ISSN: 13412051
Volume 24, Issue 03, November, 2019

Determination of prevalence and antimicrobial sensitivity pattern of *Klebsiella pneumoniae* from sputum sample of a tertiary care hospital

Tanzina Akter[1,2]*, Md Sumon Khan[3], Shamsun Nahar[2], Kaniz Fatema[3], Modhusudon Shaha[1], Mohammad Jakir Hossain[3], Shazeed-Ul-Karim[2] and Hoomyra Sultana[2]

Environmental Biotechnology Division, National Institute of Biotechnology, Savar, Dhaka-1349, Bangladesh[1]
Department of Microbiology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh[2]
Department of Microbiology, Primeasia University, Banani, Dhaka-1213, Bangladesh[3]
*Corresponding author

Abstract:

The purpose of the present study were isolation of *K. pneumoniae* from clinical samples, determination of their antibiotic sensitivity against commonly prescribed antibiotics and evaluation of their ability to produce extended-spectrum beta-lactamase (ESBL), AmpC and Carbapenemase enzymes. The study was performed at the Department of Microbiology, Primeasia University, Banani, Dhaka-1213, Bangladesh. For isolation and identification of *klebsiella pneumoniae*, cultural characteristics on blood agar and MacConkey agar was observed along with different biochemical tests and the disc diffusion assay on Mueller-Hinton agar was done for determination of antibiotic sensitivity. Statistical analysis was done by employing Statistical Package for Social Science (SPSS Version 16) software. Total 120 sputum samples were collected of them 51 (42.5%) *K. pneumoniae* were identified. Sensitivity towards 15 different antibiotics were determined by Kirby Bauer disc diffusion method. Third generation cephalosporin resistant bacteria were further analyzed to determine their extended-spectrum beta lactamases enzyme producing ability by double disc synergy test. AmpC and OXA-48 disc tests were used for detection of AmpC and Carbapenemase producer. Among 51 *K. pneumoniae*, 39 (76.5%), 31(60.8%), and 3(5.9%) were found ESBL, AmpC and Carbapenemase producer respectively. *K. pneumoniae* showed maximum sensitivity to Netilmicin (92.2%), Amikacin (90.1%), Meropenem (90.1%), Imipenem (88.2%), Ciprofloxacin (80.3%), Levofloxacin (84.3) and least sensitivity to Cefixime (47.1%), Cefotaxime (37.3%), Ceftriaxone (35.3%), Ceftazidime and Cefoxitin (31.4%). Therefore, it can be concluded that, Carbapenems (Meropenem, Imipenem), Aminoglycoside (Netilmicin, Amikacin, Gentamycin, Tobramycin) and Quinolones groups (Ciprofloxacin, Levofloxacin) antibiotics could be drug of choice against *K. pneumoniae* in this particular setting.

Key-words:

Klebsiella pneumoniae, ESBL, AmpC, and Carbapenemase.

1. Introduction:

Klebsiella, the most common causative agent of nosocomial infections, is Gram-negative, encapsulated, lactose fermenting, non-motile facultative anaerobe that belongs to the family *Enterobacteriaceae* [1]. They are ubiquitous in nature and equally found in environment and animal host [2]. Among other members of the *genus klebsiella*, *Klebsiella pneumoniae and Klebsiella oxytoca* are the most significant in commencing human disease. *K. pneumoniae* is highly responsible for causing nosocomial *pneumonia* (7-14% of all cases), septicaemia (4-15% of all cases), wound infections (2-4% of all cases) and neonatal septicaemia (3-30% of all cases) [3]. Capsular polysaccharides, lipopolysaccharide (LPS) and siderophores (iron-scavenging systems) are common virulence factors of *klebsiella* spp. that are involved in pathogenesis of the disease [4].

It was found that infection of *klebsiella* spp. is mostly common in immunocompromised patients as well as in older and middle aged people. People having diabetes, chronic obstructive pulmonary diseases (COPD), liver disease, glucocorticoid therapy, malignancy are at high risk of acquiring *klebsiella* infection [5].

Antimicrobial resistance or drug resistance to pathogenic bacteria is a common phenomenon now a day and frequently being reported worldwide [6]. However, the situation is quite alarming not only in developing

countries but also in developed countries and posing a significant challenge to public health throughout the world [6]. Inappropriate prescribing, misuse and overdose of antibiotics are the common reasons that lead antibiotic resistance [7]. Moreover, bacteria are highly susceptible to transmit and acquire resistance to this therapeutics through genetical exchange [8].

In recent years, emergence of antibiotic resistance by *klebsiella* spp. has gathered much attention. Multidrug resistant *klebsiella* spp. cause serious infections that are troublesome to eradicate with available antibiotics [4, 9]. In addition, resistance to broad spectrum beta-lactam antibiotics such as third generation cephalosporin via extended-spectrum beta-lactamase (ESBL) and AmpC production, resistance to Carbapenems due to Carbapenemase production are more common in *Klebsiella* spp. and increasing day by day [10]. However, there is insufficient information available on account of antibiotic sensitivity as well as ESBL, AmpC, and Carbapenemase production in *K. pneumoniae* isolates in Bangladesh. Therefore, present study was attempted to chalk out the prevalence and antimicrobial susceptibility pattern of *K. pneumoniae* from sputum samples and to detect ESBL, AmpC, and Carbapenemase producer from the isolated strains.

2. Materials and Methods:

2.1 Sample collection: The study was performed in Centre for Excellence Laboratory (CEL), Department of Microbiology, Primeasia University, Banani, Dhaka-1213, Bangladesh. A total of 120 sputum samples were collected from a tertiary care hospital of Dhaka city from January 2016 to May 2016. Among 120 samples, 60 samples were collected from male and the remaining 60 from female patients. All sputum samples were collected in sterile leak proof container and transported to the Microbiology laboratory of Primeasia University as soon as possible.

2.2 Isolation and identification of *K. pneumoniae*: For the isolation and identification of *K. pneumoniae* blood agar and MacConkey agar plates were used. Fresh clinical samples were cultured on blood agar and MacConkey agar plates and incubated at 37°C for 24 hours. After 24 hours of incubation large, pink colored, mucoid colonies were suspected as *klebsiella* spp. Those exhibiting mucoid colonies were further processed for biochemical testing to identify *k. pneumoniae*. Individual bacterial colony was characterized by visual observation after Gram staining.

2.3 Biochemical characterization of the isolates: For biochemical characterization Voges Proskeur (VP), Indole, Motility, Methyl red, H_2S production, Oxidase, Citrate, Catalase, Urease, Nitrate reduction, Phenylalanine deaminase, Ornithine decarboxylase, Lysine decarboxylase, Gelatin hydrolysis tests were performed. Sugar fermentation test was also done for different sugars, namely, glucose, sucrose, lactose, mannitol and maltose. All the biochemical tests were done according to the Bergey's Manual of Determinative Bacteriology [11].

2.4 Antibiotic susceptibility test: Antibiotic susceptibility pattern of the *K. pneumoniae* was examined by the disc diffusion assay on Mueller-Hinton agar against the commonly used antibiotics following the standard protocol. Commercially available laboratory grade antibiotic discs of Amikacin (30 µg), Cefixime (30 µg), Cefotaxime (30 µg), Cefoxitin (30 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Co-trimoxazole (25 µg), Gentamicin (10 µg), Imipenem (10µg), Levofloxacin (5 µg), Meropenem (10µg), Netilmicin (30 µg), Piperacillin-Tazobactam (100/10µg), and Tobramycin (10µg) were aseptically placed over the surface of Mueller-Hinton agar plates which had been previously inoculated with bacterial suspensions having turbidity compared to that of the McFarland standard of 0.5. The plates were incubated overnight at 37°C for 24 hours and zone of inhibition were measured in mm by following the recommendations of the criteria of the Clinical and Laboratory Standards Institute (CLSI) [12]. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700 603 strains were used as control in our study.

2.5 Detection of ESBLs by double disc synergy test (DDST): Isolate showed resistance to any of the third generation cephalosporin (Cefixime, Cefotaxime, Ceftriaxone, and Ceftazidime) was selected for detection of extended-spectrum beta lactamases producer (ESBL) by double disc synergy test. An inoculum of 0.5 Mcfarland standard was inoculated onto Mueller Hinton agar with sterile cotton swab to make a lawn of bacterial growth. Third generation cephalosporin disc was placed on the agar plate at a distance of 20 mm centre to centre from a Amoxicillin-clavulanic acid (20/10 µg) disc prior to incubation. The plate was incubated at 37°C for 24 hours. Enhancement of the zone of inhibition of third generation cephalosporin disc toward Amoxicillin-Clavulanic acid confirmed the presence of extended-spectrum beta-lactamases producer isolates.

2.6 AmpC beta-lactamase detection by AmpC disc test: In our study, 32 of 51 positive isolates showed resistant to Cefoxitin (30 µg) antibiotic. These isolates were further investigated for determination of the

presence of AmpC beta-lactamase by AmpC disc test. This test used Tris EDTA which permeabilized bacterial cell to release beta-lactamases into the external environment. Cefoxitin-susceptible *E. coli* strain ATCC 25922 was inoculated on a Mueller-Hinton agar plate to produce a uniform lawn. A Cefoxitin disc was placed on the bacterial lawn. Beside the Cefoxitin disc, a sterile AmpC disc containing Tris-EDTA was inoculated with several colonies of the test organism by almost touching Cefoxitin disc. Following overnight incubation at 37°C, the plates were examined for either an indentation or a flattening of the zone of inhibition. Flattening or indentation of the growth inhibition zone of the Cefoxitin disc at the side of AmpC disc containing the test strain indicated the release of AmpC beta-lactamase enzyme.

2.7 OXA-48 disc test for detection of OXA-48 Carbapenemase production: Isolates showed resistant to Imipenem and Meropenem were tested by OXA-48 disc test for the presence of OXA-48 Carbapenemase enzyme. The surface of a Mueller-Hinton agar plate was inoculated with a Carbapenem-susceptible *E. coli* ATCC 25922 at a turbidity of 0.5 McFarland standard to form a lawn. Carbapenems (Imipenem / Meropenem) antibiotic disc was placed on the inoculated plate. Test organism was heavily inoculated on the disc surface of Tris EDTA disc to provide a visible inoculum. Tris EDTA disc was then placed on the agar 1 mm from the Carbapenem disc with the inoculated side contacting the agar. After overnight incubation at 37°C, an indentation of the inhibition zone near the inoculated Tris EDTA disc indicated a positive test result and no indentation indicated a negative test result.

2.6 Statistical analysis: Statistical analysis was done by employing Statistical Package for Social Science (SPSS Version 16) software. Descriptive statistics were used for the analysis of result wherever appropriate. The Chi-square test was used to evaluate the statistical significance of differences in results. A *P* value of <0.05 was considered statistically significant.

3. Result:

Out of 120 sputum samples, *K. pneumoniae* were identified in 51 (42.5%) cases through standard cultural and biochemical tests. In our study, the sputum samples were taken from patients of age group ranging from 0 years to 90 years of both sexes. Among 51 positive samples, 34 (28.33%) samples were from male and 17 (14.17%) from female patients and the ratio of male and female is 2:1 (Table 1).

In case of male, isolation rate was highest in patient aged 61-70 years (18.33%) and in case of female , the highest rate was found (10%) in the age group of 51-60 years (Table 1). However, the Chi square ($\chi 2$) test result revealed that there was no significant variations ($P > 0.05$) between male and female in relation to the prevalence of the *K. pneumoniae* at 95% confidence interval level ($\chi 2 = 9.846$; degree of freedom = 8; $P = 0.276$) (Table 2).

Antimicrobial susceptibility test revealed that *K. pneumoniae* were highly sensitive to Netilmicin (92.2%), Amikacin (90.1%), Meropenem (90.1%), and least level of sensitive to Cefixime (47.1%), Cefotaxime (37.3%), Ceftriaxone (35.3%), Ceftazidime and Cefoxitin (31.4%) (Table 3).

45 isolates (88.2%) were selected for screening of ESBL by double disc synergy test from which 39 (76.5%) were found positive ESBL producer (Table 4).

35 out of 51 were found resistant to Cefoxitin and the remaining 16 showed susceptibility to Cefoxitin. Among 35 resistant isolates of Cefoxitin, 31 (60.8%) showed positive AmpC producer by AmpC disc test therefore 20 isolates were negative for AmpC beta-lactamase. 5 out of 6 isolates found negative for ESBL by double disc synergy were included in 31 positive AmpC beta-lactamase.

12 of 20 negative AmpC beta-lactamase exhibited a zone enhancement with Clauvulanic acid which confirmed their ESBL production. In addition, 26 of 31 isolates which exhibited Cefoxitin resistance also had zone enhancement with Clauvulanic acid, thereby indicating both ESBL and AmpC production. Hence, only 5 of 31 isolates were presumed for pure AmpC producers. 7 isolates found negative for both ESBL and AmpC.

Among 51 *K. pneumoniae*, 6 isolates were found resistant to Imipenem, and of which 4 isolates were also resistant to Meropenem. 3 isolates resistant to both Carbapenems were found positive on OXA-48 disc tests which indicated that these 3 strains were Carbapenemase producer.

Age	Male (%) (n=60)	Female (%) (n=60)	Total (%) (n=120)
0-10	0	0	0
11-20	1 (1.67)	1 (1.67)	2 (1.67)
21-30	4 (6.67)	3 (5)	7 (5.83)
31-40	2 (3.33)	1 (1.67)	3 (2.5)
41-50	5 (8.33)	1 (1.67)	6 (5)
51-60	3 (5)	6 (10)	9 (7.5)
61-70	11 (18.33)	1 (1.67)	12 (10)
71-80	4 (6.67)	3 (3.33)	7 (5.83)
81-90	4 (6.67)	1 (1.67)	5 (4.17)

Table 1: Frequency of *K. pneumoniae* according to age

Male (%) (n=34)	Female (%) (n=17)	Chi square (χ^2) value	*P* value
0	0	9.846	0.276
1(2.94)	1 (5.88)		
4 (11.76)	3 (17.65)		
2 (5.88)	1 (5.88)		
5 (14.71)	1 (5.88)		
3 (8.82)	6 (35.29)		
11(32.35)	1 (5.88)		
4 (11.76)	3 (17.65)		
4 (11.76)	1 (5.88)		

Table 2: Distribution of *K. pneumoniae* according to patient's gender

ISSN: 13412051
Volume 24, Issue 03, November, 2019

Antibiotics	*K. pneumoniae*				
	Total	**Sensitive**	**%**	**Resistant**	**%**
Netilmicin	51	47	92.2	4	7.8
Amikacin	51	46	90.1	5	9.9
Meropenem	51	46	90.1	5	9.9
Imipenem	51	45	88.2	6	11.8
Levofloxacin	51	43	84.3	8	15.7
Ciprofloxacin	51	41	80.3	10	19.7
Gentamicin	51	39	76.5	12	23.5
Tobramycin	51	39	76.5	12	23.5
Piperacillin - Tazobactam	51	33	64.7	18	35.3
Co-trimoxazole	51	28	54.9	23	45.1
Cefixime	51	24	47.1	27	52.9
Cefotaxime	51	19	37.3	32	62.7
Ceftriaxone	51	18	35.3	33	64.7
Ceftazidime	51	16	31.4	35	68.6
Cefoxitin	51	16	31.4	35	68.6

Table 3: Antimicrobial sensitivity pattern of *K. pneumoniae* against commonly used antibiotics

Total no. of *K. pneumoniae* isolates	**No. of *K. pneumoniae* selected for ESBL confirmatory tests**	**No. of positive ESBL confirmed by DDST**
51	45 (88.2%)	39 (76.5%)

Table 4: Frequency of ESBL producer of *K. pneumoniae* confirmed by DDST

4. Discussion:

Now a day's multidrug resistant *Klebsiella* has become a major public health concern across the world. Outbreaks of nosocomial infections due to *Klebsiella* spp in developing countries like Bangladesh are associated with the indiscriminate use of antibiotics [13]. These are now being recognized as one of the major threats for effective management of patients in hospital. 120 sputum samples were analyzed of which 51 (42.5%) samples showed positive growth for *k. pneumoniae.* Our result is very close to a study carried out by Bharathi DV *et al.* where the rate of culture positivity was found 50.2 % [14]. Prevalence rate of *k. pneumoniae* was 66.7% in male and 33.3 % in female and the ratio was 2:1. This finding is similar with another study where the ratio of male and female was 1.7:1 [15]. From our study it was also observed that patients in the age of above 40 were more prone to infection by *k. pneumoniae*. This result is similar with other studies [16, 15, 17, 18].

The antimicrobial susceptibility test demonstrated that *k. pneumoniae* were highly sensitive to Netilmicin (92.2%), Amikacin (90.1%), Meropenem (90.1%) and Imipenem (88.2 %). Previous studies conducted in India and Nepal where sensitivity to Netilmicin was found 62.96% and 56.36% respectively which were lower than our results [5, 19]. Sensitivity to Amikacin (92.7 %), Meropenem (100%) and Imipenem (84 %) were recorded in previous studies which are very close to our findings [4, 20,21].

76.5 % of the isolates showed sensitivity to Tobramycin which was higher than previous studies (54.37% and 41.93%) conducted in various parts of the world. [1,5]. Like Tobramycin, Gentamycin showed sensitivity to 76.5 % isolates. Sensitivity to Gentamycin (62.50%) was also recorded in 2016 by Shilpa K *et al.* in India [1]. More than 80% bacterial isolates showed sensitivity to Ciprofloxacin and Levofloxacin which indicated that antibiotics of quinolones groups also had good activity against *k. pneumoniae*. Therefore, from this study it was revealed that Carbapenems (Imipenem, Meropenem), Aminoglycoside (Gentamycin, Netilmicin, Amikacin, Tobramycin) and quinolones groups (Ciprofloxacin, Levofloxacin) antibiotics were the most sensitive drugs against the isolated strains.

k. pneumoniae showed moderate level of sensitivity (64.7%) against Piperacillin-Tazobactam. A study performed in India where sensitivity to Piperacillin-Tazobactam was recorded 37.5% which was lower than our finding [4]. Sensitivity to Co-trimoxazole was found 54.9% which is in agreement with a study conducted by Shilpa K *et al.* where they found 50% sensitivity to Co-trimoxazole [1].

In our study *k. pneumoniae* showed highest level of resistant to third generation cephalosporin such as Ceftazidime, Ceftriaxone, Cefotaxime and Cefixime. In our study, 52.9%-68.6% *K. pneumoniae* isolates were found resistant to third generation cephalosporin. Studies performed by Namratha KG *et al*. and Shah RK *et al.* reported 52%-65% and 48.33%-63.33% resistant to third generation cephalosporin respectively [15, 17]. Besides, 87%-89% and 84.8%-96% resistance to third generation cephalosporin were also recorded in Chandigarh and Nigeria respectively which are higher than our results [22, 23].

In our study, 45 (88.2%) out of 51 showed resistant to one the third generation cephalosporin which correlates to another study where the percentage was 87% [24]. These isolates were further tested by double disc synergy test (DDST) to detect ESBL producing strains. From 45 isolates, 39 (76.5%) isolates were found as ESBL producer by DDST. A study was carried out by Sarojamma V *et al.* in India, where they found 88% ESBL producer by DDST which is very close to present finding [21].

From our study, 60.8% showed positive result for AmpC producer, which was higher than a study conducted by Jennifer A. Black *et al.* in U.S. where they reported 31% AmpC producer [25]. Coproduceer of both ESBL and AmpC are becoming more common in recent years [26]. Our finding revealed that 26/51 (50.9%) isolates were coproducer of ESBL and AmpC. A study conducted by V. Hemalatha *et al.* where the percentage of coproducer was 15.8% (12/76) which was lower than our finding [27].

In health care settings, Carbapenems resistance has emerged among *Enterobacteriaceae* during the last decade, and is increasing day by day [28]. From our study, out of 6 Carbapenems non suceptible *K. pneumoniae*, 3 isolates were found OXA-48-type Carbapenemases producer. Prevalence of OXA-48-type Carbapenemases among the Carbapenem resistant isolates has also been reported in many parts of the world [28].

5. Conclusion

Prevalence of ESBL, AmpC and Carbapenemase producing isolates of *K. pneumoniae* has been increasing day by day. Indiscriminate use of extended spectrum cephalosporin against bacterial infections is mostly responsible for the emergence of these resistant isolates. Therefore, strict adherence should be followed for antibiotic selection which in turn will help to prevent emergence and spread of antibiotic resistance among *K. pneumoniae* strains.

6. Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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