

Prevalence Study of Multi-drug Resistant *Klebsiella pneumoniae* Strains Isolated from Respiratory Tract Infections

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<http://dx.doi.org/10.22207/JPAM.11.1.23>

(Received: 17 January 2017; accepted: 25 February 2017)

Resistant strains of *Klebsiella pneumoniae* are one of the most prevalent cause of nosocomial and especially respiratory tract infections al-around the world. The present investigation was carried out to study the prevalence of antibiotic resistance in *K. pneumoniae* strains of hospitalized patients suffered from RTIs. Three-hundred and fifty respiratory samples were collected from hospitalized patients of both sexes and various ages. Samples were cultured and those that were *K. pneumoniae*-positive were subjected to 16S rRNA based-PCR amplification and disk diffusion method. Of 350 samples studied, 25 samples were positive for *K. pneumoniae* (7.14%). Distribution of *K. pneumoniae* in male and female patients were 8.33% and 5.88%, respectively ($P < 0.05$). Older than 60 years old and younger than 20 years old patients had the highest prevalence of *K. pneumoniae*. Our *K. pneumoniae* isolates had the highest levels of resistance against gentamycin (96%), ampicillin (92%), erythromycin (84%), ciprofloxacin (76%), sulfamethoxazole (76%). Primary identification of *K. pneumoniae*-positive patients and their treatment with imipenem antibiotic based on the results of disk diffusion method can control distribution of *K. pneumoniae* RTIs.

Keywords: *Klebsiella pneumoniae*, Prevalence, Antibiotic resistance, Respiratory Tract Infections, Risk factors.

Respiratory tract infections (RTIs) are one of the most common types of infectious diseases al-around the world. It has been estimated that more than 15% of death in developed countries are occurred due to the occurrence of RTIs^{1,2}. RTIs are responsible for about 55,000 cases of infection and hospitalization in 2010^{1,2}. They are accounted for more than 45,000 hospital admissions with an average length of stay of 6.3 days¹.

In despite of a lot of developments in treatment and control of nosocomial infections, *Klebsiella pneumoniae* (*K. pneumoniae*) infections are remain as one of the most prevalent cause of

morbidity and mortality al-around the world (3-5). It is a Gram-negative, rod-shaped, non-motile, encapsulated, lactose-fermenting, facultative anaerobic bacterium and widely distributed in the urinary, gastrointestinal, and respiratory tracts of healthy people. *K. pneumoniae* is a causative agent of opportunistic infections mainly nosocomial and hospital-acquired infections including RTIs, pneumonia, Urinary Tract Infections (UTIs), abscesses, wound infection, diarrhea, sepsis and inflammation³⁻⁵.

In addition to the invasive nature of *K. pneumoniae*, clinical strains of this bacterium are mainly resist to several types of antibiotic agents⁶⁻⁸. Antibiotic resistant strains of *K. pneumoniae* cause more sever and dangerous infections for longer periods of time than susceptible strains (6-

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8). It has been estimated that *K. pneumoniae* strains of RTIs and also other types of nosocomial infections had a high prevalence of antibiotic resistance (30-100%) against commonly used antimicrobial agents including β -lactam antibiotics, broad-spectrum penicillins, cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and tetracyclines⁶⁻⁹.

According to the uncertain status of *K. pneumoniae* RTIs in Iran and lack of epidemiological investigations in the field of antibiotic resistance, the present investigation was carried out to study the prevalence and antibiotic resistance pattern of *K. pneumoniae* strains isolated from the respiratory samples of male and female patients of various age groups hospitalized due to the severe RTIs in Iranian hospitals.

MATERIALS AND METHODS

Samples collection

From January to November 2015, a total of 350 respiratory samples including Broncho Alveolar Lavages (BAL) (n=50) and also respiratory secretions (n=300) were sent to our laboratory center from hospitalized patients suffering from RTIs. In this study, all of respiratory samples were selected and analyzed for presence of *L. pneumophila*. At the time of sampling, information about the age, sex and clinical symptoms of the patients were recorded. Ten ml of each sample was immediately transferred to a sterile falcon tube containing ice and was immediately transferred to the laboratory.

All samples were transferred to the laboratory using peptone water (BPW, Merck, Germany). Samples were transferred to the McConkey agar (MC agar, Merck, Germany), Chocolate agar (Merck, Germany and Blood agar (Merck, Germany). The inoculum on the plate was streaked out for discrete colonies with a sterile wire loop. The culture plates were incubated at 37°C for 24 hours and observed for growth through the formation of colonies. All the bacteria were isolated and identified using morphological, microscopy of standard methods (API System; bioMérieux, Marcy-l'Étoile, France) and biochemical tests including Triple Sugar Iron agar (TSI agar, Merck, Germany), Simmons Citrate,

Methyl Red, Indole, Urease, Voges–Proskauer, motility, and H₂S production. The biochemical characteristics of *K. pneumoniae* identified were as follows: positive citrate utilization test, negative methyl red test, negative indole test, positive urease test, positive Voges–Proskauer test, sucrose, acid and abundant gas production from glucose, lactose, mannitol sugar fermentation tests, and maltose.

DNA extraction and PCR confirmation of *Klebsiella pneumoniae* isolates

Bacterial isolates were cultured another time on nutrient broth media (Merck, Germany) and incubated aerobically at 37 °C for 24 h. Genomic DNA was extracted from each *K. pneumoniae* isolate by DNA extraction kit (Fermentas, Germany) according to manufacturer's instruction. The bacteria were confirmed using the PCR method for *16S rRNA* gene of the *K. pneumoniae* (10). PCR was carried out with 2 µL template DNA, 0.25 µM of each primer (F: 5'-GCAAGTCGAGCGGTAGCACAG-3' and R: 5'-CAGTGTGGCTGGTCATCCTCTC-3')(260 bp), 0.2 mM deoxyribonucleoside triphosphates, 1x reaction buffer, 2 mM MgCl₂ and 1.5 U Taq DNA polymerase (Fermentas) in a total volume of 25 µL. The DNA was amplified using the following protocol: initial denaturation (95 °C for 5 min), followed by 30 cycles of denaturation (95 °C for 45 s), annealing (95 °C for 45 s) and extension (72 °C for 1 min), with a single final extension of 5 min at 72 °C. The programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device was used in all PCR reactions. Ten microliters of PCR products were resolved on a 1.5% agarose gel containing 0.5 mg/ml of ethidium bromide in Tris–borate–EDTA buffer at 90 V for 1 h, also using suitable molecular weight markers. The products were examined under ultraviolet illumination.

Antibiotic susceptibility test

K. pneumoniae isolates were incubated initially on the nutrient agar media (at 4 °C) and their positive colonies were transferred to the Mueller– Hinton agar (Merck, Germany). Antimicrobial susceptibility was performed on Mueller-Hinton agar by the standard disk diffusion method recommended by the Clinical and Laboratory Standards Institute¹¹. This was done by dipping a sterile swab stick in to overnight

nutrient broth and carefully swabbing the entire surface of Mueller–Hinton agar plates. Susceptibility of *K. pneumoniae* strains were determined against tetracycline (30 µg/disk), ceftazidime (30 µg/disk), ciprofloxacin (5 µg/disk), sulfamethoxazol (25 µg/disk), ampicillin (10 u/disk), trimethoprim (5 µg/disk), gentamycin (10 µg/disk), ceftriaxone (30 u/disk), amikacin (10 µg/disk), imipenem (30 µg/disk), erythromycin (15 µg/disk), and amoxicillin/clavulanic acid (20/10 µg/disc) antibiotic disk (Oxoid, UK). Plates were incubated at 37 °C for 18–24 h. The diameter of zone of inhibition was measured in millimeters and isolates were scored as sensitive or resistant by comparing with values recommended on standard charts (11). *K. pneumoniae* ATCC 43816 was used as quality control organism in antimicrobial susceptibility determination.

Statistical analysis

The results were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA) for analysis. Statistical analysis was performed using SPSS/16.0 software (SPSS Inc., Chicago, IL) for significant relationship between incidence and pattern of resistance of *K. pneumoniae* strains of RTIs. Chi-square and Fisher's exact 2-tailed tests were performed in this study. Statistical significance was regarded at a *P* value <0.05.

RESULTS

Table 1 represents the total prevalence of *K. pneumoniae* in various studied groups. Twenty-five out of 350 samples (7.14%) were positive for

Table 1. Distribution of *K. pneumoniae* in the samples taken from patients suffered from RTIs.

Samples of RTIs	No. samples collected	<i>K. pneumoniae</i> positive samples (%)	PCR confirmation of <i>K. pneumoniae</i> (%)
Male	<20	2 (10)	2 (10)
	20-40	2 (6.66)	2 (6.66)
	4-60	3 (6)	3 (6)
	>60	8 (10)	8 (10)
	Total	15 (8.33)	15 (8.33)
Female	<20	3 (12)	3 (12)
	20-40	1 (4)	1 (4)
	4-60	2 (4)	2 (4)
	>60	4 (5.71)	4 (5.71)
	Total	10 (5.88)	10 (5.88)
Total	350	25 (7.14)	25 (7.14)

Table 2. Antibiotic resistance pattern of *K. pneumoniae* in the samples taken from patients suffered from RTIs

Samples (No. <i>K. pneumoniae</i> positive)	tet30	cftz	cip5	sul25	am10	tri5	gen10	cftx	amik	imp	eryt	amclv
Male (15)	10 (66.66)	10 (66.66)	12 (80)	12 (80)	14 (93.33)	13 (86.66)	15 (100)	11 (73.33)	13 (86.66)	2 (13.33)	12 (80)	11 (73.33)
Female (10)	3 (30)	4 (40)	7 (70)	7 (70)	9 (90)	5 (50)	9 (90)	3 (30)	4 (40)	-	9 (90)	7 (70)
Total (25)	13 (52)	14 (65)	19 (76)	19 (76)	23 (92)	18 (72)	24 (96)	14 (56)	17 (68)	2 (8)	21 (84)	18 (72)

Tet30: tetracycline (30 µg/disk), cftz: ceftazidime (30 µg/disk), cip5: ciprofloxacin (5 µg/disk), sul25: sulfamethoxazol (25 µg/disk), am10: ampicillin (10 u/disk), tri5: trimethoprim (5 µg/disk), gen10: gentamycin (10 µg/disk), cftx: ceftriaxone (30 u/disk), amik: amikacin (10 µg/disk), imp: imipenem (30 µg/disk), eryt: erythromycin (15 µg/disk), and amclv: amoxicillin/clavulanic acid (20/10 µg/disc)

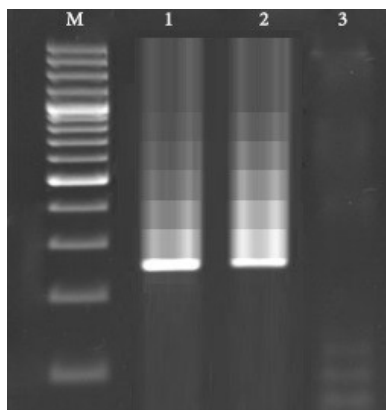


Fig. 1. Results of the gel electrophoresis for amplification of *16s rRNA* gene of the *K. pneumoniae* isolated from samples of hospitalized patients with severe RTIs. M: 100 bp ladder (Fermentas, Germany), 1: Positive sample for *16s rRNA* gene of the *K. pneumoniae* (260 bp), 2: Positive control (*K. pneumoniae* ATCC 43816) and 3: Negative control (sterile distilled water (Merck, Germany))

presence of *K. pneumoniae*. On the other hand, total prevalence of *K. pneumoniae* in male and female patients of our study were 8.33% and 5.88%, respectively. Results of the culture method were also confirmed by the *16S rRNA* gene-based PCR amplification. Figure 1 shows results of the gel electrophoresis for the PCR amplification of *16S rRNA* gene. Among all studied groups, older than 60 years old male patients (10%) and also younger than 20 years old female patients (12%) had the highest prevalence of bacteria. Statistically significant differences were seen for the prevalence of *K. pneumoniae* between various age groups ($P < 0.05$).

Table 2 represents the antibiotic resistance pattern of *K. pneumoniae* strains of patients suffered from RTIs. We found that *K. pneumoniae* strains of our study harbored the highest levels of resistance against gentamycin (96%), ampicillin (92%), erythromycin (84%), ciprofloxacin (76%), sulfamethoxazole (76%) and amoxicillin/clavulanic acid (82.28%). The lowest levels of resistance were seen against imipenem (8%). Bacterial strains which were isolated from male patients had the higher prevalence of resistance than those of females ($P < 0.05$).

DISCUSSION

The present investigation was focused on the study the prevalence rate and antimicrobial resistance pattern of *K. pneumoniae* strains isolated from the samples taken from patients suffered from RTIs. We found that 7.14% of samples were positive for *K. pneumoniae* with higher prevalence in males and also older than 60 and younger than 20 years old patients. One possible explanation for the high prevalence of *K. pneumoniae* in our study is the fact that all of the tested samples were taken in the winter season. Coldness can effect on the prevalence of RTIs and is an important predisposing factor for occurrence of pneumonia and RTIs caused by pathogenic agents like *K. pneumoniae*.

A conceivable explanation for the higher prevalence of *K. pneumoniae* in male than female is that men usually have more contact with the contaminated external environment. They work outside the house but women usually stay at home and are not in close contact with outside. In fact, most of the Iranian women prefer to work at home. In addition, higher levels of immunity in men than women caused to their infection with resistant strains of *K. pneumoniae*. Older patients had also the lower levels of immunity and therefore they had a higher prevalence of *K. pneumoniae*. The reason for the higher prevalence of *K. pneumoniae* in younger than 20 years old patients is the fact that the population of this group of patients of our study were infants and pediatrics suffered from pneumonia and RTIs. Therefore, based on their lower levels of immunity, occurrence of RTIs caused by *K. pneumoniae* were high.

Irregular and unauthorized prescription of antibiotics is the main factor for such high prevalence of antibiotic resistance in the *K. pneumoniae* strains of our study. According to this thesis, it is not surprising that *K. pneumoniae* strains of our investigation harbored the high levels of resistance against tetracycline, ceftazidime, ciprofloxacin, sulfamethoxazol, ampicillin, trimethoprim, gentamycin, ceftriaxone, amikacin, imipenem, erythromycin and amoxicillin/clavulanic acid. Higher prevalence of antibiotic resistance in isolates of male than female is due to their higher

levels of immunity. Therefore, they only be infected with resistant strains of *K. pneumoniae*.

Several investigations have been conducted in this field al-around the world. Jafari Sales *et al.* (2015)¹² revealed the high prevalence of *K. pneumoniae* in the hospital infections of Iranian hospitals with considerable levels of resistance against *et al* ceftazidime (100%), cefotaxime (98.60%), ciprofloxacin (82%), amikacin (53.30%) and gentamicin (47.60%) which was in harmony with our findings. Sanchez *et al.* (2013)⁶ reported increasing prevalence of antibiotic resistance in the *K. pneumoniae* strains of clinical infections from 1998 to 2010. They showed that the prevalence of resistance in the American isolates of *K. pneumoniae* in 2010 against tetracycline, amikacin, imipenem, gentamycin, cefepime, trimethoprim/sulfamethoxazole, piperacillin/tazobactam, tobramycin, ceftriaxone, ciprofloxacin, ceftazidime and aztreonam were 16.70%, 4.50%, 4.30%, 9.20%, 7.70%, 19.30%, 12.70%, 13.80%, 12.10%, 16.80%, 17.20% and 22.20%, respectively which was lower than our results in 2016. Kalaskar *et al.* (2012)¹³ reported that the prevalence of resistance in the *K. pneumoniae* strains of clinical infections against ampicillin, amoxicillin/clavulanate, gentamycin, amikacin, cefuroxime, ciprofloxacin, trimethoprim/sulfamethoxazole and imipenem were 100%, 100%, 100%, 37.50%, 22%, 88.60%, 86.80%, 10% and 0%, respectively which was similar with our results. Similar results have been reported from India (2011)¹⁴, Australia (2015)¹⁵, Switzerland (2016)¹⁶, South Africa (2012)¹⁷ and Brazil (2015)¹⁸.

Availability of antibiotic agents, cost of them and idea of medical practitioners for prescription of antibiotics are the main factors caused differences in the prevalence of antibiotic resistance in various studies.

CONCLUSIONS

The present investigation is one of the widespread reports of prevalence and antibiotic resistance pattern of *K. pneumoniae* isolated from samples of hospitalized patients suffered from RTIs. The most important findings of our study are higher prevalence of *K. pneumoniae* in males and also older than 60 years old and younger than 20 years old patients and also inefficiency of gentamycin,

ampicillin, erythromycin, ciprofloxacin, sulfamethoxazole and amoxicillin/clavulanic acid prescription. It seems that primary identification of sources of *K. pneumoniae* RTIs and their treatment with regular and accurate prescription of imipenem, according to the results of disk diffusion method can control distribution of *K. pneumoniae* RTIs. Study the pattern of antibiotic resistance not only for *K. pneumoniae* but also for other types of human clinical infections is necessary for each province/states, city and even each hospital.

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