Iraqi Journal of Veterinary Sciences

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Molecular characterization of extended spectrum beta-lactamase producing *Klebsiella pneumoniae* isolated from cows in Mosul city, Iraq

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Article information

Article history:
Received June 6, 2021
Accepted July 11, 2021
Available online February 8, 2022

Keywords:
Klebsiella pneumonia
ESBLs
Beef

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Abstract

Cows are considered as reservoir hosts for many pathogenic bacteria that are resistant to broad-spectrum beta lactamase (ESBL). Presence of ESBL resistant *K. pneumoniae* in nasal of cows and beef meat constitutes a risk for public health due to transfer of antibiotic resistance gene from cows to environment, humans and farm animals. Therefore, the current study was concluded the detection of molecular characteristics of ESBL producing *Klebsiella pneumoniae* that was isolated from both cow's nostrils and from local beef samples. Fifty nasal swabs were collected from farms cows in Mosul city, and 50 samples of beef from local butcher shops for the period from February to August 2020. Bacterial isolation and identification tests were conducted for ESBL resistant *Klebsiella pneumoniae* using MacConkey agar medium with 1 µg/ml cefotaxime. PCR was carried out to confirm the results using special primers SSKP1F and SSKP1R for *Klebsiella* of the target gene 16srRNA. Then, a molecular examination was performed using the precursors CTX-M, TEM and SHV. Through bacterial isolation, 36(72%) and 28(56%) isolates were belonging to ESBL resistant *Klebsiella pneumoniae* from nasal samples and beef meat respectively. The CTX-M, TEM and SHV genes formed 100, 89.2 and 85.7% and 100, 72.2 and 71.4% for each the meat and nasal samples respectively. This study showed that cows play a major role in transferring ESBL producing *Klebsiella pneumoniae* from cows to humans as a result of environmental handling or consumption contaminated meat.

DOI: 10.33899/ijvs.2021.130341.1803,

Introduction

Cows are considered a potential reservoir for bacteria that resistance against antibiotic, which lead to cause human infection. Cows may be exposed to antibiotics resistance from various ways such as contact with humans and feeding on raw feed, Thus, the prevalence of resistance against antibiotics between livestock can reflect the bacterial distribution among humans, animals and the environment, therefore it is necessary to know the effect of animals as potent source for the prevalence of pathogenic bacteria that are related in public health (1). Beef is one of the most important sources of animal protein that essential to human nutrition, and it is one of the most consumed types of meat globally, and formed an essential component of the food chain. Many animals, including cows, carry *K. pneumonia*e naturally in the nostrils and intestine, which leads to lung invasion when predisposing factor are available to infection, thus cause high risks to meat production and represent a great threat to public health as it is a reservoir of *Klebsiella* spp**.** strain that resist to many antibiotics which cause human infection. *K. pneumonia*e causes severe infections as pneumonia, urinary tract infection and otitis in humans even in developed countries, and it is associated frequently with mastitis and pneumonia in cows (2,3). The increasing use of broad beta-lactam as treatment in field of veterinary medicine has cause the proliferation of genetic determinants, especially in

Klebsiella spp., ESBLs are enzymes that degrade beta-lactam antibiotics and thus can influence on penicillin resistance, 3rd and 4th generation of cephalosporin's, in addition to aztreonam and monobactams that considered actively against Enterobacteriaceae (4,5). *K. pneumoniae* are Grams negative, capsule forming bacteria, belong to the family Enterobacteriaceae. *Klebsiella* spp. are one of the bacteria that has the ability to acquire resistance genes, and because cows are natural hosts of *Klebsiella* spp., Therefore, human acquired infection has been related to consume of impure meat or handling, also the ESBLs producing *K. pneumoniae* can be contaminated the meat when operating methods and hygiene practices are not applied accurately in slaughterhouse and farms (6,7). Recent studies focused to compare *Klebsiella* spp. from different source to evaluate the potent of the foodborne pathway to human (8,9). ESBLs *K. pneumoniae* were first reported in 1983 in Germany, ESBLs enzymes genes are commonly found in each the plasmids and chromosomes in species of Enterobacteriaceae (5). The *bla*CXT-M, *bla*TEM, *bla*SHV genes are the most common resistance determinants of *K. pneumoniae.* ESBLs producing *K. pneumoniae* isolates possessing these resistant agents especially *bla*CXT-M that lead to severe complications in humans if they consume polluted animal products (10,11). CTX-M enzymes (cefotaximases) are recently discovered, it is the more effective enzyme that related to resistance, these enzymes are carried on plasmid and considered the rapid growing ESBLs family (12). Chromosomally encoded SHV-1 β-lactamase are present in the most *K. pneumonia*e strains (13). The mutations of CTX and SHV genes were responsible for producing these enzymes in family Enterobacteriaceae (14) Klebsiella species were difficult to eradicate due to fastly converted to ESBL resistant bacteria. The TEM-genes are carried on plasmid that created by mutations in classic genes of TEM by substitution a single amino acid or multiplier around the active site of enzyme (4,9).

Rarely study for ESBLs producing *K. pneumonia*e in Iraq, therefore Current study aimed to know the molecular characterization of ESBLs producing *K. pneumonia*e that isolated from nasal swabs of farms cows and beef meat from the supermarket and butchers and detect the ESBLs gene *bla* SHV, CTX -M and TEM.

Materials and methods

Sampling

A total of 50 nasal swabs from cows and 50 samples of beef meat were collected from butcher's shops in Mosul city in the period between February to August. The samples were transported under cooled and sterile condition to Microbiology lab /College of Veterinary Medicine, University of Mosul. All Nasal swabs were inoculated on MacConkey agar (OXOID) supplemented with -1 µg/ml of cefotaxime (15), then incubated the plates for 24 h at 37°C. While 25g of meat samples were prepared by using a stomacher homogenizer then a swab from homogenized meat were taken and incubated for 18 h at 37°C in Nutrient Broth. One loopfull of inoculated broth was cultivated on MacConkey agar with 1 µg/ml cefotaxime (24 h at 37°C) as a selective medium for the resist bacteria to cefotaxime (14,16).

Identification of *K. pneumoniae*

K. pneumoniae isolates were identified microscopically by classical stains (Gram and capsule stain) and based on colonies morphology on MacConkey agar. Biochemical tests were used for suspected colonies included urease, oxidase, catalase test, H_2S production and citrate utilization test, these tests were done according to McFadden (17).

Extraction of DNA

Twenty -four hr. culture of positive ESBLs *K. pneumonia*e colonies on BHIA (HIMEDIA) were suspended in 1.5 ml Eppendorf tubs and used for DNA extraction, according to the DNA Preparation Kit were supplied by (Jena Bioscience, Germany). Then stored the extracted DNA under -20°C for following using (18,19).

PCR assay procedure

All primer sequences used for PCR and thermocycling conditions which is used in current study were listed in (Table 1). All DNA samples of *k. pneumoniae* were tested for 16S rRNA gene by using the (*K. pneumoniae*: SSKP 1 F and SSKP 1 R) primers (19).

CTXM, SHV, TEM genes were confirmed by using (CTX-M, SHV, TEM) Uni-f and Uni-r primers. The PCR reaction mixture for all protocols were done according the manufactured company instructions.

2XTaq Premix (12.5 µl of HS Prime) that supplied by (GeNet Bio, Korea). Thermal cycler (Bio-Rad, T100, Bio-Rad - USA) was used for PCR, the cycling conditions were done as shown in table 1. 1.2% (of (Promega - USA) agarose gel that contain the Prime Safe Dye by (Ge, Net-bio, Korea) was used to separate PCR products by electrophoresis.

The condition of electrophoresis includes (75 V, 300 mA ,50 min by using the system of (Wide Mini -Sub Cell GT). After that, the gel was noticed by using (Bio-Rad-USA) (Gel doc Ez) system to revealing the specific bands.

Table1: primer sequences for PCR and thermocycling conditions

Primers	Sequence (5′-3′)	size (bp)	PCR conditions	Reference
KP-16S rRNA-f KP16S rRNA-r	GGAACTGAGACACGGTCCAG CCAGGTAAGGTTCTTCGCGT	770	1 cycle (10) min at 94°C, (35) cycles (30) second at 94°C, annealing at 57°C for30sec.one cycle 5min at 72°C.	19
CTXM-f CTXM-r	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	One cycle ,5 min, (94°C). (35)cycles, 30 sec (94°C) , annealing at (54°C) for 30 sec. (1)cycle 5 min (72°C).	19
SHV-f SHV-r	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	753	One cycle ,5 min, (94°C), (35)cycles 30 sec at (94°C), annealing temp (57°C)for 30sec and one cycle 5min (72°).	20
TEM-f TEM-r	AAACGCTGGTGAAAGTA AGCGATCTGTCTAT	822	One cycle ,5 min, (94°C), (35)cycles 30 sec (94°C) , annealing (45°C)for 30sec, and(1)cycle 5min (72°).	2

Results

Through bacterial isolation of the ESBL resistant *K. pneumoniae,* we obtained 36 (72%) and 28 (56%) isolates from nasal swabs and beef meat respectively (Table 2).

Table 2: The number and percentage of *K. pneumoniae* isolated from beef meat and nasal swabs of cows

Samples	No. of isolates	Percentage %
Nasal swabs	36/50	72
Beef meat	28/50	56
Total	64/100	64

All *Klebsiella pneumoniae* suspected isolates were confirmed by PCR by using 16sRNA were give positive result 100% as shown in figure 1.

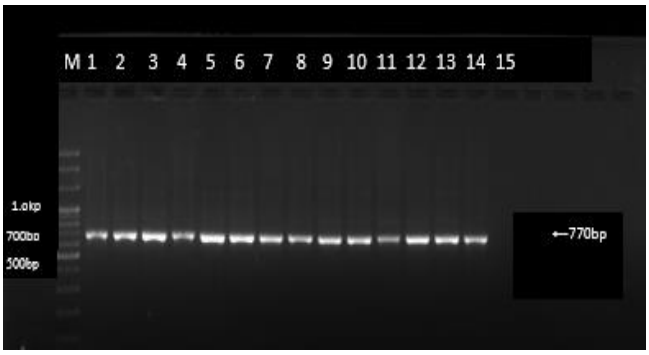

Figure 1: Gel electrophoresis of PCR final products for *Klebsiella pneumoniae*. Lane M= DNA marker 100 bp. Well 1-14 positive K. *pneumoniae* samples giving, 770 bp product size. Well 15 negative control.

Regarded PCR products for universal CTX-M gene the result showed that 100% of samples from beef and nasal cows were possess this gene as in figure 2.

While the percentage of SHV-gene formed 89.2%, 72.2% from all isolates of beef and nasal samples respectively as shown in the figure 3. TEM gene formed 85.7%, 71.4% beef and nasal samples which observed in the figure 4.

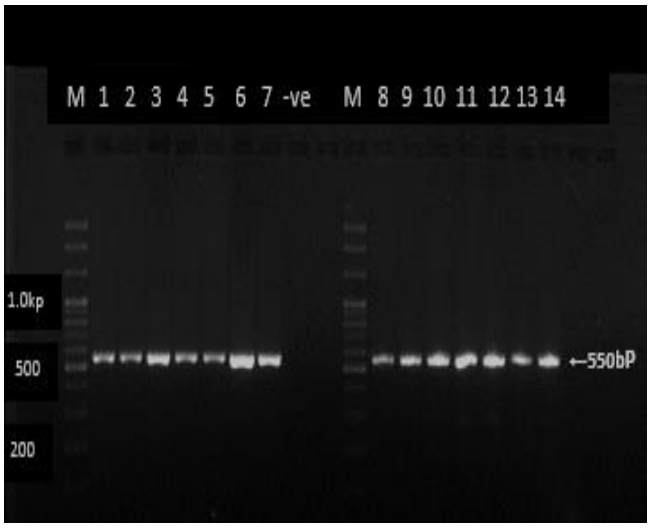

Figure 2: PCR products for universal CTX-M gene. Lane M, DNA marker. Well 1-7 positive tested meat samples and Well 8-14 positive tested nasal caws samples, 550 bp product size. -ve: negative control.

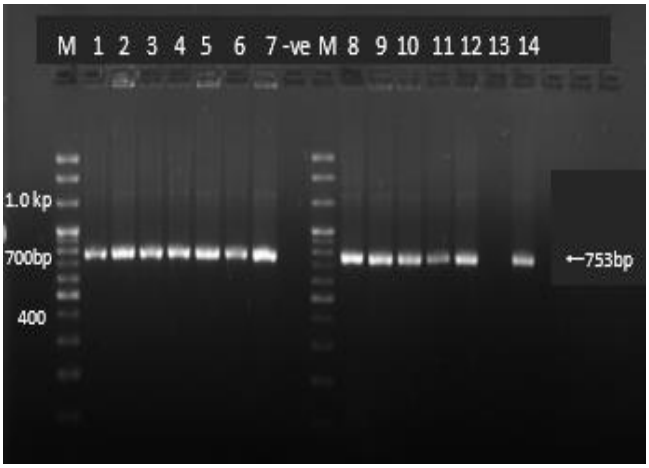

Figure 3: PCR products for universal SHV gene. Lane M, DNA marker; Well 1-7 positive tested meat samples and well 8-14 positive tested nasal caws samples giving 753 bp product size. -ve negative control.

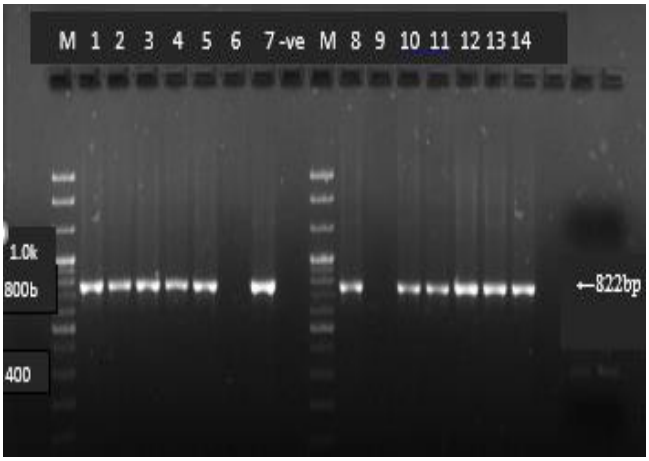

Figure 4: PCR products for universal TEM gene. Lane M, DNA marker. Well 1-7 positive tested meat samples and well 8-14 positive tested nasal samples, 822 bp product size. -ve negative control.

Discussion

Cows are considered as reservoir hosts of bacteria that are resistant to broad-spectrum beta lactamase. Therefor increasing consumed of beef meat are formed high risk for human worldwide, the prevalence of resistance to antibiotics between cows reflect the bacterial distribution among animals, humans, and the environment (1,2). Through bacterial isolation of the ESBL resistant *K. pneumoniae* from 50 beef meat samples and 50 nasal swabs, we obtained 36 (72%) and 28 (56%) for nasal swabs and beef respectively, these finding are closely with results of Klaif *et al.* (8) who isolated *K. pneumoniae* from beef at percentage 38 (76%), While Das *et al.* (21) isolated it at percentage 21.43%, from beef. Also ESBLs resistant *K. pneumoniae* isolates constituted 15% from the abattoir meat samples as in the publication of (21).

Current study showed that 72% of nasal samples were positive for ESBLs producing *K. pneumoniae*, these finding disagreements with the results of Bradford (22) who obtained 33/213 (15.5%) of nasal swabs samples for these bacteria. All ESBLs producing *K. pneumonia*e isolated from meat and nasal samples were confirmed by molecular diagnosis with universal 16sRNA gene.

For detection the ESBLs genes this study showed that all meat and nasal swabs isolates were carried the CTX-M gene 100%. Many studies indicated that CTX-M gene is the most predominant gene and formed the high percentage in nasal swabs of cattle and animal's products as meat and milk (19,23). This finding is in identical with the result of (21), who obtained the *bla* CTX-M gene at percentage 100% from beef samples. Also Bariz et al. (6) and Anbazhagan *et al.* (24) showed that all ESBLs producing K. *pneumonia* strains were tested possess CTX-M gene from clinical samples. The *bla* CTX-M gene is represent the more incidence of ESBLs type in cattle in twenty countries, and CTX-M1, CTX-M14, CTX-M15 are formed the higher prevalence for CTX-M (2). Therefore, the emergence the ESBLs producing bacteria and spreading worldwide are cause a risk to human and animals.

In present study the SHV gene formed 89.2 ,72.2% from cow meat and nasal isolates respectively. While TEM gene formed 85.7% and 71.4% from cow's meat and nasal isolates respectively. These results are relatively variable with the finding of Ugwu *et al.* (23) who isolated ESBLs producing *K. pneumoniae* from beef at percentage 21.43% but *bla*SHV gene formed 77.97%, and *bla*TEM gene formed 55.93% for these bacteria (24). While the results of Babini and Livermore (25) indicated that ESBLs genes formed 93.4% of *K. pneumoniae* strains, and the *bla*TEM gene was the most frequent 94% then *bla* CTX-M 57.6% and *bla*SHV 39.4% but Shin *et al.* (1) indicate that *bla*SHV 94.1% and TEM 100% were the most frequent ESBL genes were diagnosed in *K. pneumoniae* strains in companion animals.

The variation in results in most studies are belonging to the production of ESBLs genes are differed regionally, and may be related to highly used of ESBL antibiotic, or the methods that choice for detect them (20). Generally, *K. pneumoniae* are considered as neglected pathogenic bacteria in veterinary medicine, environmental health, and it is constituted a dangerous for human infection related with cows contact and meat consumption. Therefore, the distribution of ESBLs-producing bacteria is constituting source of concern and a highly risk to public health and it is necessary to know the role of cows as a potent source for the spreading the pathogenic bacteria to humans and other animals (2,3).

Conclusion

Cows are considered the one of the most important source for distribution of ESBLs producing *K. pneumoniae* to human due consume contaminated meat and by handling, being a carrier of broad spectrum antibiotic resistance genes.

Acknowledgments

I would like to thanks the Microbiology Department-College of Veterinary Medicine-University of Mosul-Iraq for the facilities they provided to me to conduct the research.

Conflict of interest

I declare that no conflict of interest.

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التوصيف الجزيئي لجراثيم الكلبسيلا الرئوية المنتجة للبيتالا كتاميز الواسعة الطيف المعزولة من الأبقار في مدينة الموصل، العراق

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الخلاصة

تعد الأبقار مضيفا خازنا للعديد من الجراثيم المرضية المقاومة للبيتالاكتام ذات الطيف الواسع. إن تواجد الكلبسيلا الرئوية المقاومة للبيتا لاكتام ذات الطيف الواسع في مناخير الأبقار ولحومها يشكل خطرا على الصحة العامة نتيجة لنقل جينات المقاومة من الأبقار إلى البيئة والأنسان والحيوانات الحقلية الأخرى. تناولت الدراسة الحالية التحري عن الصفات الجزيئية لجراثيم الكلبسيلا الرئوية المقاومة للبيتا لاكتام ذات الطيف الواسع المعزولة من مناخير الأبقار ولحومها. جمعت ٥٠ عينة من مناخير الأبقار في الحقول التابعة لمدينة الموصل و٥٠ عينة من لحوم الأبقار من محلات القصابة المحلية للفترة من شباط إلى أب ٢٠٢٠. أجريت فحوصات العزل الجرثومي والتوصيف لجراثيم الكلبسيلا الرئوية ذات الطيف الواسع باستخدام وسط أكار الماكونكي المضاف له السيفوتاكسيم ١ ميكروغرام/مل. اجري فحص البلمرة المتسلسل لأثبات نتائج العزل باستعمال بادئات خاصة لجراثيم الكلبسيلا الرئوية SSKP1F and 1R للجين 16srRNA.ثم اجري فحص جينات المقاومة باستخدام البادئات CTX-M، TEM و SHV ومن خلال العزل الجرثومي تم الحصول على ٣٦(٧٢ %) و ٢٨ (٥٦%) عزلة تعود لجراثيم الكلبسيلا المقاومة للبيتا لاكتام ذات الطيف الواسع من لحوم الأبقار ومناخيرها على التوالي. ظهرت جينات CTX-M، TEM و SHV بنسبة ١٠٠، ٨٩,٢، ٧٥,٧% و ١٠٠، ٧٢,٢، ٧١,٤% لكل من عينات اللحم والمناخير على التوالي. أوضحت هذه الدراسة أن الأبقار تلعب دورا رئيسا في نقل الكلبسيلا الرئوية المقاومة للبيتا لاكتام ذات الطيف الواسع من الأبقار إلى الأنسان نتيجة التعامل معها في البيئة أو عن طريق استهلاك اللحوم الملوثة.