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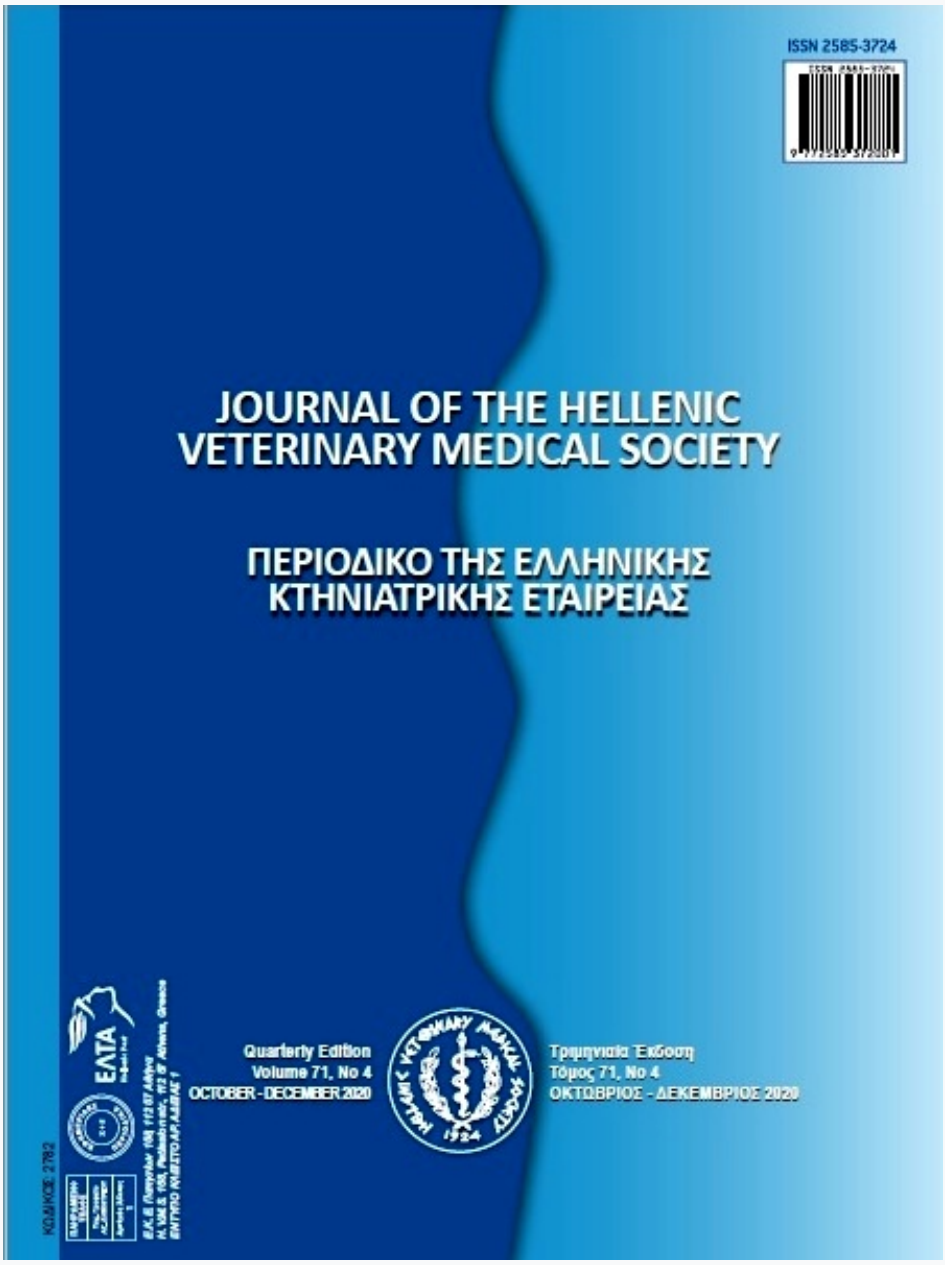

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Research article
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Antibiotic resistance pattern and frequency of some beta lactamase genes in *Klebsiella pneumoniae* isolated from raw milk samples in Iran

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ABSTRACT: *Klebsiella pneumoniae* have become an important cause of mastitis in dairy cows. Resistance to beta lactam antibiotics resulted from beta lactamases enzyme production. The aim of this study was to investigate the antibiotic resistance pattern and frequency of some beta lactamase genes in *Klebsiella pneumoniae* isolated from raw milk samples in Iran. 200 raw cow milk samples were collected from different villages of north west of Iran. The samples were cultured and biochemical tests were performed for phenotypic diagnosis. Then, antibiotic resistance pattern was determined by antibiogram test. Finally, the presence of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* isolates was found by PCR method. Of total 200 raw cow milk samples, 80 samples (40%) contained *Klebsiella pneumoniae*. The frequency of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* isolates was 50 (62.5%), 34 (42.5%) and 70 (87.5%), respectively. 14 *Klebsiella pneumoniae* isolates (17.5%) possessed all three intended genes simultaneously. All strains of *Klebsiella pneumoniae* (100%) were resistant to ampicillin. The most strains were resistant to ceftriaxone (75%), gentamicin (70%) and nitrofurantoin (70%). 4 *Klebsiella pneumoniae* strains (5%) were resistant to all of tested antibiotics. The results showed high frequency of ESBLs and antibiotic resistance in *Klebsiella pneumonia* samples isolated from raw milk. It may occur exchange of resistance genes within and across species and with commensal bacteria of the human and animals.

Keywords: *Klebsiella pneumoniae*; Raw milk; Antibiotic resisrance; Beta lactamase genes

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INTRODUCTION

Klebsiella pneumoniae is a gram-negative intestinal bacterium that forms part of the natural microflora of the human body. This bacterium is responsible for a wide range of diseases including bacteremia, pneumonia and urinary tract infection in human (Hashemi et al., 2014). Studies worldwide have revealed that Klebsiella can contaminate meat (Messaoudi et al., 2009) and dairy products (Yilma et al., 2007) and contribute to disease and spoilage (Clegg and Sebghati, 2002). Raw milk can be contaminated with ESBLs (Extended Spectrum Beta Lactamases) producing enterobacteriaceae in several entities such as mastitis, directly by animal feces or indirectly during milking (Dahmen et al., 2013). Clinical mastitis resulting from *Klebsiella pneumoniae* infection causes high milk losses and mortality of the affected cows (Grohn et al., 2004). The sources of *Klebsiella* spp. in dairy operations include organic bedding material such as wood by-products (Hogan et al., 1989). In addition, fecal shedding by cows contributes to the presence of a large variety of *K. pneumoniae* strains in dairy herds (Munoz et al., 2006; Munoz and Zadoks, 2007). Humans may become colonized or infected by ESBL producing *K. pneumoniae* upon contact with blood, saliva, feces and urine of ESBL carrier animals or consumption of contaminated water or food products (Founou et al., 2016). *Klebsiella pneumoniae* are more capable than most strains of *E. coli* to overcome the inhibitory effects of lactoferrin and infect involuted mammary glands (Todhunter et al., 1990). According to different studies, 1- 5% of food borne intoxications are associated with milk consumption and dairy products (Mansuri Najand and Ghanbarpour, 2006). Unfortunately, the majority of the population in Iran, especially in rural families, still consume raw dairy products without pasteurization including traditional Lighvan cheese (most popular soft white cheese). The general assumption is that raw milk is generally safer and has more beneficial health effects, and that pasteurization would drastically affect the milk quality (Zeinhoma and Abdel-Latefb, 2014). The entry of ESBL-producing enterobacteriaceae in the food chain and environment could be considered a possible interface for the exchange of resistance genes between humans and animals (Walsh and Fanning, 2008). ESBL-producing *Klebsiella* spp. and *E. coli* are now listed among the six drug-resistant microbes for which new therapies are urgently needed (Shah et al., 2004). Approximately 20% of *K. pneumoniae* infections in intensive care units in the United States are now caused by strains not susceptible to third-generation cephalosporins (Paterson 2006). The most frequent and clinically relevant ESBL genes belong to *CTX-M*, *TEM*, and *SHV* families, with CTX-M enzymes emerging as the predominant type. *K. pneumoniae* commonly produces all three groups of enzymes (Perovic et al., 2016). These resistance genes are generally carried on mobile genetic elements (MGEs) facilitating their dissemination within and between bacterial species (Founou et al., 2016). 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. pneumonia*, and today this resistance mechanism is recognized globally. During the past few years, there has been an increase in the detection of ESBL-producing strains in the general community (Mesa et al., 2006). The aim of current study was to investigate the antibiotic resistance pattern and frequency of some beta lactamase genes in *Klebsiella pneumoniae* isolated from raw milk samples in Iran.

MATERIALS AND METHODS

Sample collection

This cross-sectional study, was performed from April to October 2018. A Total of 200 raw cow milk samples from 35 dairy farms (which had not received antibiotics for at least five days) were collected randomly from different villages in north west of Iran. The breasts of the studied cows were apparently healthy. First, each teat was disinfected with ethanol 70̊, then the first few showers of milk were thrown away and 30-50 ml of milk was taken from the animal separately and 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 Eosin Methylene Blue (EMB) agar and MacConkey agar (Merck, Germany) and the plates were incubated at 37$^{\circ C}$ for 24 h. Then, separation of pure colonies were performed by streaking onto sterile nutrient agar slants as pure culture and subjected for standard morphological (Gram staining) and biochemical tests such as oxidase, catalase, methyl red, voges proskauer, citrate, indole and urease.

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 2017) (Padtan teb, Iran) (Kirby-Bauer method). In addition, all isolates were screened in terms of ESBL presence by using combined disk method. Cefotaxime + clavulanic acid (*CTX*/CLA) and Ceftazidime + clavulanic acid (CAZ/CLA) were used to confirm ESBL-producing isolates (Becton, USA). The strains in which the diameter of growth inhibition zone of cephalosporin + clavulanic acid disc were ≥ 5 mm compared to cephalosporin discs only were considered as ESBL-producing bacteria (Coudron et al., 2000).

DNA extraction

Genomic DNA was extracted using the boiling method (Chen et al., 2009). DNA extraction was performed on 80 cultured isolates of *Klebsiella pneumoniae* in brain heart infusion (BHI) agar (Merck, Germany) medium at 35℃ for 24 h. 3-5 colonies of each sample were poured in 1.5 ml eppendorf tube containing 200 µl of sterile TE buffer and were mixed thoroughly using a shaker. Then, the vials were boiled in boiling ban Marry (100 ℃) for 10 minutes, so that the boiling water level covered two-thirds of the vials. Finally, the vials were centrifuged at 9000 g for 5-10 minutes. The supernatant of vials containing DNA was transferred to sterile ependorf for PCR test. The quantity and quality of DNA extracted were investigated by nano-drop and electrophoresis apparatuses.

PCR test to detect intended genes

The polymerase chain reaction (PCR) method was done in 25 µl, including 11µl of Master mix PCR, 1 µl of each specific primers (25 nano moles) (Table 1), 1 µl (50 ng) of DNA template and 11 µl of double distilled water. Timetable and thermal schedule for each gene is presented in Table 2. The amplified products were run on 1% agarose gel and staining with ethidium bromide (0.5 mg/ml) in a dark room.

Table 1. Characteristics of specific primers related to the genes under investigation

Gene	Primer sequence	Amplicon size (bp)	Reference
CTX	5′-CGCTTTGCGATGTGCAG-3′ 5′-ACCGCGATATCGTTGGT-3′	550	Lin et al., 2012
SHV	5′-TACCATGAGCGATAACAGCG-3′ 5′-GATTTGCTGATTTCGCTCGG-3′	450	Doosti et al., 2015
TEM	5′-TCCGCTCATGAGACAATAACC-3′ 5′-ATAATACCGCACCACATAGCAG-3′	296	Doosti et al., 2015

Table 2. PCR test conditions for *Klebsiella pneumoniae* samples for replication of the tested genes

Stage	Number of cycles	Tested gene Time	Temperature (℃)
		CTX/*SHV*/*TEM*	
Primary denaturation	1	5′	95
Denaturation	32	60”	94
Annealing	32	40”	55/55/58
Extension	32	40”	72
Terminal extension	1	5′	72

RESULTS

Of total 200 raw cow milk samples, 80 samples (40%) contained *Klebsiella pneumoniae*. The results showed that 50 (62.5%) samples of *Klebsiella pneumoniae* isolates harbored *CTX* gene (Figure 1).

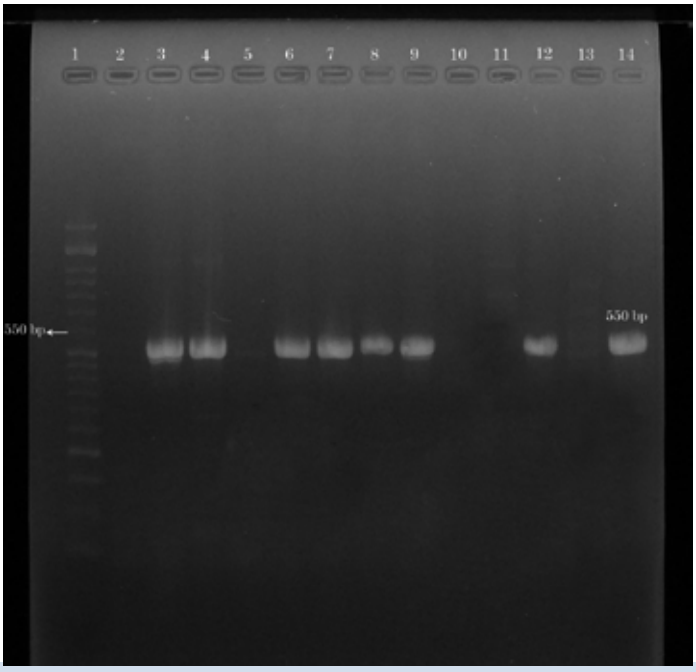

Figure 1. Lane 1 is marker (50 bp). Lane 2 is negative control (Double distilled water). Lane 3 indicates *Klebsiella pneumoniae* PTCC 1290 as positive control that bands within 550 bp and is associated to *CTX* gene. Lanes 4, 6-9, 12 and 14 are positive samples. Lanes 5, 10, 11 and 13 are negative samples

34 (42.5%) samples of *Klebsiella pneumoniae* isolates harbored *SHV* gene (Figure 2).

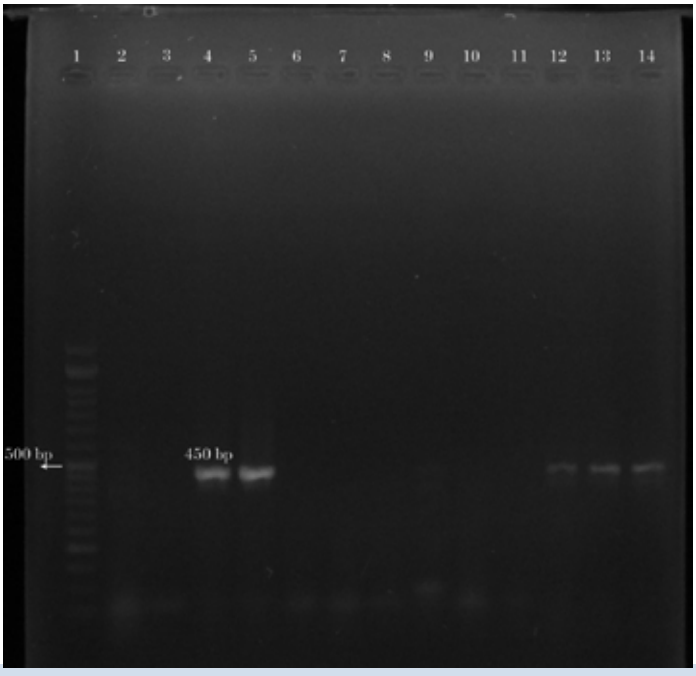

Figure 2. Lane 1 is marker (50 bp). Lane 2 is negative control (Double distilled water). Lane 4 indicates *Klebsiella pneumoniae* ATCC 700603 as positive control that bands within 450 bp and is associated to *SHV* gene. Lanes 5, 12-14 are positive samples. Lanes 3, 6-11 are negative samples

70 (87.5%) samples of *Klebsiella pneumoniae* isolates harbored *TEM* gene (Figure 3).

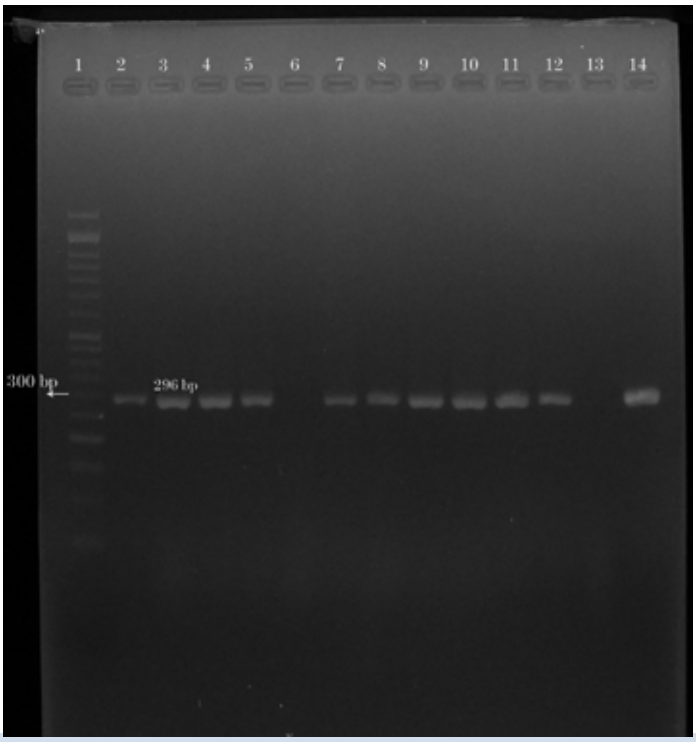

Figure 3. Lane 1 is marker (50 bp). Lane 6 is negative control (Double distilled water). Lane 2 indicates *Klebsiella pneumoniae* PTCC 1290 as positive control that bands within 296 bp and is associated to *TEM* gene. Lanes 3-5, 7-12 and 14 are positive samples. Lane 13 is negative samples

Furthermore, 14 (17.5%) *Klebsiella pneumoniae* isolates possessed all three intended genes simultaneously. 4 (5%) *Klebsiella pneumoniae* isolates lacked the tested genes. On the basis of Chi square test, the frequency of *CTX+TEM* genes was significantly higher than the other double combination genes ($P< 0.05$).

The results revealed that the frequency of studied genes within *TEM* and *SHV* genes showed statistical significance ($p< 0.05$). Moreover, the frequency of studied genes among gene groups showed statistical significance ($p< 0.05$) (Table 3).

Table 3. Frequency of intended genes in the studied bacterial samples

	CTX	TEM	SHV
Yes	50	70	34
No	30	10	46
Total	80	80	80
Chi square	0.8	45.0	7.2
P value	0.371	0.0001	0.007

The obtained results recorded in Table 4 releaved that, all strains of *Klebsiella pneumoniae* (100%) were resistant to ampicillin. Most of the strains were resistant to ceftriaxone (75%), gentamicin (70%) and nitrofurantoin (70%). Much of the sensitivity of *Klebsiella pneumoniae* strains was to amikacin (47.5%) and chloramphenicol (47.5%). Of total 80 *Klebsiella pneumoniae* isolates, only 56 samples (70%) were considered as ESBL producing strains in combined disk test. The percentages of resistance of *Klebsiella pneumoniae* isolates to cefotaxime and ceftazidime were 55% and 67.5%, respectively.

The antibiogram results showed that 4 *Klebsiella pneumoniae* strains (5%) were resistant to all of tested antibiotics (Table 5).

Table 4. Antibiogram results obtained from *Klebsiella pneumoniae* samples isolated from milk

Antibiotic	Abbreviation	Concentration (µg)	Susceptibility R(%)	S(%)	I(%)
Ampicillin	AM	10	0 (0)	0 (0)	80 (100)
Amikacin	AN	30	8 (10)	38 (47.5)	34 (42.5)
Ceftriaxone	CRO	30	14 (17.5)	6 (7.5)	60 (75)
Chloramphenicol	C	30	6 (7.5)	38 (47.5)	36 (45)
Ciprofloxacin	CP	5	12 (15)	32 (40)	36 (45)
Cotrimoxazole	SXT	23.75	4 (5)	26 (32.5)	50 (62.5)
Gentamicin	GM	10	6 (7.5)	18 (22.5)	56 (70)
Imipenem	IMP	10	16 (20)	12 (15)	52 (65)
Nitrofurantoin	FM	300	10 (12.5)	14 (17.5)	56 (70)
Tetracycline	TE	30	14 (17.5)	22 (27.5)	44 (55)
Cefepime	FEP	30	8 (10)	20 (25)	52 (65)

R: Resistant, S: Sensitive, I: Intermediate

Table 5. Frequency and percentage distribution of multidrug resistant (MDR) ***Klebsiella pneumoniae*** **isolates**

Resistant isolates (%)	1 fold	2 fold	3 fold	4 fold	5 fold	6 fold	7 fold	8 fold	9 fold	10 fold	11 fold
	80 (100)	78 (97.5)	76 (95)	70 (87.5)	66 (82.5)	56 (70)	50 (62.5)	34 (42.5)	26 (32.5)	16 (20)	4 (5)

DISCUSSION

Klebsiella pneumoniae is a causative agent of coliform mastitis. Mastitis is one of the major diseases affecting livestock breasts and is considered to be the most costly disease of dairy cattle worldwide, causing major damage to the livestock industry worldwide annually. In the US, milk production decline due to subclinical mastitis costs about 1 billion dollars (110 $ per cow) annually in the dairy industry, and 70% of cases of milk production decline in the herd is associated with subclinical mastitis (Seegers et al., 2003). The incidence of mastitis has been between 0.5-25 percent per month in Iran. It has been estimated that milk production decline from subclinical mastitis alone in 2006 had been approximately 150,000 tons in national level in Iran (Bolourchi et al., 2008). The results of current study revealed that the percentages of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* strains isolated from raw cow milk were 62.5%, 42.5% and 87.5%, respectively. In a study in Kenya, it was reported that the percentage of *SHV* and *CTX* genes in *Klebsiella pneumoniae* strains isolated from raw camel milk were 97.1% and 57.1% , respectively (Njage et al., 2012). In another study in Sudan, it was revealed that 61% of *Klebsiella pneumoniae* strains isolated from cow milk harbored *CTX* gene. They also showed that 23% and 16% of these strains possessed *SHV* and *TEM* genes, respectively (Badri et al., 2018). In a research in India, 1.5% of *K. pneumoniae* strains was identified as ESBL from raw milk (Koovapra et al., 2016). Significant differences in the frequency of *CTX*, *SHV* and *TEM* genes in different studies may be due to different sources of isolation in different regions, their methods of investigation and sensitivity, and number and types of samples. The results of the present study indicate the difference in dispersion of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* strains; this difference probably originates from geographical diversities and also differences in the ecological origin of the isolated strains (milk, human and different animals). In present study, the antibiogram results showed that, all strains of *Klebsiella pneumoniae* (100%) were resistant to ampicillin and 4 (5%) strains were resistant to all of tested antibiotics. In a study in Egypt, it was reported that all of *Klebsiella pneumoniae* strains (100%) isolated from buffalo and cow milk were resistant to ampicillin and most of the strains (82.6%) were resistant to chloramphenicol. Furthermore, much of the sensitivity of *Klebsiella pneumoniae* strains was to gentamicin (100%) and nitrofurantoin (91.3%) (Osman et al., 2014). In another study, the resistance of *Klebsiella pneumoniae* strains isolated from cow milk to ampicillin, ciprofloxacin, gentamycin, amikacin and cefepime was 94%, 89.2%, 46%, 82,5% and 92%, respectively (Badri et al., 2018). The cause of mismatch between the results of the antibiogram test in several studies is probably due to differences in geographical area, type of treat-

ment regimen and measure of antibiotic use in different regions. In present study, the results of combined disk test showed that 70% of *Klebsiella pneumoniae* isolates were considered as ESBL producing strains but PCR test revealed that 95% of *Klebsiella pneumoniae* isolates were ESBL producing strains. This inconformity in the results of these two methods may be due to insufficient sensitivity of the antibiogram discs or errors in performing test. Prolonged and wasteful use of antibiotics in the treatment of livestock diseases such as mastitis as well as the use of antibiotics as growth promoters has created antibiotic resistant strains. This raises concerns about the introduction of bacterial resistant strains into the food chain.

CONCLUSION

The results of current study showed high frequency of ESBLs and antibiotic resistance in *Klebsiella pneumonia* samples isolated from raw milk. It may occur due to the exchange of resistance genes within and across species and with commensal bacteria of the human and animals. Therefore, in order to control infection and prevent distribution of antibiotic resistance genes among clinical or foodborne isolates, correct management of treatment is necessary.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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