

Presence of Extended Spectrum Beta Lactamase, Virulence Genes and Resistance Determinants in Biofilm Forming *Klebsiella pneumoniae* Isolated from Food Sources: A Potent Risk to the Consumers

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Abstract

Foodborne diseases and infection caused by associated pathogens is a public health concern. Majority of the investigations focus on common foodborne pathogens like *Vibrio parahaemolyticus*, *Escherichia coli*, *Listeria monocytogenes*, *Shigella*, *Salmonella* and *Staphylococcus aureus*. Limited knowledge has been accounted on *Klebsiella pneumoniae*. Presence of multidrug-resistant *K. pneumoniae* in the food supply is disturbing. Hence, this study assessed the presence of *K. pneumoniae* isolates from food samples (fresh vegetables and chicken), ascertained the presence of drug-resistant phenotypes, extended spectrum beta lactamase production, antibiotic resistance determinants, genes associated with virulence and their ability to form biofilm. Resistance towards ceftazidime and tetracycline was noted among all the isolates in the study, while they exhibited sensitivity to chloramphenicol and cotrimoxazole. All the isolates were potent ESBL producers carrying at least one ESBL encoding genes. Plasmid mediated quinolone resistance gene was detected in one isolate each from onion and chicken respectively. The isolates marked the absence of tetracycline and chloramphenicol resistance genes. Multiple virulence genes (*ureA*, *khe*, *fimH*, *mrkD*, *wabG*, *uge* and *elt*) were possessed by each of the isolates. *K. pneumoniae* from chicken and cucumber were moderate biofilm formers and those from tomato exhibited weak biofilm formation. Increased expression of the *mrkA* gene and reduction in the expression of the biofilm forming gene *fimH* gene was observed among the biofilm formers. One of the moderate and non-biofilm formers exhibited increased *mrkD* gene expression. The results from our study stipulate, that raw vegetables and meat serve as dormant source of drug-resistant and virulent *K. pneumoniae*.

Keywords: *Klebsiella pneumoniae*, Multidrug-resistance, ESBL production, Resistance Determinants, Virulence Factors, Biofilm Formation

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INTRODUCTION

Klebsiella pneumoniae is acknowledged as a momentous pathogen causing various infections in humans that include diarrhoea, pneumonia, septicaemia and liver abscesses. Besides, the clinical environment *K. pneumoniae* has been isolated from several foods including fish, meat, powdered infant food, raw vegetables and street foods.¹ Extensive use of antibiotics in agriculture, veterinary and clinical practices has resulted the emergence of drug resistant *K. pneumoniae* strains.

Genetic elements *viz* plasmids, integrons and transposons further aid the spread of the resistance genes. In addition, persistence of these genes in the environment makes the control of drug-resistant pathogens strenuous. Currently, *K. pneumoniae* is resistant towards broad spectrum drugs like fluoroquinolones and aminoglycosides.² Extended spectrum β -lactamases (ESBLs) offer broad spectrum of resistance towards penicillin's, tetracyclines cephalosporins, sulfonamides, chloramphenicol and trimethoprim. *K. pneumoniae* are ready receptors of the R plasmids and found to have significant involvement in several nosocomial epidemic diseases. The plasmid mediated multi-drug resistant genes could possibly be transferred from pathogens of fish, animal and human origin to the susceptible strains of a diverse ecological niche.³ Therefore, the issue of resistance among the bacterial populations persists to be a critical public health concern.

Among all the other possible ESBL producers, *K. pneumoniae* is one of the dominant organisms. Although carbapenems are believed as the first line of drugs in the treatment of ESBL producing *K. pneumoniae* infections, the resistance has been steadily increasing.⁴ The ESBL producing *K. pneumoniae* is to be considered a critical affair due to limitations in the treatment choices and alarming rates of morbidity and mortality. Both humans and animals exposed to the ESBL producing strain by the consumption of contaminated water or food sources, could be at a greater risk of colonization and infection by the pathogen. In *K. pneumoniae*, ESBL genes belonging to the CTX-M, SHV and TEM families are familiar and clinically relevant, with CTX-M being the most predominant.

Formation of biofilms by food-related pathogens is a matter of consideration, in addition a predisposing factor for contamination of food sources. Microbes in a community secrete cement-like substances that serve as a glue, protecting them against host macrophages and antibiotics.⁵ Because, of which eradication of the biofilm producing pathogens from the indwelling surfaces. Pathogenic microorganisms like *K. pneumoniae* can attach, grow on food surfaces by forming biofilms. Formation of biofilms are one of the major virulence factors causing chronic infections.⁶ Inadequate hygiene in the food processing sectors facilitate bacterial adherence, biofilm formation and serve as reservoirs for various infectious agents.⁷ Thereby, food contamination continues to be a threat and burden on local and global markets.

Besides biofilms, fimbrial adhesins, production of siderophores, nature and synthesis of the polysaccharide capsule collectively contribute to the pathogenicity of *K. pneumoniae*. In addition, resistance to drugs, production of phospholipase and hemolysin are considered to be the potent virulence intensifiers of the pathogen. Conventionally, the food sector is considered to be a subtle affair that provokes fear to the food industry and also the common man, if contaminated.

The reciprocity amidst the resistance mechanisms and presence of virulent traits in *K. pneumoniae* is not substantiated. However, it was previously established that drug-resistance comes with a fitness cost and decreased virulence. At present, reports have proposed increased virulence to the developing resistance. Additionally, virulence is noted to evolve naturally as a response to resistance acquired, or possibly shared among bacteria.⁸

In country like India, where antibiotics are used indiscriminately not only in the hospital settings, but also in the agriculture and food industries, drug-resistant pathogen like *K. pneumoniae* becomes prevalent and is a public health implication, often neglected.

Hence, the present study aimed to isolate and characterize *K. pneumoniae* from commonly used food sources. Antibiotic susceptibility pattern, ESBL production, antibiotic resistance

genes and virulence factors of the isolates were also investigated. Ability of the isolated bacteria in forming biofilms and their associated gene expression were also studied.

MATERIALS AND METHODS

Sample Collection

In total 30 samples derived from chicken (10), onion (10), tomato (5) and cucumber (5) collected from a local market in Mangaluru were used for the isolation of *K. pneumoniae*. Samples collection was done in pre-sterilized containers under aseptic conditions. The samples were stored in 4°C and were processed further.

Isolation of the *K. pneumoniae* and Phenotypic Identification

Collected samples (25 g) were weighed and enriched in 250 ml of heat sterilized nutrient broth (HiMedia, India) following an incubation of 37°C for 24 h. A loopful of the inoculum was streaked onto MacConkey agar (HiMedia, India) and further incubated at 37°C for 24 h. Pink mucoid colonies indicative of typical *K. pneumoniae* colonies were selected and re-grown on Luria Bertani (LB) broth (HiMedia, India). Gram-staining, motility, capsule staining, series of biochemical tests (Indole, methyl-red, Voges-Proskauer, citrate-utilization, catalase and oxidase) were performed for re-conformation of *K. pneumoniae* isolates. Phenotypically confirmed isolates were stored at -80°C in LB broth containing 30% glycerol.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out for all the confirmed isolates *K. pneumoniae* by Kirby Bauer's disc diffusion following the guidelines of Clinical and Laboratory Standards Institute (CLSI).⁹ Antibiotics (HiMedia, India) included in the study were chloramphenicol (30 µg), cefotaxime (30 µg), ceftazidime (10 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), imipenem (10 µg), meropenem (10 µg), tetracycline (30 µg), co-trimoxazole (25 µg) and. Based on their zone of inhibition *K. pneumoniae* isolates were assigned as resistant, intermediate and susceptible. As a reference strain *Escherichia coli* ATCC 25922 was used.

Double Disk Diffusion to Assay Production of ESBL

Phenotypic confirmation of the ESBL production by potent ESBL producing *K. pneumoniae* from food samples was affirmed by the use of and by a combination of both ceftazidime (30 µg) and clavulanic acid (10 µg) placed 25mm away from each other.

Isolates of *K. pneumoniae* exhibiting ≥5 mm increase around combined clavulanate discs in comparison to the ceftazidime disc alone were confirmed to produce ESBL.

Molecular Confirmation

Isolates obtained were genotypically confirmed as genus *Klebsiella* and species *K. pneumoniae* by extraction of DNA from the isolates by cetyl trimethyl ammonium bromide (CTAB) as reported by Ausubel et al.¹⁰ and subjecting them to polymerase chain reaction. The gene *gyrA* was used as a target for the identification of isolates as genus *Klebsiella*, while the 16S rDNA -23S rDNA internal transcribed spacer (ITS) region was used for the species-specific confirmation. Details of the primers used is provided in Table S1.

Identification of Antibiotic Resistance Determinants and Genes Associated with Virulence

All the *K. pneumoniae* isolates were investigated for their respective antibiotic resistance determinants and virulence genes by PCR. Genes encoding for ESBL (*bla*_{TEM'}, *bla*_{CTX-M} and *bla*_{SHV}). Tetracycline resistance (*tetA*, *tetB* and *tetG*), plasmid mediated quinolone resistance (*qnrA*, *qnrB*, *qnrS*), chloramphenicol resistance (*catA1*), sulphonamide resistance gene (*sul1* and *sul2*) (Table S2).

Presence of nine different virulence genes *viz ureA* (urease), *uge* (epimerase), *wabG* (transferase), *kfu* (iron uptake system), *khe* (haemolysin), *bfp* (bundle forming pilli), *elt* (enterotoxin), *fimH* and *mrkD* (fimbrial adhesins) were investigated by PCR in the *K. pneumoniae* isolates (Table S3 and S4).

Biofilm Formation Assay 96-well Microtiter Plate Method

Biofilm forming ability of *K. pneumoniae* food isolates was assessed by microtiter plate method. To brief, (10 µl) *K. pneumoniae* isolates

grown overnight were allowed to form added biofilm by incubated them at 37°C for 24 h in wells containing LB broth (190 µl). Sterile distilled water was used to wash and remove the cells from the well. Methanol was used to fix the cells followed by crystal violet (1%) staining and incubation for 15 min. Glacial acetic (30%) acid was used to solubilize the stain adhering the cells forming biofilm, followed by measuring their absorbance at 630 nm. True OD values were calculated by deducting the control value. The isolates were classified as follows (i) $4 * O.D.c > O.D$ =strong biofilm former (ii) $2 * O.D.c < O.D \leq (4 * O.D.c)$ moderate biofilm former (iii) $O.D.c < O.D \leq (2 * O.D.c)$ weak biofilm former (iv) $O \leq O.D.c$ non-biofilm former.¹¹

Expression of Biofilm Related Genes

K. pneumoniae isolates were grown for 24 h at 37°C in a 6 well cell culture plate. As a negative control LB broth and as positive control *K. pneumoniae* ATCC 10031 were used following incubation, Trizol reagent (1 ml) was added to lyse the cells. Chloroform (200 µl) was added following centrifugation (12000xg at 4°C for 15 min). Isopropyl alcohol (0.5 ml) was used to precipitate the RNA from the aqueous phase. Ethanol (75%) was used to pellet the cells and was resuspended in nuclease-free water. Concentration and purity of the obtained RNA were examined by using a spectrophotometer (Biospectrometer, Eppendorf, Germany). The RNA samples obtained were stored (-80°C) for the synthesis of cDNA.

DNase Treatment and cDNA Synthesis

Thermo scientific kit was used to convert RNA to cDNA. To 0.5 g of the RNA sample reaction buffer (1 µl) with MgCl₂ (10 x) and DNase (1 µl) was added. DEPC- treated water was used to make up the volume to 10 µl. Incubation step for 30 min at 37°C following deactivation at a temperature of 65°C for 10 min. For cDNA synthesis the RNA samples obtained were normalized to 500 nanograms. Prime Script one-step reverse cDNA synthesis kit (TaKaRa, Japan) was used to reverse transcribe the RNA samples. The cDNA samples were stored at -20°C for the expression studies.

Real-time PCR (q-PCR) Studies to Quantify Associated Biofilm Genes in *K. pneumoniae*

Relative gene expression (*fimH*, *mrkA*,

and *mrkD*) of the biofilm forming genes in *K. pneumoniae* food samples were quantified. PRIMER 3 software was used to design primers for the biofilm-related genes in *K. pneumoniae* (Details of the primers used are given in Table S4). Relative expression of three target genes were standardized in comparison to the house keeping gene (*rpoA*). Standardization of primer concentration for all the biofilm genes was performed and used henceforth. The cycling conditions were as follows: initial denaturation of 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 10 sec, 55°C for 20 sec and extension of 72°C for 20 sec. Melt curve was analysed to exempt the amplification untargeted regions. Relative quantification of all the genes was calculated using the $2^{-\Delta\Delta Ct}$ formula.¹²

RESULTS

Isolation and Identification of *K. pneumoniae*

All the isolates formed lactose fermenting typical pink mucoid colonies on MacConkey agar. Gram-negative rods were observed, upon Gram staining. The isolates showed positive for capsular staining, Voges-Proskauer, citrate production and catalase test. Negative for indole, methyl-red, oxidase, motility test and spore staining.

Antibiotic Susceptibility Testing and ESBL Production

All the isolates tested for their antibiotic susceptibility were resistance towards ceftazidime and tetracycline. Among the 5 isolates resistance was observed towards cefotaxime (3), ciprofloxacin (2) and imipenem (1) respectively. Isolates exhibited intermediate susceptibility towards nalidixic acid (2), meropenem (2) and cefuroxime (3). All the isolates were sensitive to chloramphenicol and co-trimoxazole followed by imipenem (4). The antibiogram of the isolates is represented in Table (1).

All the *K. pneumoniae* isolates in the study were potent ESBL producers.

Genotypic Confirmation of *K. pneumoniae*

Genus confirmation of the isolates as *Klebsiella* was done by targeting the gene *gyrA*, while 16S rDNA -23S rDNA internal transcribed spacer (ITS) region of *K. pneumoniae* was targeted

Table 1. Isolation source, antibiogram and biofilm formation by *K. pneumoniae* food isolates

Isolates	Source of isolation	Biofilm forming ability	Antibiotic susceptibility Pattern									
			CAZ	CIP	C	COT	CXM	CTX	IPM	MRP	NA	TE
KP1	Chicken	Moderate	R	R	S	S	I	S	S	S	I	R
S1B	Tomato	Weak	R	I	S	S	S	R	S	I	S	R
S2B	Tomato	Weak	R	I	S	S	S	R	S	S	S	R
S9	Cucumber	Moderate	R	R	S	S	S	R	R	I	I	R
S13	Onion	Non	R	S	S	S	S	S	S	S	S	R

CAZ- ceftazidime, CTX- cefotaxime, CIP- ciprofloxacin, COT- co-trimoxazole, CXM- cefuroxime, C- chloramphenicol, IPM- imipenem, NA- nalidixic acid, MRP- meropenem, TE- tetracycline, R= Resistant, S= Sensitive, I=Intermediate.

Table 2. *K. pneumoniae* isolates harbouring ESBL encoding and antibiotic resistance determinants

Isolates	<i>bla</i> _{SHV}	<i>bla</i> _{CTX-M}	<i>bla</i> _{TEM}	<i>tetB</i>	<i>tetG</i>	<i>qnrA</i>	<i>qnrB</i>	<i>qnrS</i>	<i>catA1</i>	<i>sul1</i>	<i>sul2</i>
KP1	+	-	-	-	-	-	-	+	-	+	-
S1B	+	+	-	-	-	-	-	-	-	-	-
S2B	+	+	+	-	-	-	-	-	-	-	-
S9	+	-	-	-	-	-	-	-	-	-	-
S13	-	+	-	-	-	-	-	-	-	+	-

Table 3. Details of *K. pneumoniae* isolates carrying virulence genes

Isolates	<i>ureA</i>	<i>uge</i>	<i>wabG</i>	<i>kfu</i>	<i>khe</i>	<i>bfp</i>	<i>elt</i>	<i>fimH</i>	<i>mrkD</i>
KP1	+	+	+	-	+	-	+	+	+
S1B	+	+	+	+	+	-	+	+	+
S2B	+	+	+	-	+	-	+	+	+
S9	+	+	+	-	+	-	+	+	+
S13	-	-	-	-	+	-	-	+	+

to confirm the isolates as *K. pneumoniae*. All the isolates included in the study were confirmed as *K. pneumoniae*.

Detection of Antibiotic Resistant Determinants

All the ESBL producers in the study carried either one of the ESBL encoding genes. None of the isolates harboured tetracycline resistance genes nor the chloramphenicol resistance genes. One isolate each from chicken (KP1) and onion (S13) harboured the plasmid mediated quinolone resistance (*qnrS*) and sulphonamide resistance genes (*sul1*) respectively. Details of the antibiotic resistance genes harboured by the isolates is depicted in Table 2.

Determination of Virulence Genes

All the *K. pneumoniae* isolates in the study carried the genes *ureA*, *khe*, *fimH* and *mrkD*. None

of the isolates harboured the *bfp* gene. The genes *wabG*, *uge* and *elt* were detected in four of the isolates. One of the isolates from tomato (S1B) carried the *kfu* gene. Four isolates (KP1, S1B, S2B and S9) found to harbour multiple virulence genes in them. Virulence genes carried by each of the isolates is represented in Table 3.

Ability of the *K. pneumoniae* from Food Sources to Form Biofilms

Among the five isolates of *K. pneumoniae* from food sources, four of the isolates could form biofilms (KP1, S9, S1B, S2B). Of which two isolates could form moderate (KP1, S9) and weak (S1B, S2B) biofilm formers respectively. *K. pneumoniae* isolated from onion (S13) did not show any biofilm formation. Isolation source and their biofilm forming ability has been represented in Table 1.

Co-relation among Biofilm Forming Ability and Antibiotic Resistance of *K. pneumoniae* Food Isolates

In our study, the moderate biofilm formers (KP1, S9) were resistant to ciprofloxacin and tetracycline, while they exhibited sensitivity towards co-trimoxazole and cefuroxime respectively. On the other hand, weak biofilm formers (S1B, S2B) were found resistant to tetracycline, cefotaxime and ceftazidime, and sensitive to imipenem, nalidixic acid, co-trimoxazole and chloramphenicol.

Strikingly imipenem, co-trimoxazole, meropenem, nalidixic acid, ciprofloxacin, chloramphenicol, ceftazidime and cefuroxime were effective against the non-biofilm former (S13) in the study.

Quantification of Associated Biofilm Genes in *K. pneumoniae*

Relative gene expression of *fimH* (type I) and *mrkA* and *mrkD* (type III) fimbrial adhesins in the *K. pneumoniae* biofilm forming food isolates were analysed (Figure). Weak biofilm formers showed marginal level expression of all the three biofilm related genes. The gene *mrkD* which facilitates dense biofilm formation in *K.*

pneumoniae exhibited enhanced expression in the non and one of the moderate biofilm formers. The other moderate biofilm former in the study exhibited reduced expression of all the three fimbrial genes (*fimH*, *mrkA* and *mrkD*).

DISCUSSION

Klebsiella pneumoniae is an opportunistic pathogen encountered from diverse microbiological niches, for instance, food, soil, skin, intestine and faeces of mammals.¹³ The bacterium has been substantiated to cause pneumonia, bacteraemia and urinary tract infections.

Through the years, resistance towards broad spectrum drugs like the third and fourth generation cephalosporins and fluoroquinolones have been gradually acquired by *K. pneumoniae* prevalent in both community and hospital settings.¹⁴ Carbapenems, being the last line of resort has failed in treating the pathogen and is a matter of utmost concern.

The Centre for Disease Control and Prevention (CDC) stated millions of deaths occurring worldwide annually, is due to the foodborne pathogens. In addition, indiscriminate use of antibiotics in treating the foodborne

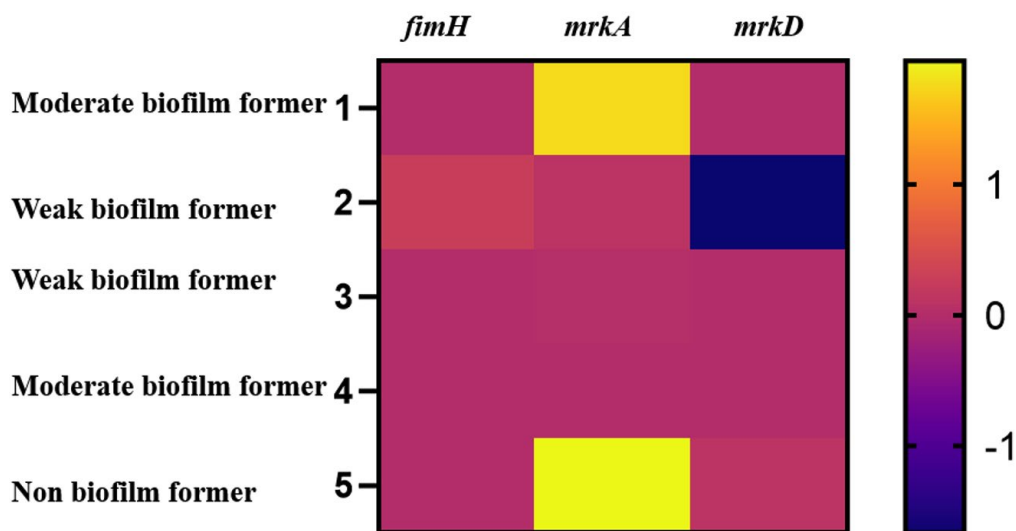


Figure. Expression levels of biofilm associated genes in the *K. pneumoniae* isolated from food sources. Levels of biofilm gene expression among the food isolates of *K. pneumoniae* is depicted by fold-changes transformed to log2 in comparison to the control. The fold-change (log2) value and the colour scale are indicated towards the right of the heat map.

diseases has increased the resistance offered by the pathogens, towards the antibiotics, eventually leading to ineffectiveness of the drugs.

The present study reports *K. pneumoniae* from raw vegetables and poultry sources resistant to multiple antibiotics, harbouring ESBL and virulence associated genes, capable of forming biofilms.

The food isolates of *K. pneumoniae* in our study exhibited higher rates of resistance towards third generation cephalosporins, ceftazidime and cefotaxime. Resistance to third-generation cephalosporins among *K. pneumoniae* isolates from food have also been reported in other studies.^{15,16}

Cephalosporins have a major role in the hospital settings as therapeutics to *Klebsiella* related infections. In comparison to other beta-lactam antibiotics, cephalosporins exhibit less sensitivity to inhibition by beta-lactamase enzymes, possessing a greater spectrum of activity.¹⁶ World health organization, has entitled the third generation cephalosporins into “watch” category of antibiotics, i.e., drugs crucial in treating bacterial infections in humans and thus must be given precedence as objective of antibiotic resistance investigation and stewardship bulletin.¹⁷

K. pneumoniae food isolates exhibiting tetracycline resistance were reported in some of the studies.^{15,18} In our study there was a discrepancy between the resistance pattern of tetracycline phenotypically and genotypically. Though all the isolates exhibited phenotypic resistance to tetracycline, the tested corresponding genes were not present. This could be due to several reasons, (i) In this study we could test for the presence of *tetB* and *tetG*, while the isolates could harbour other variants of tetracycline resistance determinants (ii) resistance towards tetracycline among these isolates could involve other mechanisms like enzymatic inactivation, efflux pumps, mutations in the plasmids etc (iii) resistance to a particular antibiotic phenotypically is due to several genetic mechanisms, reasons of which remain unknown.¹⁹ Over and above, *tet* genes have been proved to persist in the environments, even in the absence of selection pressure established by the constant use of tetracycline.²⁰

Finer understanding of the mechanisms of tetracycline resistance conferred by the

pathogens, could direct the design of modified next generation tetracyclines that could cautiously evade inactivation and maintain the efficacy against pathogens.

The jeopardization of antibiotic resistance could magnify by lack of awareness, self-medication and abuse of antibiotics. Confoundingly, all the isolates in the study were susceptible to chloramphenicol and co-trimoxazole, which is in line with the few studies involving *K. pneumoniae* from raw vegetables, meat and poultry dishes.^{13,21} Contrary to our findings few authors report complete resistance towards chloramphenicol and co-trimoxazole by the *K. pneumoniae* isolates from enteral diets.^{22,23} According to the findings of the current study, chloramphenicol and co-trimoxazole could be the drugs effective on *K. pneumoniae* isolates from food samples.

ESBLs are rapidly evolving plasmid borne enzymes that can catalyse monobactams and cephalosporins, thereby contributing to a major threat. It was exceptional to note that all the isolates were potent ESBL producers and carried at least one beta lactamase encoding gene (Table 2). Potent ESBL producing *K. pneumoniae* isolates from food sources were also reported by few other studies.^{15,24}

Dietary intake could be considered one of the possible routes for the introduction of antibiotic resistance genes in bacteria into human gut. Furthermore, such pathogens can transfer the antibiotic resistance determinants to the other strains.

Different food sources including the meat products could be a potential route through which the MDR and ESBL-producing strains of the *Klebsiella* species could be transmitted. The clinical importance of *K. pneumoniae* as a pathogen is prevalent, while its dominance as a public health hazard in food requires attention.

Presence of virulence factors *viz* siderophores, endotoxins and iron scavenging systems in the pathogen have collectively seem to have enhanced its pathogenicity. Assessment of virulence factors in our study suggested *ureA*, *khe*, *fimH*, *mrkD*, *wab*, *uge*, *elt* and *kfu* were some of the genes harboured among the *K. pneumoniae* isolates from food.

All the *K. pneumoniae* food isolates carried multiple virulence genes (Table 3). Other

reports also demonstrate multiple virulence genes in *K. pneumoniae* food isolates.^{25,26} These genes aid the bacterium evade the immune system causing diversified infections in animals and humans. One of the isolates in our study from tomato (S1B) harboured the gene *kfu*. The gene is identified to be involved with capsule formation, hypermucoviscosity virulent phenotype, tissue infections. The gene *kfu* is recognized as a pivotal gene in up take from the host cell.²⁷

Though the isolates in the current study were sensitive to few antibiotics, they were found to harbour virulence genes in them (Table 1 & 3). In order to improvise treatment and prevention of *Klebsiella* related infections, an epidemiological relationship amidst the food and clinical isolates must be scrutinized.

Foremost step in the establishment of infections by pathogens like *K. pneumoniae* is, their competence in forming biofilms on the surface of the host. Biofilm formation significantly scales down the activity of the antimicrobials making it less effective on the bacterium. Few distinctive genes involved in the formation of biofilms are type I fimbriae (*fimA* and *fimH*), type III fimbriae (*mrkA* and *mrkD*), allantoin (*allS*), capsular polysaccharide (CPS) (*k2A*, *wzyk2*, *treC*, *wabG*, *cpsD*, *wcaG*, and *wzc*), aerobactin (*iutA*), colonic acid (*wcaI*) and quorum sensing (QS) (*luxS*).²⁸ The type 1 fimbriae are significant in the establishment of urinary tract infections in bacteria like *E. coli* and *Klebsiella*.²⁹ Binding of the pathogen to the epithelial and endothelial cells of respiratory tract and urinary tracts is mediated by the type III fimbriae.³⁰

In our study, the moderate biofilm formers were from chicken and cucumber respectively. Weak biofilm formers were found to be from tomato and the non-biofilm former from onion.

The gene *mrkA* showed increased expression among the biofilm formers. It has been investigated that the expression of the type 1 fimbriae is regulated by the *fim*-switch regulatory mechanism that contains the promoter for the major fimbrial subunit *fimA*. Nullification in the expression of the type I fimbriae is compensated by the upregulation of the type III fimbriae and vice versa, indicating a strong cross-regulation of the two fimbrial gene clusters.³¹ Complementary

to the above hypothesis it was also suggested that the type I and type III fimbriae are able to compensate for each other and both promote biofilm formation.³² Mutational studies have demonstrated that the type III fimbriae have seem to promote biofilms in *K. pneumoniae*.³³ Whereas, type 1 fimbriae have been reported to aid formation of biofilm in *E. coli*. However, in the present study, there was no impact found on the formation of biofilms as a result of reduced expression of the fimbrial genes. Per contra, there is a contradictory in the conclusion with respect to the genes involved in the biofilm formation. Further studies are necessary to augment on the association between the biofilm formation and associated genes.

The investigations from the study favours the fact that *K. pneumoniae* is considered a commensal of the upper respiratory and the gastro-intestinal tract in both mice and humans. However, the ability of the food pathogen to produce ESBL, presence of multiple virulence genes aids the pathogen to evade and infect the GI tract is the most panicking. Multiple virulence genes harboured by the isolates might provide an explanation for infections caused by *K. pneumoniae* and a major threat for the microbiological security. Furthermore, the drug-resistant phenotypes of the *K. pneumoniae* from food samples make the treatment regimens firm.

Thereby, the present study aimed to generate knowledge on the prevalence, resistance genes, virulence and biofilm forming ability as a potential factor of vulnerability in the ESBL producing *K. pneumoniae* from raw vegetables and chicken available for human consumption.

CONCLUSION

Current study detects the presence of various virulence genes in the food isolates of *K. pneumoniae* conferring resistance to antibiotics. It is worrisome to observe drug-resistant and potentially virulent *K. pneumoniae* in foods that could be consumed raw (onion, cucumber and tomato). *K. pneumoniae* from chicken and vegetables exhibiting resistance to antibiotics is a state of concern, as choices exposed to their antibiotic treatment stands straitened, should these isolate cause infections in humans. On this

account, our study elucidates the significance in controlling the dissemination of antibiotic resistance determinants by judiciously prescribing antibiotics in the clinical settings, avoiding haphazard recommendation of antibacterial for treatments. Upgrading the hygiene and sanitation standards with appropriate food handling protocols and food safety practices would contribute towards the same.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.16.3.66>

Additional file: Additional Table S1-S4.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

ETHICS STATEMENT

Not applicable.

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