM) by modified Kirby Bauer disc diffusion method and interpreted as per Clinical and Laboratory Standards Institute (CLSI M100-S20) guidelines [13].

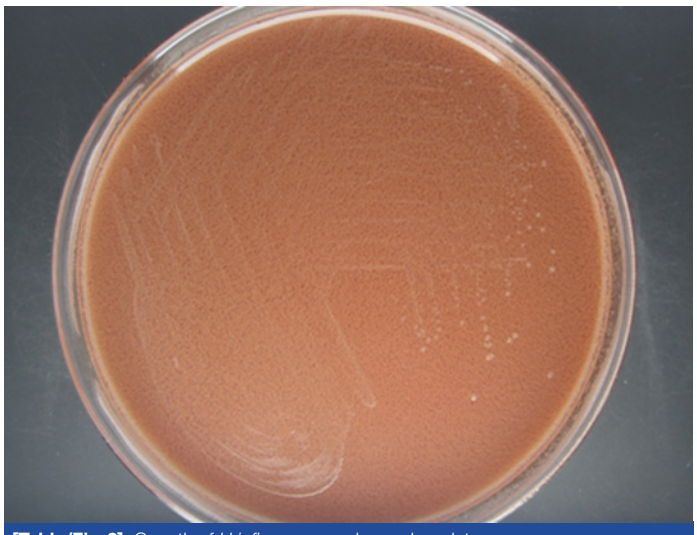
Biotyping was done for all *H. influenzae* isolates based on their ability to produce urease and indole and to decarboxylate ornithine. The isolates were grouped into 8 biotypes (I-VIII) [14].

Capsular serotyping and genotyping was performed only on same 50 randomly selected *H. influenzae* strains.

Capsular serotyping of *H. influenzae* isolates was performed by slide agglutination test by using polyclonal and type specific (a-f) antisera (Difco Laboratories, BD, USA) as per manufacturer's instructions. *H. influenzae* isolates were divided into typeable and non-typeable strains based on their ability to agglutinate the polyclonal antisera. Typeable strains were further treated with type specific antisera (a-f) to look for six type specific serotypes [9].



[Table/Fig-1]: Gram stain of sputum showing pleomorphic gram negative coccobacilli.



[Table/Fig-2]: Growth of *H.influenzae* on sheep chocolate agar.

For genotyping, DNA extraction was done from fresh overnight cultures of *H. influenzae* grown on sheep chocolate agar. In a micro centrifuge tube, 150µL of distilled water was taken to which six to eight isolated colonies from the sub cultured plates were inoculated. The tubes were placed in 80 ˚c dry bath for 20 minutes. After cooling, tubes were centrifuged at 12,000 rpm for 3 minutes. The supernatant was aspirated and stored at -20˚C for further use. PCR was performed with a pair of primers HI-1 & HI-2, specific for bex-A gene to differentiate typeable strains from non-typeable strains. PCR was done as per the protocol mentioned by Luong DC et al., where 25µl of reaction volume, contained 0.2µL of each oligonucleotide primer, 2.5µL of 10xEXTaq buffer, 2µL of dNTP mix, 1.5µL of template DNA, 1.0 U of Taq polymerase and 16µL of distilled water. ATCC 10211 *H. influenzae* type b, biotype I was used as positive control for PCR.The end products were visualized by agar gel electrophoresis [15].

RESULTS

A total of 8995 respiratory samples (BAL, ET aspirates and Sputum) were received for culture from patients with suspected LRTI in the Microbiology laboratory. Among these, 2848 samples yielded significant growth. *H. influenzae* was isolated from 175 (6.14%) patients of all age groups in whom a clinical diagnosis of respiratory tract infection was made. Males were more commonly affected than females and *H. influenzae* respiratory tract infections were more observed in the age group of 41-60 years [Table/Fig-3]. Majority (138, 78.9%) of the patients presented with acute exacerbations of chronic obstructive pulmonary disease (COPD) followed by pneumonia (19, 10.8%). LRTI due to *H. influenzae* was also observed as super-added infection in patients previously diagnosed with pulmonary tuberculosis (9, 0.6%) and Interstitial Lung Disease (4, 2.3%). Majority of the *H. influenzae* strains were isolated from sputum samples (155, 88.5%) followed by bronchoalveolar lavage (12, 6.85%) and endotracheal aspirate (8, 4.57%). Out of 175 isolates, 150 strains (85.7%) were grown as mono-microbial flora. Among the rest, *H. influenzae* was isolated more commonly in association with *Streptococcus pneumoniae* and *Klebsiella pneumoniae* [Table/Fig-3].

Character (n=175)	No. of strains	Percentage
Age wise distribution		
15-25	14	8.0
26-40	23	13.14
41-60	71	40.57
>60	67	38.28
Gender wise distribution		
Male	120	68.57
Female	55	31.42
Specimen wise distribution		
Sputum	155	88.57
BAL	12	6.85
ET	8	4.57
Monomicrobial/polymicrobial distribution		
Monomicrobial growth	150	85.71
Polymicrobial growth	25	14.28
Haemophilus influenzae with *S. pneumoniae*	17	9.7
Haemophilus influenzae with *K. pneumoniae*	17	9.7
Haemophilus influenzae with *Moraxella catarrhalis*	4	2.2
Haemophilus influenzae with MRSA	2	1.1
Haemophilus influenzae with *Streptococcus* spp	1	0.5

[Table/Fig-3]: Demographic characterization of *Haemophilus influenzae* isolates.

On Bio typing, 62 (35.42%) strains belonged to biotype II followed by biotype III (31, 17.81%) and biotype VII (18, 10.34%) [Table/Fig-4]. On capsular serotyping, only four isolates were typeable which were of type b (n=2), type e and f (n=1 each) and the rest 46 isolates were found to be non-typeable. All the 50 isolates tested were non-typeable by PCR for capsular genotyping. Majority of the *Haemophilus* isolates (148, 84.6%) were found susceptible to the antibiotics tested. Maximum resistance was observed to ampicillin (17, 9.71%) followed by amoxicillin-clavulanic acid (9, 5.14%) and ceftriaxone (1, 0.57%) [Table/Fig-5].

Character	No. of strains	Percentage
Biotyping (n=175)		
1	16	9.2
2	62	35.42
3	31	17.81
4	17	9.77
5	13	7.47
6	03	1.72
7	18	10.34
8	15	8.04
Capsular serotyping (n=50)		
Non-typeable *H. influenzae*	46	92
Typeable *H. influenzae*	04	08
Type b	02	04
Type e	01	02
Type f	01	02
Capsular genotyping by PCR (n=50)		
Typeable	-	-
Non-typeable	50	100

[Table/Fig-4]: Results of typing of pathogenic *Haemophilus influenzae* isolates.

Antibiotics	Sensitivity Number of isolates (%)	Resistance Number of isolates (%)
Amoxicillin/clavulanate	165 (94.28)	9 (5.14)
Ampicillin	158 (90.28)	17 (9.7)
Ceftriaxone	174 (99.42)	1 (0.5)

[Table/Fig-5]: Antibiotic susceptibility pattern of *Haemophilus influenzae* isolates (n=175).

DISCUSSION

H. influenzae though a component of normal respiratory tract flora, is well recognized to be an important cause of community acquired respiratory infection. A combination of bacterial pathogenic features and deficiency of host defence may permit this bacterium to establish infection in the lower respiratory tract resulting in inflammation and clinical disease [16].

Out of 8995 samples from patients with suspected LRTI, significant growth was observed in 2848 (31.7%) samples. The frequency of *H. influenzae* lower respiratory tract infection was found to be 6.14% which was comparatively lower than the reports of Rai R et al., (18.05%), Mishra SK et al., (21%), Ayyangari et al., (21.9%) and Khan et al., (27.6%) [17-20]. However, *H. influenzae* was also recognized as etiological agent in 6-10% of community acquired pneumonia [21]. The low prevalence in our setup could be due to difference in geographical distribution of the organism, host immune conditions and over the counter usage of antibiotics. In our study most of the isolates were recovered as Monomicrobial flora (150, 85.5%) which was found to be consistent with other studies [18,20].

In this study, more number of cases were found in the age group of 41-60 years, and males were commonly affected than females. This could be due to occurrence of more number of COPD cases in our study group [18]. However, Rai R et al., found children less than 10 years as the commonly affected age group, followed by 41-50 years [17]. Males (68.5%) were more affected than females which is mirroring the findings of Luong DC et al., (59.8%) and Puig C et al., (69.6%) [15,22].

Isolation of *H.influenzae* isolates was observed maximally from sputum (n=155, 88.6%) followed by bronchoalveolar lavage (n=12, 6.9%) and endotracheal aspirate (n=8, 4.5%). Likewise, other studies also showed the maximum recovery from sputum [18]. COPD (138, 78.9%) was the predominant underlying cause for *H. influenzae* lower respiratory tract infections and this could be one of the reasons for isolation of more number of isolates from sputum.

Majority (92%) of the tested *H. influenzae* isolates were found to be non-typeable by capsular serotyping. Four (8%) isolates were typeable which included type b (n=2), type e (n=1) and type f (n=1). But the capsular genotyping results revealed all 50 strains to be non-typeable. This variation in results could be due to the number of inconsistencies associated with capsular serotyping with slide agglutination test [8,9,14,15]. Hence PCR for capsular serotyping could be more useful [15].

If PCR was performed on larger number of isolates in our study, the probability of getting typeable strains would have been more. Obtaining more non-typeable strains could be attributable to the involvement of more number of COPD cases [9,22] in our study population. Puig C et al., found that 33.3% of the isolates were non-typeable [22]. There is scarcity of reports depicting capsular genotyping of *H. influenzae* from India. With capsular serotyping by PCR, Luong DC et al., found 96.7% of 122 clinical isolates from paediatric patients to be NTHi [15]. King P et al., noted, NTHi as the most common cause of bacterial colonization in patients with COPD and was recognized as major bacterial cause for severe exacerbation of COPD [16]. Imad K et al., in their analysis of *H. influenzae* from nasopharyngeal isolates in asymptomatic school children found 33.3% of isolates as non-typeable, 42.8% were serotype b and 23.8% were non-type b capsulated isolates [23]. Mohd-Zain Z et al., observed that majority of the NTHi was isolated from sputum. NTHi not only adhere and invade the respiratory epithelial cells but also form biofilms which reduces the effectiveness of antibiotic therapy [1].

Among the isolated *H. influenzae*, biotype II (35.42%) was the most frequent followed by biotype III (17.8%). Biotype VI (1.7%) was the least frequent. Mojgani N et al., and Imad K et al., found biotype III as the commonly prevalent biotype [14,23]. Jain A et al., have reported biotype III and Das BK et al., noted biotype I as the most frequent biotype from nasopharyngeal carriage [7,24].

Resistance of *H. influenzae* to antibiotics remains growing problem worldwide with high impact on cost and longer hospital stays. The pattern of antibiotic resistance varies from region to region [1]. As a result of ampicillin resistance, the usage of cephalosporins has increased. Over the counter administration of cephalosporins and their unintended usage can result in the development of drug resistance in developing countries [7]. Lower rates of resistance to beta lactam antibiotics were seen among the *H. influenzae* isolates in comparison with earlier studies across the globe [Table/Fig-6].

LIMITATION

Capsular serotyping and molecular work was done only on 50 randomly selected isolates of *H. influenzae* due to financial constraints. While performing the antibiotic susceptibility testing, β-lactamase detection test was not done.

CONCLUSION

Capsular genotyping has shown that most of LRTI were caused by non-typeable *H.influenzae*. These non-typeable strains shouldn't be neglected as colonizers. Increased resistance was seen against beta lactam antibiotics which are the main stay of treatment in

Prior studies	Percentage of resistance reported
Saikia KK et al., [3]	81.25
Das BK et al., [24]	56.8
Mojgani N et al., [14]	43.6
Mohd-Zain Z et al.[1]	29.4
Jain A et al., [7]	22.9
Rai R et al., [17]	19.23
Imad K et al., [23]	19
Puig C et al., [22]	10.5
Present study	9.74

[Table/Fig-6]: Ampicillin resistance in *Haemophilus influenzae* reported in previous studies.

LRTI. It highlights the possibility of treatment failure with beta lactam drug usage.

REFERENCES

[1] Mohd-Zain Z, Kamsani NH, Ismail IS, Ahmad N. Antibiotic susceptibility profile of *Haemophilus influenzae* and transfer of co-trimoxazole resistance determinants. *Trop Biomed*. 2012;29:372-80.

[2] Ramachandran P, Fitzwater SP, Aneja S, Verghese VP, Kumar V, Nedunchelian K. Prospective multi-centre sentinel surveillance for *Haemophilus influenzae* type b & other bacterial meningitis in Indian children. *Indian J Med Res*. 2013;137:712-20.

[3] Saikia KK, Das BK, Bewal RK, Kapil A, Arora NK, Sood S. Characterization of nasopharyngeal isolates of type b *Haemophilus influenzae* from Delhi. *Indian J Med Res*. 2012;136:855-61.

[4] King PT, Sharma R. The lung immune response to nontypeable *Haemophilus influenzae* (Lung Immunity to NTHi). *J Immunol Res*. 2015;2015:706376.

[5] Moghaddam SJ, Ochoa CE, Sethi S, Dickey BF. Nontypeable *Haemophilus influenzae* in chronic obstructive pulmonary disease and lung cancer. *Int J Chron Obstruct Pulmon Dis*. 2011;6:113-23.

[6] Falla TJ, Crook DWM, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of *Haemophilus influenzae*. *J Clin Microbiol.* 1994;32:2382-86.

[7] Jain A, Kumar P, Awasthi S. High ampicillin resistance in different biotypes and serotypes of *Haemophilus influenzae* colonizing the nasopharynx of healthyschool-going Indian children. *J Med Microbiol*. 2006;55:133-37.

[8] Maaroufi Y, De Bruyne JM, Heymans C, Crokaert F. Real-time PCR for determining capsular serotypes of *Haemophilus influenzae*. *J Clin Microbiol.* 2007;45:2305-08.

[9] LaClaire LL, Tondella ML, Beall DS, Noble CA, Raghunathan PL, Rosenstein NE, et al. Active bacterial core surveillance team members identification of *Haemophilus influenzae* serotypes by standard slide agglutination serotyping and PCR-based capsule typing. *J ClinMicrobiol*. 2003;41:393-96.

[10] Invasive Bacterial Infections Surveillance (IBIS) Group of the International Clinical Epidemiology Network. Are *Haemophilus influenzae* infections a significant problem in India? A prospective study and review. *Clin Infect Dis*. 2002;34:949-57.

[11] Bartlett RG. Medical Microbiology: Quality, costs and clinical relevance. New York: John Wiley and Sons, Inc.; 1974; Pp. 24-31.

[12] Howard AJ, Ison CA. Haemophilus, Gardnerella and other bacilli. In: Collee JG, Fraser AG, Marmion BP, Simmons A, editors. Mackie and McCartney Practical Medical Microbiology. 14th ed. New York: Churchill Livingstone; 2006; Pp. 883—92.

[13] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twentieth informational supplement M100-S20. Clinical and Laboratory Standards Institute, Wayne. 2010.

[14] Mojgani N, Rahbar M, Taqizadeh M, Ashtiani MP, Mohammadzadeh M. Biotyping, capsular typing, and antibiotic resistance pattern of *Haemophilus influenzae* strains in Iran. *Jpn J Infect Dis*. 2011;64:66-68.

[15] Luong DC, Ishiwada N, Takeda N, Kohno Y. Serotypes of *Haemophilus influenzae* strains isolated from paediatric patients with respiratory tract infections. *Tohoku J Exp Med*. 2004;202:245-54.

[16] King P. *Haemophilus influenzae* and the lung (Haemophilus and the lung). *ClinTransl Med.* 2012;1:10.

[17] Rai R, Kumar KV, Ramanath G, Madle SK, Hosdurg S, AshaPai KB. *Haemophilus influenzae*, an underdiagnosed cause of Respiratory Tract Infections. *J Clin Diagn Res.* 2012;6:385-87.

[18] Mishra SK, Kattel HP, Acharya J, Shah NP, Shah AS, Sherchand JB, et al. Recent trend of bacterial aetiology of lower respiratory tract infections in a tertiary care centre of Nepal. *Int J Infect Microbiol*, 2012;1:3-8.

[19] Ayyagari A, Sharma P, Chakrabarti A, Agarwal KC. Isolation and detection of *Haemophilus influenzae* from patients of respiratory tract infections and their antibiotic susceptibility pattern in Chandigarh. *Indian J Chest Dis Allied Sci*. 1985;27(4):230-35.

[20] Khan S, Singh P, Gurung K, Ansari M. Bacteria etiological agents causing lower respiratory tract infections in the western part of Nepal. *Ibnosina J Med BS*. 2014;6:3-8.

[21] Garcia-Vidal C, Carratalà J, Fernández-Sabé N, Dorca J, Verdaguer R, Manresa F, et al. Aetiology and risk factors for, recurrent community-acquired pneumonia. *Clin Microbiol Infect.* 2009;15(11):1033-38.

[22] Puig C, Calatayud L, Marti S, Tubau F, Garcia-Vidal C, Carratala J, et al. Molecular epidemiology of nontypeable *Haemophilus influenzae* causing community-acquired pneumonia in adults. *PLoS One*. 2013;8:e82515.

[23] Imad K, Monzer H, Fouad D. Molecular characterization and resistance of *H. influenzae* isolated from nasopharynx of Students in North Lebanon. *The International Arabic Journal of Antimicrobial Agents*. 2012;2:5.

[24] Das BK, Arora NK, Mathur P, Ostwal P, Mandal S, Kabra SK, et al. Nasopharyngeal carriage of *Haemophilus influenzae*. *Indian J Paediatr*. 2002;69:775-77.

PARTICULARS OF CONTRIBUTORS:

1. Assistant Professor, Department of Microbiology, Kasturba Medical College, Manipal University, Manipal, India.
2. Professor and Head, Department of Microbiology, Kasturba Medical College, Manipal University, Manipal, India.
3. Associate Professor, Department of Microbiology, Kasturba Medical College, Manipal University, Manipal, India.
4. Post Graduate Student, Department of Microbiology, Kasturba Medical College, Manipal University, Manipal, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Kiran Chawla,
Professor and Head, Department of Microbiology, Kasturba Medical College, Manipal University, Manipal-576104, India.
E-mail: kiran.chawla@manipal.edu

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Dec 29, 2015**
Date of Peer Review: **Feb 16, 2016**
Date of Acceptance: **Mar 25, 2016**
Date of Publishing: **May 01, 2016**