

# Prevalence of Extended Spectrum Beta Lactamase (ESBL) Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Raw Milk Samples in Al Jazirah State, Sudan

Ali M Badri<sup>1\*</sup>, Ibrahim T Ibrahim<sup>2</sup>, Sameer G Mohamed<sup>3</sup>, Mohamed I Garbi<sup>2</sup>, Ahmed S Kabbashi<sup>2</sup> and Mohamed H Arbab<sup>2</sup>

<sup>1</sup>Molecular Biology Research Lab, International University of Africa, Khartoum, Sudan

<sup>2</sup>Department of Microbiology, Faculty of Medical Laboratory Sciences, International University of Africa, Khartoum, Sudan

<sup>3</sup>Medical Laboratory Sciences, International University of Africa, Khartoum, Sudan

\*Corresponding author: Ali M Badri, Molecular Biology Research Lab, International University of Africa, P.O. Box 2469, Khartoum, Sudan, Tel: +249922233425; E-mail: ali.almhasi@gmail.com

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## Abstract

Milk play a major role in human sources of nutrition and remain as the most important prominent in the Sudanese diet. *Escherichia coli* and *Klebsiella pneumoniae* are humans and animals opportunistic pathogens, responsible for a wide range of infections. The aim of this study was to evaluate the quality of the commercial available milk and to detect ESBL producing *E. coli* and *K. pneumoniae* from raw milk samples of cow in Al Jazirah state, Sudan. Seventy fresh row cow milk samples were collected and examined using standard microbiological methods, ESBL detection was performed on all the isolates by Ceftazidime screening test, those shows positive results by screening method were subjected to ESBL confirmatory test using Double-Disk Synergy Test and Molecular base detection using conventional PCR. Out of the 70 collected samples, 58 (82.8%) showed positive isolating result, the highest prevalence of the isolates was *K. pneumoniae* 36 (62%) followed by *E. coli* 22 (38%). The most resistance antibiotics against isolates was Ampicillin (98%), ESBL production was detected among 17 out of the 22 isolated *E. coli* (29.3%) and 26 (44.8%) out of the 36 isolated *K. pneumoniae*. The ESBL gene encoding the ESBL isolates was CTX- M gene representing 61% fellows by SHV gene (23%) and TEM gene (16%). ESBL-producing bacteria may also be transferred via waste milk to calves, thus further spreading antibiotic resistance in the farm environment.

**Keywords:** *E. coli*; *K. pneumoniae*; Raw milk; ESBL; Al Jazirah State; Sudan

## Introduction

Milk is a major part of human food and plays a prominent role in the Sudanese diet.

It's considered as nature's single most complete food Moreover; its high nutritive value makes it an ideal medium for the rapid multiplication of bacteria, particularly under unhygienic production, production and storage at ambient temperatures [1,2]. Microorganism in raw milk can originate from different sources such as air, milking equipment, feed, soil and grass [3,4]. Largely depends on fecal contamination and the presence of pathogen in feces mainly originates from feed contamination. The presence of pathogenic bacteria in milk is of considerable public health concern, especially for those individuals who still drink raw milk [5]. Enterobacteriaceae are the significant causes of serious infection. *Escherichia coli* and *Klebsiella pneumoniae*, an opportunistic pathogen of humans and animals responsible for a wide range of infections, such as diarrhea, urinary tract infections, pneumoniae, wound infections, septicemia, hemolytic uremic syndrome and nosocomial infections especially meningitis in infants [6,7]. The appearance of ESBL stated in the 1980s and widely distributed in the world [8,9] and conferred increased resistance to beta lactams except carbapenems and cephamycins [10,11]. ESBLs are plasmid mediated and the genes encoding these enzymes are easily transferable among different bacteria [12]. Most of these plasmids not only contain DNA encoding ESBLs but also carry genes conferring

resistance to several non-β-lactam antibiotics [13]. ESBL can hydrolyse penicillins first, second and third-generation cephalosporins and aztreonam (but not cephamycins or carbapenems). Resistance to beta lactam antibiotics is most commonly found in *E. coli* and *K. pneumoniae* and today, this resistance mechanism is recognized globally, in the past few years, there has been an increase in the detection of ESBL-producing strains in the general community [14,15]. The antibiotic resistance leads to increased morbidity, mortality and the cost of treating infections, in particular, those caused by ESBL producing bacteria [16]. Microbiological assessments have an important role to play in the dairy industry to protect the public health and can reduce economic losses. The objective of this study was to isolate and identify *E. coli* and *K. pneumoniae* from raw milk samples of cow and to evaluate the antibiotic sensitivity pattern.

## Materials and Methods

### Study area and sampling

This was a health facility based cross-sectional study, performed from March to August 2017. A Total of 70 raw cow milk samples were collected from different villages in Al Jazirah State-Sudan, all samples were collected aseptically, transported to the laboratory under chilled conditions and processed for microbiological analysis.

### Isolation and identification of bacteria from raw milk samples

The samples were inoculated into MacConkey's broth tubes (HiMedia, Mumbai, India) and incubated at 37°C for 18-24 h. A loopful inoculum from MacConkey's broth was streaked onto Eosin Methylene Blue (EMB) agar (HiMedia, Mumbai, India) and MacConkey's agar; plates were incubated at 37°C for 18-24 h. After that separation of pure colonies were take place by seeding it onto sterile nutrient agar slants as pure culture and subjected for standard morphological and biochemical tests as well as PCR [17].

### Antimicrobial susceptibility testing and ESBL detection

The antimicrobial susceptibility testing of all identified isolates were done according to the criteria of the Clinical and Laboratory Standards Institute method (CLSI). All isolates were screened for ESBL production by using Cefotaxime (CTX 30 µg).

Ceftazidime (CAZ 30 µg) and Ceftriaxone (CRO 30 µg). Each isolates which showed resistant to one or more of these antibiotics were confirmed for ESBL production by Double Disk Synergy Test (DDST) recommended by the CLSI guidelines [18].

### Molecular detection

DNA for molecular detection was extracted after bacterial lysis according to the extraction protocol prepared by the Community Reference Laboratory for Antimicrobial Resistance, 2009. Briefly, a few colonies taken from fresh culture medium and transferred to phosphate buffered saline (pH 7.3). The suspension was heated at 100°C for 30 min. Boiled suspension was transferred directly on ice. The suspension was then centrifuged at 12,000 rpm for 30 min and the supernatant containing DNA was transferred to new Eppendorf tubes. PCR method was used for resistance encoding genes detection (Table 1). PCR Master mix components, Dream Taq Green PCR Master Mix, Nuclease free water and DNA marker "Gene Ruler" were provided by Thermo Scientific (Lithuania). PCR protocol was described by Community Reference Laboratory for Antimicrobial Resistance, 2009.

Primer name	Sequence (50–30)	PCR product size (bp)
CTX-MF	ATGTGCAGYACCAGTAARGT	593
CTX-MR	TGGGTRAARTARGTSACCAGA	
blaSHV-F	CAAAACGCCGGGTTATTC	937
blaSHV-R	TTAGCGTTGCCAGTGCT	
blaTEM-F	GAGTATTCAACATTTTCGT	857
blaTEM-R	ACCAATGCTTAATCAGTGA	

**Table 1:** Primers used for PCR protocols.

### Results

A Total of 70 raw cow milk samples were collected from different villages in Aljazira state. Out of them, 58 (82.8%) showed positive isolating result, after morphological and biochemical identified, the highest observed prevalence of the isolates was *K. pneumoniae* (62%) followed by *E. coli* (38%) (Table 2).

Isolated Microorganisms	Frequency	Percent
<i>Escherichia coli</i>	22	38
<i>Klebsiella pneumoniae</i>	36	62
Total	58	100

**Table 2:** Prevalence of organism contaminating row cow milk.

All isolated (n=58) were assessed to antibiotics sensitivity test against Ampicillin Amikacin, Ciprofloxacin, Gentamycin, Cefepime, Imipenem, using Kirby-Bauer disc diffusion method. The most resistance antibiotics tested against isolated were Ampicillin (98%), Cefepime (95%), Ciprofloxacin (91%), followed by Gentamycin (67.5%), Amikacin (63.2%) and Imipenem (19.9%) as described on (Table 3).

Antibiotics	% Resistance among bacterial isolates	
	<i>E. coli</i>	<i>K. pneumoniae</i>
Ampicillin	98	94
Ciprofloxacin	91	89.2
Gentamycin	67.5	46
Amikacin	63.2	82.5
Cefepime	95	92
Imipenem	19.9	49.4

**Table 3:** Antibiotic resistance against isolated *E. coli* and *K. pneumoniae*.

Total of 58 isolates (22 *E. coli* and 36 *K. pneumoniae*) were tested for screening and confirmatory tests, 52 (89.6%) were positive ESBL and 6 (10.4%) were negative ESBL using screening test, while 43 (74.1%) were positive ESBL and 15 (25.9%) were negative for ESBL production when DDST used and the final confirmation were take place using PCR representing 40 (68.9%) were positive and 18 (31.1%) were showed negative for ESBL.

The most ESBL gene encoding the ESBL isolates was CTX-M gene representing 61% fellows by SHV gene (23%) and TEM gene (16%) (Table 4).

Test	Positive ESBL		
	<i>E. coli</i> N (%)	<i>K. pneumoniae</i> N (%)	Total
ESBL screening	20 (90.9)	32 (88.8)	52
ESBL DDST	17 (47.2)	26 (72.2)	43
PCR	16 (40)	24 (60)	40

**Table 4:** Distribution of ESBL strains according to screening and confirmation test.

### Discussion

In this study, we screened for ESBL-producing *E. coli* and *K. pneumoniae* from raw-cow-milk sample. The results highlighted that 52 isolates were positive for ESBL-producing. ESBL producing *E. coli*

(90%) was the most frequent species; a combination of CTX- M gene representing 61% follows by SHV gene (23%) and TEM gene (16%). High contamination of raw-cow-milk was also reported by [19-28]. *E. coli* is accepted as a fecal contamination indicator in foods because of its presence in the intestinal tract. The gastrointestinal tract and the hands of personnel were reported as principal reservoirs of *Klebsiella* spp. and *E. coli* [29]. Drug resistance in Enterobacteriaceae has increased dramatically worldwide. This increase is mainly the result of an increased prevalence of ESBL-producing Enterobacteriaceae and has increased the use of last resort antimicrobial drugs [30]. In this study, all of the *E. coli* and *Klebsiella* spp. isolates were resistant to ampicillin. Previous studies reported that ampicillin was the least effective among the antimicrobials tested against *E. coli* [31] and similar findings have been reported in a study of swine *K. pneumoniae* isolates in China, where 100% of all the isolates were resistant to ampicillin [32]. According to our results, most of the *E. coli* and *Klebsiella* spp. isolates were susceptible to imipenem. This result is consistent with those obtained in previous studies [33,34]. The observed resistance of *E. coli* and *Klebsiella* spp. isolates to ciprofloxacin was 91% and 89.2%, respectively. High prevalence of ciprofloxacin resistance (84%) has been reported in *E. coli* in Chinese [35,36]. As resistance to ciprofloxacin emerged, resistance to  $\beta$  lactam antibiotics became prominent. This resistance was largely a result of ESBLs, which mediate resistance to newer  $\beta$ -lactam agents, such as ceftazidime, ceftriaxone, cefotaxime and aztreonam, that have an oxyamino group [37]. In this study, all isolates were higher resistance than the study conducted in North America by Sader et al. [38]. The use of cephalosporins in food-producing animals could be selective factor for the appearance of ESBL-producing and multiple-antimicrobial-resistant bacteria in such animals [39]. On the other hand, increasing resistance to third generation cephalosporins (for example, cefotaxime, ceftazidime, ceftiraxone) has become a cause for concern about Enterobacteriaceae [40]. In recent years, ESBL-producing Enterobacteriaceae isolates have shifted from the hospital to the community and the environment [41]. ESBL-producing Enterobacteriaceae have been recovered from different sources in the community, including cattle, chickens, pigs, raw milk and lettuce [42-44]. Recent study from India reported that a substantial number of tap water samples were contaminated with carbapenemase bla<sub>NDM-1</sub> producing organisms [45]. Most of the studies on this subject have been conducted in developed countries, but the major epicenters of ESBL expressing bacteria are located in Asia, Africa and the Middle East [46].

However, both milk and milk products the methods of production, handling, transportation and marketing of these products are entirely depend upon traditional system. Such system could pose favorable environment for bacterial contamination. The unclean hands of worker, poor quality of milk, unhygienic conditions of manufacturing unit, inferior quality of material used and water supplied for washing the utensils could be the source of accelerating the bacterial contamination of milk products and the post manufacturing contamination. [47-51]. However, there remains a need for continued surveillance and judicious use of these antibiotics. The increasing prevalence of resistance in the isolates from animal origin may have important therapeutic implications. Thus, monitoring of ESBL-producing enter bacteria should be continued at various level (animals, human and environment), should be reconsidered because it does not only contribute to the spread of pathogenic bacteria but also is a vehicle to spread antibiotic resistance. While investigating the factors that contribute to their selection and dissemination.

## Conclusion

The results obtained in this study concluded that the milk available for consumer have a high bacterial contamination. Thus, the results of the present study warn the need for stricter preventive measures. For this, regular sterilization of dairy equipment, washing of utensils, milker's hands, udders, eradication of diseased animals, pasteurization/boiling of milk is required before collection and distribution for consumption and product making. The magnitude of the problem of bacterial contamination deserves more elaborative studies from the point of production of milk and milk products to the point of consumption and at all intermediary levels. Thus present study suggests isolating and characterizing the *E. coli* and *Klebsiella* spp. which may cause the pathogenicity in milk products.

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