Occurrence of ESBL-producing *Klebsiella pneumoniae* in ready-to-eat street foods in Bouaké, Côte d'Ivoire

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ABSTRACT

Aims: This study aimed to estimate the occurrence of ESBL-producing *Klebsiella pneumoniae*(*K. pneumoniae*) in ready-to-eat street foods.
Study design:it's a prospective study
Place and Duration of Study:foods samples were collected from February 2016 to June 2017 from street vendors in Bouaké, Côte d'Ivoire
Methodology:A total of 300 foods samples were collected from February 2016 to June 2017 from street vendors in Bouaké, Côte d'Ivoire. After a microbiological analysis, bacterial identification was performed according to the conventional microbiological tests. Antibiotic sensitivity of *K. pneumoniae* isolates to 18 antibiotics was determined by the disk diffusion method and by VITEK 2. Phenotypic and molecular detection of producing extended Spectrum Beta-Lactamase (ESBL) was performed by Chromogenic medium and double disk synergy test and by PCR method.
Results: Four percentages (4 %) of foods samples were contaminated by *K. pneumoniae*. Most of the 12 *K. pneumoniae* strains isolated expressed resistance to the various classes of antibiotics used. Of the 12 strains, one was ESBL-producing, representing a prevalence of 8 %. This strain was isolated from cooked cow's milk and resistant with all 18 antibiotics tested except gentamicin and sulfamethoxazol-trimepothrim. This strain harbored only bla_{SHV} gene.
Conclusion: The results of this study indicate that RTE foods are a reservoir of resistant bacteria. Thus, they may play a role in spreading antimicrobial resistant bacteria and ESBL genes to humans.

Keywords: Ready-to-eat street foods, Klebsiella pneumoniae, antibiotic, resistance gene.

1. INTRODUCTION

"Street foods are ready-to-eat foods and beverages prepared and/or sold by street vendors, especially on the streets and in other similar locations. They represent an important part of the daily urban food consumption of millions of low- and middle-income consumers in urban areas"[1]."For many people with limited resources, street foods are often the least expensive and most accessible way to obtain a nutritionally balanced meal away from home, provided that the consumer is informed and able to choose the appropriate food combination"[1]. The preparation and selling of these foods provides a regular source of income for millions of people in developing countries, but whose education and skills in food processing are often limited, and who enter the business primarily to escape poverty, especially because it requires little initial investment. In Africa, this street food phenomenon has developed strongly over the last thirty years, under the combined effect of the rural exodus and the demographic growth of the countries. The labor pool has increased significantly, while the distance between home and work has become much longer. So, finding a solution to eat on the same site has become crucial.

“Nowadays, local authorities, international organizations and consumer associations are increasingly aware not only of the socio-economic importance of street foods but also of their risks. The main concern is food safety. The risk of food poisoning associated with street foods remains a threat in many parts of the world, with microbiological contamination being one of the major problems. It is recognized that foodborne pathogens pose a serious health danger, with the risk depending primarily on the type of food, and the method of preparation and storage”[2]. “The ignorance of street vendors about the causes of foodborne illness is a risk factor that cannot be ignored. Lack of hygiene, inadequate access to safe water supply and waste disposal, and unsanitary environments (such as proximity to sewers and garbage dumps) further increase public health risks”[2]. “An estimated 91 million people in Africa in a year consume contaminated food that renders them ill, and around 137,000 people die”[1]. Food contaminated with bacteria, viruses, parasites or harmful chemicals causes illnesses ranging from acute diarrhea to permanent conditions, including some cancers.
“Klebsiella pneumoniae is one of the bacteria responsible for foodborne infections”[3]. “*K. pneumoniae* is a natural member of the microbiome of the gastrointestinal tract of humans and healthy animals”[4]. “It is associated in extra-intestinal infections, including urinary tract infections, cystitis, pneumonia, surgical wound infections, and life-threatening infections such as endocarditis and sepsis. It is also an important cause of serious community-acquired infections such as necrotizing pneumonia, pyogenic liver abscesses and endophthalmitis”[4]. *K. pneumoniae* represents one of the most worrisome pathogens involved in antibiotic resistance. Antibiotic-resistant *K. pneumoniae* isolates have been isolated from a variety of foods including raw meat [5, 6], raw vegetables ([7, 8], fruit juices [9], and ready-to-eat foods [3, 10].
The presence of antibiotic-resistant strains of *K. pneumoniae* in the food chain and its potential contribution to the resistome, including resistance in clinically relevant bacteria, is a significant risk for human health because of its epidemiological importance. Hence the need to screen these ready-to-eat foods in order to limit their spread in the environment, especially in food. The objective of the present study was to determine antimicrobial resistance profiles of *Klebsiella pneumoniae* strains isolated from few ready-to-eat street foods.

2. METHODOLOGY

A total of 300 foods samples, consisting of 100 porridge, 100 cooked cow's milk and 100 samples of dêguê and juice, were purchased from February 2016 to June 2017 from street vendors in Bouaké, Côte d’Ivoire. After purchase, the samples were transported to the laboratory in sterile sachets, maintained under refrigeration (< 4 °C), and tested within 24 h.

2.1. Microbiological analysis

Once arrived at the laboratory, 1 ml of each sample was aseptically mixed into tube containing 9 ml of physiological water (10^{-1} dilution) and diluted serially up to a 10^{-4} dilution. Then, 1 ml of each tube of previously dilutions and of the original sample has put aseptically into sterile Petri dishes (Thermo Scientific™). Thereafter, 15 ml of Violet Red Bile Glucose Agar (~ 45°C) (Bio-Rad, France) were mixed with inoculums. After complete solidification, Petri dishes incubated at 37 °C for 24 h. The colonies grown were counted (30 to 300 colonies per dish).

2.2. Identification of isolates

Colonies of characteristic shape (4 mm of diameter, curved, shiny, opaque and often confluent) on Violet Red Bile Glucose Agar were subjected to Gram staining. After microscopic observation, colonies were plated on nutrient agar (Oxoid) and incubated at 37

°C for 24 h. Then, *K. pneumoniae* strains were confirmed using various biochemical tests such as H_2S and gas production, lactose, and glucose fermentation, mobility, catalase/oxidase/indole/urea production and mannitol/citrate utilization. Isolates were subsequently transferred to Homburg, Germany for confirmatory testing using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Maldi Biotyper, Bruker Daltonics; Bremen, Germany).

2.3. Antibiotic susceptibility profile of *K. pneumoniae* isolated strains

"The antibiotics susceptibility pattern of the isolates was determined using the disk diffusion method on Mueller-Hinton agar (Oxoid, England) and also by VITEK 2, bioMérieux; Marcy L'Étoile, France). Resistance patterns were interpreted according to break- points put forth by the European Committee on Antimicrobial Susceptibility Testing"[11]. Eighteen (18) antibiotics Oxoid Ltd (Basingstoke, Hampshire, UK) were used in this study such as ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), piperacillin/tazobactam (PTZ, 30/6 µg), cefalexin (CFX, 30 µg), cefuroxim (CXM, 30 µg), cefoxitin (FOX, 30 µg), cefotaxim (CTX, 5 µg), ceftazidim (CAZ, 10 µg), cefixim (CFM, 5 µg) cefepim (FEP, 30 µg), meropenem (MEM, 10 µg), imipenem (IPM, 10 µg) aztreonam (ATM, 30 µg), gentamicin (GM, 10 µg), ciprofloxacin (CIP, 5 µg), norfloxacin (NOR, 10 µg), trimethoprim/sulfamethoxazol (SXT, 1.25/23.75 µg), chloramphenicol (C, 30 µg).

2.4. Phenotypic detection of producing Extended Spectrum Beta-Lactamase (ESBL)

"The screening of *K. pneumoniae* strains producing ESBL was performed by Chromogenic medium (CHROMagar ^{TM} ESBL) and double disk synergy test"[12]. "The screening by Chromogenic medium was performed by direct inoculation of agar. Double disk synergy test was performed as recommended by the European Committee on Antimicrobial Susceptibility Testing"[11]. "The antibiotic discs used to perform this test are amoxicillin + clavulanic acid and the third generation Cephalosporins namely Cefotaxim (30 µg) and Cefixim (30 µg). Amoxicillin + clavulanic acid disc was placed at the center of the inoculated Mueller-Hinton agar petri dish whereas the cefotaxime and cefixim discs were placed at both sides (about 15 to 20 mm) of the amoxicillin + clavulanic acid disc. After incubation at 37 °C for 18 h, the enhancement of the zones of inhibition of any of the cephalosporin disc towards the clavulanic acid disc confirms the strains as an ESBL producer"[13].

2.5. Extraction of Bacterial DNA

"Klebsiella pneumoniae isolate DNA was extracted by the simple boiling method. Briefly, *Klebsiella pneumoniae* isolates were cultured on Mueller Hinton agar (Merck, Germany) and incubated at 37 °C. After 24 hours, one to five colonies were suspended in distilled water, and suspension was boiled for 30 min at 95 °C in water bath"[14]. The suspension was centrifuged at 14,000 rpm for five min and the supernatant transferred to filter columns. After a final centrifugation at 1200 rpm for five min, the supernatant was transferred to filter columns. After a final centrifugation at 1200 rpm for five min, the supernatant was transferred to a new microtube and stored at -20 °C.

2.6. Molecular characterization of ESBLs genes

Presence of bla_{TEM}, bla_{SHV} and bla_{CTX} genes was detected by simplex PCR using primers specific for each gene (Table 1). PCR amplification was performed in a reaction mixture of 25 µL, which contained 5 µL template DNA, 12.5 µL of 2x GoTaq® G2 Hot Start Colorless

Master Mix (Promega, Madison, USA), 2.5 µL of each primer (10 µM) and 2.5 µL nuclease free water. The amplification of all these resistance genes was performed in a thermal cycler (Applied Biosystems, Inc., CA) whose conditions consisted of an initial denaturation cycle of amplification at 94 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s, extension at 72 °C for 90 s, and a final cycle of amplification at 72 °C for 7 min. PCR products were analyzed on 1.5 % agarose gel at 115 V for 80 min in TBE 1X containing GelRed® Nucleic Acid Stain 10,000X using 100-bp DNA ladder (Promega, USA) as a size marker. The visualization of the bands was done under UV (ultra violet) in a Gel Documentation System. All positive PCR products were sent to the BGI Corporation, Ltd (Hong Kong, China) for sequencing. The nucleotide sequences were analyzed with the basic local alignment search tool online.

Table 1. Primers used for the detection of ESBL genes

Genes	Primers	Sequences (5'-3')	Size (pb)	References
***bla*$_{TEM}$**	TEM-F	ATGAGTATTCAACATTTCCGTG	840	[15]
	TEM-R	TTACCAATGCTTAATCAGTGAG		
***bla*$_{SHV}$**	SHV-F	TTTATGGCGTTACCTTTGACC	1051	[16]
	SHV-R	ATTTGTCGCTTCTTTACTCGC		
***bla*$_{CTX}$**	CTX-F	TTTGCGATGTGCAGTACCAGTAA	544	[17]
	CTX-R	CGATATCGTTGGTGGTGCCATA		

3. RESULTS

3.1.Street foods contamination by mesophilic Microorganisms

The rate of street foods contamination by mesophilic microorganisms showed that, cooked cow's milk samples are more contaminated by mesophilic microorganisms ($1.5.10^3$ UFC/mL) followed by “dêguê-juice” ($0.092.10^3$ UFC/mL). However, no microorganism was isolated from the porridge.

3.1.2. Street foods contamination by *K. pneumoniae* strains

Four percentages (4 %) of foods samples (300) were contaminated by *K. pneumoniae*. Twelve strains of *K. pneumoniae* consisting of ten strains from cooked cow's milk (10 %) and two strains from dêguê and juice (2 %) were isolated.

3.1.3. Susceptibility of *K. pneumoniae* strains to antibiotics

The susceptibility of 12 *K. pneumoniae* strains isolated from different street foods was variable according to the 18 tested antibiotics (Figure 1). *K. pneumoniae* strains expressed strong resistance to antibiotics of 1st and 2nd generation cephalosporin such as Cefalexin (83 %) and Cefuroxim (83 %). Strong resistance was too observed with the fluoroquinolones antibiotics as Norfloxacin (92 %) and Ciprofloxacin (92 %). Low resistance (8 %) was observed with ceftazidim, cefepim, cefixim, cefotaxim, aztreonam, chloramphenicol and with

carbapenems as meropenem and imipenem. As for amoxicillin + clavulanic acid and piperacillin + tazobactam, 3 % of strains were resistant. However, gentamicin was effective on all strains.

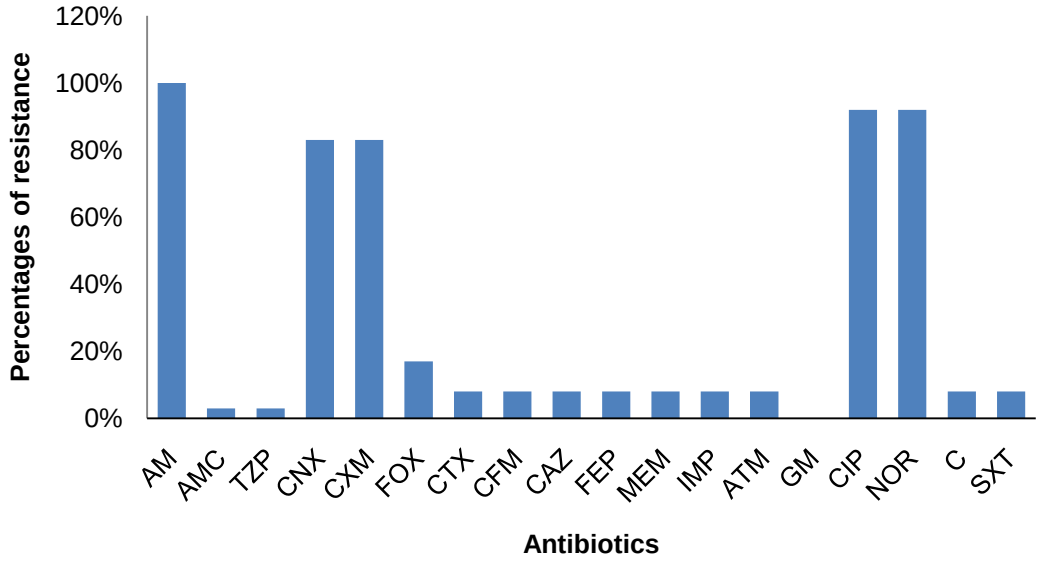

Figure 1. Resistance profile of *K. pneumoniae* strains isolated from street foods
FEP : cefepim, ATM : aztreonam, CTX : cefotaxim, CAZ : ceftazidim, AMP : ampicillin, AMC : amoxicillin- clavulanic acid, IPM : imipenem, FOX : cefoxitin, CFM : cefixim, MEM : meropenem, CFX : cefalexin, PTZ : piperacillin-tazobactam, GM : gentamicin, NOR : norfloxacin, CXM : cefuroxim, C : chloramphenicol, CIP : ciprofloxacin and SXT : sulfamethoxazol-trimepothrim.

3.1.4. Prevalence and pattern susceptibility of ESBLS-producing strains

Among the 12 isolated *K. pneumoniae* strains, one produced ESBL, a prevalence of 8 %. It was isolated from cooked cow's milk (figure 2 and 3.). This strain was resistant with all 18 antibiotics tested except gentamicin and sulfamethoxazol-trimepothrim.

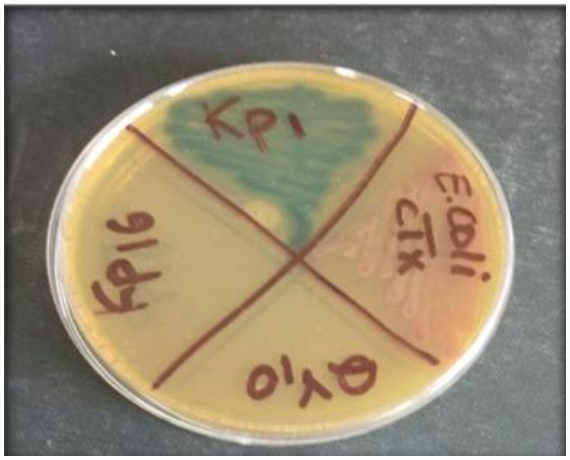

Figure 2. Production of ESBL on CHROMagar ESBL medium
QV 10 : ESBL négatif control ; E. coli CTX : ESBL positif control ; Kp1 et Kp16 : Klebsiella pneumoniae test *strains*

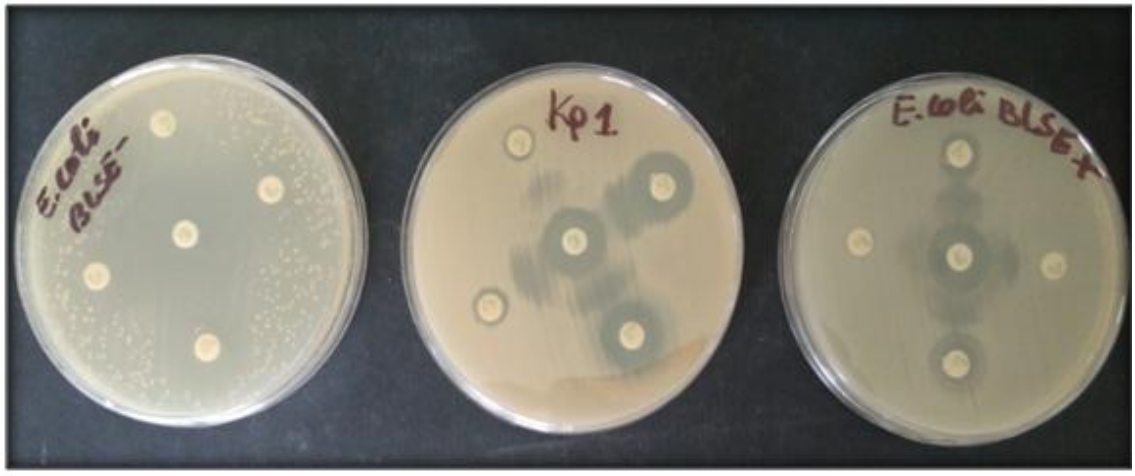

Figure 3. Appearance of ESBL production (picture in the form of a champagne cork)on Mueller-Hinton medium
E. coli BLSE- : ESBL-negative Escherichia coli strain ; Kp1 : K. pneumoniae strain to test ; E. coli BLSE+: ESBL-positive Escherichia coli strain

3.1.5. Molecular characterization of ESBLs genes

The total DNA of ESBL-producing strain, were used to explore the presence or no of the *bla*$_{TEM,}$ *bla*$_{SHV}$and *bla*$_{CTX-M}$ genes. This investigation showed that this strain carried the *bla*$_{SHV}$gene (figure 4).

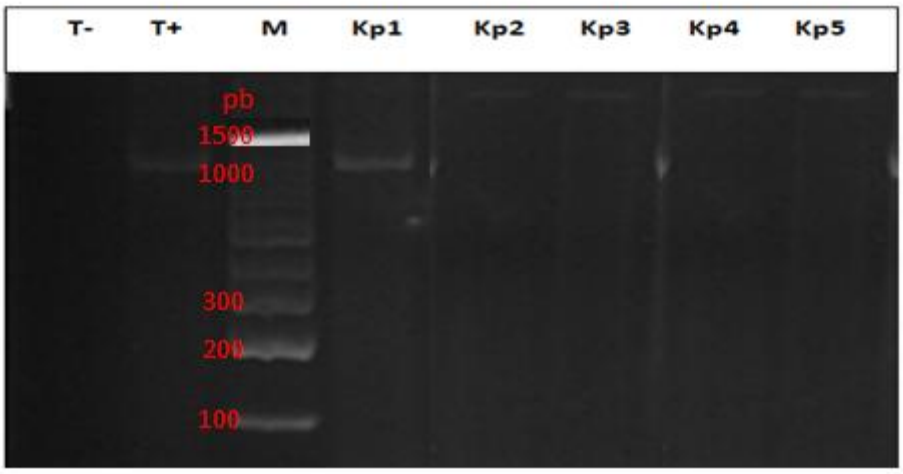

Figure 4. Agarose gel electrophoresis of PCR SHV genes of food isolates of *Klebsiella pneumoniae.*

Lane M: DNA Marker (100-1500 bp), T+: positive control, T-: negative control, lane Kp1: positive strain (1051 pb)*; lanes Kp2, Kp3, Kp4 and Kp5: negative strains.*

4. DISCUSSION

This study attempted to examine the bacteriological quality of ready-to-eat street foods at selected vending few places in Bouaké, Côte d'Ivoire. Accordingly, 28 % (83/300) of investigated ready-to-eat street foods are contaminated by mesophilic microorganisms. A low rate of ready-to-eat street food contamination (28 %) by mesophilic microorganisms was revealed in this study. This rate found in our study was different from those reported in Ethiopia [18] and Saudi Arabia[19], whichwere of 52% and 80.73% respectively.Such variations are largely attributed to differences in food contents, methods of preparation, personal hygiene, ways of handling and serving practices of vendors. The diversity in the environments/climatic conditions could be additional explanation for the observed differences.

Among these microorganisms, we found *K. pneumoniae*. The presence of *K. pneumoniae* in various ready-to-eat street foods such as leafy vegetables, chocolate drink, macaroni, and popsicles has also been reported in Ghana by [20] and in India by [10]"in vegetarian and non-vegetarian salads, different sandwiches, a variety of chutneys, sauces and dressings, instant noodles and pasta, vegetarian and non-vegetarian fritters, paani puri water and potato mix (a local Indian street food), mayonnaise, samosas and cutlets, chaats like bhelpuri (mixture of puffed rice with sweet and sour chutney) and potato chaat (mixture of potatoes with green and red chutney, curd, cut vegetables and some dry masala powder), patties with the filling of eggs/ vegetables /chicken, chicken sausages and salami, wet and dry pickles, fruits and vegetable juices, cakes and muffins, and different types of cheese". Its presence has also been reported on hamburger in Houston, Texas by [21], in dried bush okra (Corchorus olitorius) and leafy vegetables (Cleome gynandra) in Botswana by Mpuchane and Gashe (1996), and in fruit juice sold in Tripoli by [9]. This same finding was also reported by [18] in Ethiopia in various street foods such as local bread ('ambasha' and 'kita'), raw fish, chilli ('awaze'), avocado and cooked potato. Factors contributing to food contamination could be contaminated hands of the vendor, unhygienic environment where food is prepared and sold, such as sewage runoff in open gutters and inadequate waste disposal system [18]. This observation has also noticed by [22]to Ghana and [23] to Bangladesh. The lack of hygiene in the commercialization process of street foods leads to the microbial foodborne disease that can reach one or more people at a time. The lack of formal education and food service training for the majority of vendors are factors that may influence their ignorance of the impact of good handling practices on the quality of food for Human consumption [24]. Furthermore, the presence of *K. pneumoniae* in food indicates a potential health risk, so cross-contamination to other ready-to-eat products sold on the street is possible with this bacterium. In this regard, food handlers must be trained in food safety and personal hygiene to know the precautions to take to avoid any form of contamination.

A low rate of ready-to-eat street food contamination (4 %) by *K. pneumoniae* was observed in this study. This rate is lower than those obtained in the studies of[3]to Benin, [18] in Ethiopia, [19] in Saudi Arabiaand [10] in India had shown respectively the rate of 20, 10, 21 and 32 %, respectively. This difference in levels could be due to variation in the type of food samples investigated as reported by [3]. All twelve *K. pneumoniae* strains isolated were resistant to at least 3 antibiotic families. The presence of antibiotic-resistant pathogens in street vended foods has been mentioned by several authors notably [3]to Benin, [10] in India and [18]in Ethiopia. "Food stained with antibiotic-resistant bacteria is a threat to public health as it encourages persistence and dissemination of resistance determinants. Multiple resistances of the isolated strains could be due to laxity in the enforcement of public health measures. Of the 18 antibiotics tested, only Gentamicin was effective on all *K. pneumoniae* strains. The intramuscular mode of administration possibly could be limits the use of these

antibiotics and thus contributes to the preservation of the antimicrobial activity"[25]. In the present study, the frequency of antibiotic resistance to oral antibiotics was high and ranged from 92 % for fluoroquinolones to 3 % amoxicillin + clavulanic acid. The high level of resistance to oral antibiotics is possibly due to availability and convenient mode of administration.

Of these 12 *K. pneumoniae* strains isolated, one was ESBL-producing, representing a prevalence rate of 8 %. Contrary to most of the studies screening for ESBL producers, our study reports lower prevalence of ESBL in *K. pneumoniae*[10, 19, 26, 27]. However, in the studies by [3], "no strain was ESBL-producing". "Primary source of ESBL producers in this study was cooked cow's milk. Presence of cooked food sample harbored ESBL was reported" by [28]. "Presence of ESBL producing bacteria in cooked foods is of public health significance. It has been proven from the previous studies that the foods are a transmission vector for ESBL producing bacteria, probably from reservoirs, food animals and food handlers and once infected it can cause an outbreak"[29, 30]. In addition to ESBL resistance, ESBL strain was also resistant to fluoroquinolones and especially to carbapenems. This represents a danger for human health because carbapenems are antibiotics of last resort.

"Screening for the presence of ESBL genes revealed the presence of bla_{SHV} gene. *K. pneumoniae* isolates from ready-to-eat foods could be ingested by consumers. These *K. pneumoniae* isolates might subsequently colonize humans or might transfer resistance genes to other bacteria during passage through the intestinal tract"[31]. The presence of the ESBL genes CTX-M, TEM and SHV in food samples has been reported in Saudi Arabia[19].In other previous studies, bla_{SHV} gene was isolated from human clinical strains [32-34]. This would suggest human contamination of food. Genetic homogeneity among isolates of human and food origin was showed by [35]) in their study.

5. CONCLUSION

RTE foods are a reservoir of resistant bacteria. Thus, they may play a role in spreading antimicrobial resistant bacteria and ESBL genes to humans. In this regard, food handlers must be trained in food safety and personal hygiene to know the precautions to take to avoid any form of contamination.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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