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RESEARCH ARTICLE

CHARACTERIZATION OF *KLEBSIELLA PNEUMONIAE* FROM FRESH VEGETABLES MARKETED IN PUDUCHERRY

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ABSTRACT

Multidrug resistant *Klebsiella pneumoniae* is an increasingly difficult problem in India. Carbapenems have been the grounding of drug treatment for serious infections caused by these pathogens and also the carbapenems resistant increasing in other side. Vegetables are known as source of contamination with *K. pneumoniae*. Raw vegetables are usually consumed in salads and other dishes. The aim of this study was to investigate the occurrence and characterization of *K. pneumoniae* in raw vegetables marketed in Puducherry and 100 commonly used salad vegetables (tomato, carrot and cucumber) were collected. Antimicrobial resistance profile, Metallo-beta-lactamases detection, modified Hodge's test for carbapenemase, Minimum inhibitory concentration for colistin in resistant isolates, and biofilm production on congo red agar were done. A total of 15 antibiotics were tested. Sulfamethoxazole- trimethoprim, norfloxacin and chloramphenicol was displayed 100.0% sensitivity. Ampicillin, Cephalothin, Ciprofloxacin and Tetracycline was produced resistance 34.3%, 45.7%, 66.0% and 22.5%, respectively. About 40.2% isolates were resistant to meropenem, 41.0% to imipenem and 10% were resistant to colistin. None of them were Metallo-beta-lactamases producers. Six isolates showed Modified Hodge's test positive with dissimilar clonal types. All the isolates (100.0%) were produced biofilm on modified congo red agar.

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INTRODUCTION

Klebsiella pneumoniea is a rod shaped non motile, Gram negative, lactose fermenting and facultative anaerobic bacterium which is usually found in the normal flora of skin, mouth, and intestines. Besides *Klebsiella* is found to cause infections in the urinary and lower biliary tract (Lopes *et al.,* 2005). The species *K. pneumoniae* encompasses three subspecies namely, *K. pneumonia* subsp *pneumoniae* (*K. pneumoniae*), *K. pneumonia* subsp *ozaenae* (*K. ozaenae*) and *K. pneumonia* subsp *rhinoscleromatis* (*K. rhinoscleromatis*) among which *K. pneumoniae* being the most common (Taxonomic style according to Le Mino, 1992). Due to the high nutritional value, vegetables are considered as important components in every healthy human diet. Regular consumption of vegetables can reduce risk of some important disease such as cancers, stroke and cardiovascukar diseases (Van Duyn and Pivonka, 2000). Vegetables can be contaminated with harmful enteric bacteria in farm, pre-harvest, harvest and post-harvest activities and even in transportation and processing line. Untreated wastewater and animal/human faeces are considered as usual sources of contamination (Beuchat, 2002; Gupta *et al*., 2009). Foodborne pathogens primarily belong to Enterobacteriaceae which are considered as the common intestinal flora of human beings and animals. They are mainly transmitted through fecal-oral route, which known to cause diarrhoea, vomiting, and gastrointestinal infections. One of the foodborne pathogen is *Klebsiella*, which is ubiquitous in nature. They are common inhabitants of sewage, soil, respiratory and intestinal tracts of human beings and animals. *Klebsiella pneumoniea* is the most important species of this genus which are significant in both communities acquired (foodborne) and hospital acquired infections, (Halda and Alija, 2004). A number of reports showed that there was an increase in the number of outbreaks of food-borne diseases associated with consumption of fresh produce (Beuchat, 1995; De Roever, 1998). After the introduction of extended- spectrum cephalosporins, extended spectrum β-lactamase (ESBL)-producing *Klebsiella* pneumonia have become an increasingly serious problem worldwide (Jacoby and Han, 1996). Their management has become complicated by the generation of a variety of ESBLs production and is considered as an important threat till now (Livermore, 1998). ESBL-containing plasmids

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often carry resistance genes for other antibiotics thus; aminogycosides and fluoroquinolones may be ineffective (Paterson *et al*., 2000). Although β lactam/ β-lactamase inhibitors combinations have been suggested as an option for ESBL producers, these drugs must be given in high doses (Gold *et al*., 1996). Carbapenems were first introduced in 1980 and they are now frequently used as the last choice in treating serious infections caused by multidrug-resistant strains of Gram negative bacilli in intensive care units (ICUs) and in high risk wards. These are stable to β-lactamase including the ESBLs and AmpC produced by Gram negative bacilli (Zhanel *et al*., 2007). The carbapenems are a class of beta-lactamase antibiotics that differ from the penicillins by the substitution of a carbon atom for a sulfur atom and by the addition of a double bond to the five member ring of the penicillin nucleus. They bind the bacterial penicillin-binding proteins, which are responsible for elongation and cross link the peptidoglycan of the bacterial cell wall. This results in impairment of construction of the cell wall, inhibition of cell growth frequently, cell lysis and death (Halliger *et al*., 1999). Multi-drug resistance (including carbapenem) in *Klebsiella* spp is an increasingly difficult problem in Indian hospitals due to the lack of therapeutic options and the potential transfer of antibiotic resistance to other pathogens. Carbapenems have been the grounding of drug treatment for serious infections caused by these pathogens. Meropenem and Imipenem are the two carbapenems available for use in India (Tribuddaret *et al*., 2008). *K. pneumoniae* was reported to be able to grow *in vitro* as a biofilm since the end of the 1980s (Lechavalliar *et al*., 1998), but clear evidence of an *in vivo* biofilm was provided only in 1992 by Reid and coworkers, who investigated by scanning electron microscopy some bladder epithelial cells of a spinal cord injured patient with an asymptomatic urinary tract infection caused by *K. pneumoniae* (Reid *et al*., 1992). Later, *in vitro* studies have demonstrated that about 40% of *K. pneumonia* isolated not only from urine, but also from sputum, blood and wound swabs, were able to produce biofilm (Yang and Zhang, 2008), as well as that about 63% of *K. pneumonia* isolates from urine samples of catheterized patients suffering from UTIs were positive for *in vitro* biofilm production (Niveditha *et al*., 2012). Recently, also a high rate of *K.pneumoniae* strains isolated from endotracheal tubes (ETT) of patients affected by ventilator-associated pneumonia (VAP) were reported to be able to form an *in vitro* biofilm (Sanghai *et al*., 2012). Biofilm formation on abiotic surfaces was shown to be more consistent at 40 °C than 35 °C, using atomic force and high-vacuum Scanning Electron Microscope (Korres *et al*., 2013).

MATERIALS AND METHODS

Collection of samples

A total of 100 salad vegetable samples from *Solanum lycopersicum* (Tomato=40), *Dacus carota* (Carrot=38), and *Cucumis sativus* (Cucumber=22) were purchased from different markets in and around, Puducherry. All fresh samples were transported on ice and analyzed immediately. All the chemicals used in this study were procured from Hi media. Mumbai.

Laboratory Analysis and Techniques

All materials needed for the study were thoroughly sterilized using a combination of the most appropriate methods of sterilization such as flaming, chemical sterilization, Ultra Violet light, hot air ovens and autoclaving to ensure that experimental materials brought in from the two major markets were not contaminated from the laboratory.

Sample preparation and isolation

The sample preparation and isolation was followed as per Babu *et al*., 2015 with slight modification. Ten ml of the surface wash was homogenized and added to sterile 90 ml Brain Heart Infusion broth and incubated overnight at 37 °C. One ml of homogenate was plated and purified on Mac Conkey agar, supplemented with 50 mg per liter carbenicillin. Identification of the isolates was done as per standard microbiological procedure (Veterinary Microbiology and Microbial Disease, 2007). The pure cultures were also stored at -80 °C in 15% glycerol for further studies.

Antimicrobial resistance profiling of the isolates

The drug susceptibility of isolates was performed on Mueller Hinton agar plates by disc diffusion method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). The bacterial isolates was inoculated into nutrient broth and incubated for 18 hours at 37°C. About 0.1 ml of each bacterial isolate was seeded onto a separate Petri dish containing Mueller-Hinton agar and allowed to stand for about 5 min. The details of the antibiotics given in Table no: 6. The interpretation of the measurement as sensitive and resistant was made according to the manufacturer standard zone size interpretative table. The percentage resistance was calculated using the formula PR=a/b×100, where 'PR' is percentage resistance, 'a' is the number of resistant isolates and 'b' is the number of isolates tested with the drug. Generally, colonies with clear zone of diameter between 0-13mm will be classified as resistant (CLSI, 2012; Akinjogunla and Enabulele, 2010).

Metallo β-lactamase (MBL) detection, Modified Hodge's Test was performed as per Lee *et al*., 2001 with slight modification mentioned below:

Metallo β-lactamase (MBL) detection

EDTA disks were prepared using 0.5M EDTA (Sigma Chemicals) solution dissolved in distilled water; pH was adjusted to 8 and sterilized by autoclaving. Ten µl (10 µl) of the EDTA solution was added to sterile blank 6mm disk (Whatman filter paper 4) and dried without overflowing. The disk contained approximately 1,900µg of EDTA. The test was performed on Muller-Hinton agar plate by disk diffusion method. A 0.5McFarland adjusted suspension of the test organism was inoculated on MHA plated. A 10 µg meropenem disk was placed at the center of the plate and the EDTA disk was place at a distance of 10mm, centre to centre, from the meropenem disk and the plate was incubated at 37ºC overnight. The zone around the meropenem disk would be extended on the side nearest the EDTA if the organism is a MBL producer.

Modified Hodge's Test

This is a phenotypic test which could be used to determine if reduced susceptibility to carbapenems is mediated by a carbapenemase production. Mueller-Hinton agar plate was

inoculated with a 0.5McFarland suspension of *E.coli* and streaked for confluent growth using a swab. A 10 µg imipenem disk was placed in the center, and each test isolate was streaked from the disk to the edge of the plate and the plate was incubated at 37ºC overnight. After incubation the plates were examined for a clover leaf-type indentation at the intersection of the test organism and the *E. coli*, within the zone of inhibition of the carbapenem susceptibility disk. A positive test has a clover leaf-like indentation of the *E.coli* growing along the test organism growth streak within the disk diffusion zone. A negative test has no growth of the *E .coli* along the test organism growth streak within the disc diffusion.

AmpC detection

This method was done as per Black *et al*., 2005. AmpC enzyme production was tested by the AmpC disk. 0.5 McFarland suspension of *E. coli* was inoculated on the surface of Mueller-Hinton agar plate. A 30 µg cephoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cephoxitin disc almost touching it, with the inoculated disk face in contact with the agar surface. The plate was then inverted and incubated overnight at 37°C. After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cephoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cephoxitin (negative result).

MICs of colistin

MICs of colistin was done as per Kalem *et al*., 2016 and obtained using colistin sulphate powder (Hi-media., Mumbai) by the agar dilution method performed according to CLSI methods by a 2-fold concentrations ranging from 0.25 to 128 mg/L. *Escherichia coli* and *P. aeruginosa* were used as quality control, and test values obtained were in line with published standards.

Table 1. The growth characteristics of *Klebsiella pneumonia* on different medium

Sl.no	Selective agar	Colony characters
01	Mac Conkey's agar (MCA)	Lactose fermenting pink colonies
02	Eosin and Methylene blue (EMB)	Non - metallic sheen colonies

Table 2. The results biofilm on modified congo red agar

Sl.no	Selective agar	Biofilm	
		Positve (100.0%)	Negative (0%)
01	Modified congo red agar (MCRA)	Black colored colonies with dry crystallization	Red colored colonies were noticed

Biofilm production assay

Biofilm/Slime production assay was performed as per Vijayalakshmi and Lalitha, 2015. Briefly, Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared and autoclaved. The isolates were inoculated and incubated aerobically for 24 to 48 hours. The ability of the isolates to produce bio-films was indicated by black colonies with a dry crystalline consistency. In case of negative results red colour colonies were noticed.

RESULTS AND DISCUSSIONS

Carbapenem resistance in *Klebsiella* spp., is an emerging problem and is a cause of concern as many nosocomial *Klebsiella* spp., are detected to be resistant to most other antibiotics. Improper food handling practices, poor hygienic condition of places where vegetables were displayed use of contaminated equipments and containers during transportation can contribute to contamination with *K. pneumoniae*. According to some researchers (Ponniah *et al*., 2010; Tunung *et al*., 2010; Usha *et al*., 2010; Yang *et al*., 2008), poor hygienic practices and improper handling are considered as major factors for contamination of food at markets level. Though the clinical significance of *Klebsiella* strains isolated from the food and the environment is unclear, these habitats are thought to be potential reservoirs for the growth and spread of these pathogens which may colonize animals and human.

A total of 52(52.0%) isolates were recovered from 100 (tomato = 40, carrot= 38 and cucumber=22) fresh vegetables sample. Among 52 isolates tomato was found to be 60.0% (40) followed by carrot 52.6% (38) and cucumber 36.6% (22). This study result is higher than Puspanathan *et al*., 2012. Who reported 50.0% from tomato, 30.0% from carrot in malaysia. Author also reported the 82.2% from cucumber which is higher than the present report. Possibly these vegetables have high relative humidity which favours the spread and survival of bacteria on plant surfaces (Adams *et al*., 1989). Isolation of Enterobacteriaceae and other bacterial species from vegetables has been reported by several researchers (Bennik *et al*., 1998; Brocklehurst *et al*., 1987; Ercolani, 1976; Garg *et al*., 1990; King *et al*., 1991; Sahilah *et al*., 2010; Tunung *et al*., 2011). The main sources for these contaminations are as follows; animal waste fertilizers, contaminated irrigation water and post harvest washing using contaminated water. Usually the upper layer of the soil (30 $cm^2$from the ground) contains 106- 107 bacteria/g as some farmers use animal manure or faecal as a fertilizer to enrich soil. On the other hand, contamination may occur through the systemic contamination starting from cultivation site to storage and handling. Contamination in such places can lead to the development of biofilms (Blackman and Frank, 1996).

This is because of the presence of normally present microorganisms on the surface of cucumber the close contact of it with soil. Presence of soil bacteria or fungi can result in growth and colonization of microorganism on the vegetable and the hygienic quality of fresh vegetables. Coliforms are associated with both soil and decaying vegetation. The survival of enteric bacteria in soil is particularly one of the main reasons in the contamination of raw vegetables (Geldreich *et al*., 1962). According to Rao and Rao (1983), *Klebsiella* cannot be easily dislodged from the surface of vegetables by gentle washing. Washing vegetables using disinfectants is one of the suggested ways to reduce risk. Sodium hypochloride or potassium permanganate solutions can be used against a wide number of microorganisms (Adams *et al*., 1989; Albrecht

et al., 1995; Beuchat *et al*., 1998; Brackett, 1992; Garg *et al*., 1990; Lisle *et al*., 1998; Reynolds *et al.*, 1989). Carbapenems have been the grounding of drug treatment for serious infections caused by these pathogens.

Table 3. The results of primary identification tests

Sl.no	Tests	Results
01	Capsule	+ve
02	Catalase	+ve
03	Citrate	+ve
04	Flagella	-ve
05	Gas	+ve
06	Gelatin Hydrolysis	-ve
07	Gram Staining	-ve
08	Growth in KCN	+ve
09	H2S	-ve
10	Indole	-ve
11	Motility	-ve
12	MR (Methyl Red)	-ve
13	MUG Test	+ve
14	Nitrate Reduction	+ve
15	OF (Oxidative-Fermentative)	Fermentative
16	Oxidase	-ve
17	Pigment	-ve
18	Shape	Rod
19	Spore	-ve
20	TSIA (Triple Sugar Iron Agar)	A/A
21	Urease	+ve
22	VP (Voges Proskauer)	+ve

Table 4. Results of secondary identification tests

Sl.no	Tests	Results
01	Adonitol	+ve
02	Arabinose	+ve
03	Arabitol	+ve
04	Cellobiose	+ve
05	DNase	-ve
06	Erythritol	-ve
07	Esculin Hydrolysis	+ve
08	Glucose	+ve
09	Glycerol	+ve
10	Inositol	+ve
11	Lactose	+ve
12	Maltose	+ve
13	Mannitol	+ve
14	Mannose	+ve
15	Melibiose	+ve
16	Mucate	+ve
17	MyoInositol	+ve
18	Raffinose	+ve
19	Rhamnose	+ve
20	Salicin	+ve
21	Sorbitol	+ve
22	Sucrose	+ve
23	Tartrate	+ve
24	Trehalose	+ve
25	Xylose	+ve

Meropenem and imipenem are the two carbapenems available for use in India (Tribuddharat *et al*., 2008). According to Parveen *et al*., 2010 meropenem was the frequently used antibiotics to treat infections by multidrug resistant bacteria in ICUs and high risk wards and soon within a short period. Among the 52 isolates 40.2% percent isolates were resistant to meropenem and 41.0% to imipenem. Gupta *et al*., from Delhi reported 6.9% of meropenem resistance and 4.3% of imipenem resistance in *Klebsiella* and a study from Kanpur reported no carbapenem resistance among *K.pneumoniae* tested from human samples. High-Level carbapenem resistance in a *K.pneumoniae* is due to the combination of β-Lactamase production, porin OmpK35/36 insertional inactivation, down-regulation of the phosphate transport porin and changes in penicillin-binding proteins (Frank *et al*., 2006).

Table 5. List of antibiotics used in this study

S.no	Antibiotics	Content / disk
1	Ampicillin	25µg
2	Amphotericin B	10 units/disk
3	Colistin	10µg
4	Cephalothin	30µg
5	Cefoxitin	30µg
6	Cefriaxone	30µg
7	Ciprofloxacin	10µg
8	Chloramphenicol	30µg
09	Gentamicin	120µg
10	Imipenem	30µg
11	Meropenem	10µg
12	Norfloxacin	10µg
13	Nalidixic acid	30µg
14	Sulfamethoxazole- trimethoprim	25µg
15	Tetracycline	30µg

Table 6. Results of antibiotic resistance profile of the isolates

S.no	Antibiotics	Results (%)		
		R	I	S
1	Ampicillin	34.3	0	65.7
2	Amphotericin B	10.8	0	89.2
3	Colistin	10.0	0	90.0
4	Cephalothin	45.7	0	54.3
5	Cefoxitin	20.0	20.0	60.0
6	Cefriaxone	17.0	26.0	57.0
7	Ciprofloxacin	66.0	14	20.0
8	Chloramphenicol	0	0	100.0
09	Gentamicin	17.5	12.5	70.0
10	Imipenem	41.0	0	59.0
11	Meropenem	40.0	0	60.0
12	Norfloxacin	0	0	100.0
13	Nalidixic acid	50.0	20.0	30.0
14	Sulfamethoxazole- trimethoprim	0	0	100.0
15	Tetracycline	22.5	0	77.5

R – resistance, I- intermediate, S- sensitive

Sulfamethoxazole- trimethoprim, norfloxacin and chloramphenicol was displayed 100.0% sensitivity. Ampicillin, Cephalothin, Ciprofloxacin and Tetracycline was produced resistance 34.3%, 45.7%, 66.0% and 22.5%, respectively. Parveen *et al*., 2010 reported the 100.0% resistance of tetracycline, gentamicin, cephoxitin and trimethoprim/ sulfamethoxazole. The metallo-β-lactamases VIM and IMP are scattered globally, with VIM predominating in southern Europe and IMP in the Far East, and NDM being widespread in India and Pakistan (Poirel *et al*., 2004). The metallo-β-lactamases VIM, IMP, and NDM. Carbapenemase-producing *K. pneumonia* (CPKP) isolates have undergone extensive dissemination in many countries, and continues to spread in new geographical locations, indicating an ongoing dynamic process (Gundmann, 2010). In this study none of them were Metallo-beta-lactamases producers but 59.2% of AmpC production was there. This result is agreed with the findings of Parveen *et al*., 2010 from clinical isolates in Puducherry.

The modified Hodge's test differentiates carbapenemases (MHT positive) from *Klebsiella* strains showing resistance to carbapenems by a combination of porin loss and ESBL or AmpC production (MHT negative). Two strains from tomato were modified Hodge's test positive which optimistically indicate that they could posses the KPC gene which mediated the carbapenem resistance. Recently, colistin an older polymyxin antibiotic with a reputation for nephrotoxicity and neurotoxicity, has emerged as a salvage therapy for nosocomial infections caused by multidrug-resistant pathogens in the ICU. However, colistin-resistant strains have recently

been reported. Eighteen specimens containing colistin-resistant *K. pneumoniae* were cultured from 13 ICU patients in 2004 and 2005. Result is differing with our study where a maximum of 10.0% of colistin resistance was recorded. The formation of biofilms on leaf surfaces of some vegetables (lettuce, celery, spinach, parsley, Chinese cabbage, basil, leek and endive) has been reported (Morris *et al*., 1997). In our study all the isolates (100.0%) were found to produce biofilm on modified congo red agar. *In vitro* studies have demonstrated that about 40% of *K. pneumoniae* isolated not only from urine, but also from sputum, blood and wound swabs, were able to produce biofilm (Yang and Zhang, 2008) and 63% of *K. pneumonia* isolates from urine samples of catheterized patients suffering from UTIs were positive for *in vitro* biofilm production (Nivedhitha *et al*., 2012). Also a high rate of *K.pneumoniae* strains isolated from endotracheal tubes (ETT) of patients affected by ventilator-associated pneumonia (VAP) were reported to be able to form an *in vitro* biofilm (Singahai *et al*., 2012).

Conclusion

Vegetables are known as source of contamination with *K. pneumoniae*. Raw vegetables are usually consumed in salads and other dishes, so there is a threat persisting in salad vegetables. The biofilm forming ability demonstrated by these isolates reveals the pathogenic status of the organisms. From the findings, *Klebsiella* spp., use multiple hosts as channel to human or other animals and also multiple antibiotic resistances are found against different classes of antibiotics.The microbiological risk assessment of *K. pneumonia* associated with raw vegetables consumption in Puducherry will be useful for further studies.

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