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Research Article

Determination of antibiotic resistance and biofilm formation in *Klebsiella* strains isolated from bovine mastitis cases

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ABSTRACT

Mastitis is diseases of dairy cows with a high economic impact. Bovine mastitis is caused by a wide range of bacterial pathogens. As one of the major environmental pathogens Klebsiella spp. was investigated in this study by some phenotypic characteristics like antibiotic resistance patterns and biofilm formation properties. A number of 483 cows by dairy farms around the Konya were examined by California Mastitis Test (CMT) producing 36 positives in terms of subclinical mastitis. A further 19 samples from clinical mastitic udders were also collected. Samples were inoculated onto Trypticase Soy Agar medium enriched with sheep blood and incubated aerobicaly for 24-48 h at 37 °C. By morphological, biochemical and cultural charcateristics 14 isolates out of 37 coliforms were identified as Klebsiella spp. The double disc synergy method and Congo Red Agar test were used to perform antibiotic susceptibility and in vitro biofilm forming properties, respectively. Resistances to the Ampicillin, Carbenicillin, Cephotaxime, Chloramphenicol, Erythromycin, Gentamicin, Neomycin, Oxytetracycline, Sulphamethoxazole/Trimpethoprim, Amoxicillin-Clavulanate and Imipenem antibiotics were 78.5%, 78.5%, 35.7%, 42.8%, 100%, 7%, 7%, 50%, 14%, 21% and 7%, respectively. Three of the total isolets produced biofilm. This appears to the first report on ESBL producing Klebsiella spp. from subclinical cases of bovine mastitis in Konya, Türkiye. Presently, two numbers of antimicrobial combinations to treat bovine cases are recommended by this work. In conclusion, because of costly challange nature of Klebsiella caused bovine mastitis implemantation of an effective mastitis control program should be used in local farms from Konya.

INTRODUCTION

Mastitis, the infection of the mammary gland and one of the most significant diseases of the cattle. It has been leading to the highest economic losses in many national dairy industries (Erdoğdu, 2019). In Türkiye, annual economic loss of 41.5 million TL caused by mastitis was roughly estimated. This amount is mainly due to the decrease in milk production, veterinarian and treatment costs, labor expenses and removal costs of diseased animals (Sabuncuoğlu & Çoban, 2006). Mastitis is caused by a variety of microorganisms, mostly bacteriae. Of these, *Staphylococcus aureus*, *Streptococcus* spp. and *Enterobacteriaceae* are the dominant aetiological factors of bovine cases in many countries of the World including Türkiye (Ajose et al., 2022; Arslan et al., 2009; Kırkan et al., 2005; Tel et al., 2009; Uçan et al., 2005). Of the *Enterobacteriaceae* members, *Klebsiella* spp. is classified as both a major pathogen and an environmental agent. Additionally, in terms of milk loss, *Klebsiella* spp. is the agent that causes the most evident losses both in the first lactations and in the followings (Nielsen, 2009). On the other hand it is the fact that emergence of multi-drug resistance bacteria has been growing problem due to indiscriminate uses of chemotherapeutics in livestock, recently (Feiyang et al., 2021). Although *Klebsiella pneumoniae* (*K. pneumoniae*) which was rather known to cause clinical form of bovine mastitis also causes subclinical form and is presently considered as an emerging issue related to contaminated environments. Although primary species is the *K. pneumoniae* occasionally *K. oxytoca* aslo causes intramammary infections (IMI) in cows (Kleinhenz et al., 2019). Pathogenecity of the *Klebsiella* mastitis in the bovine udder is not fully understood, yet (Cheng et al., 2020). In the virulence of *K.pneumonia* infection in human origin, the factors associated with the infection were reported as those of varieties or presence of capsular serotypes, iron scavenging systems, and fimbriae (Holt et al., 2015). Alternatively,, some factors involved in virulence of *K. pneumoniae* in bovine mastitis were reported as being capsular polysaccharites especially K1 (Capsule) and K2 (Hypermucoviscisity), vmpA, kfu, uge, magA and aerobactin (Osman et al., 2014). Intruguingly, absence of *Klebsiella* spp. isolates from subclinical mastitis was considered that the role of *Klebsiella* spp. in subclinical mastitis was insignificant (Katsande et al., 2013; Swartz & Novello, 1984). However, bedding products (especially materials made by wood) can be a source of *Klebisella* since healthy cows shed *Klebsiella* in their excrements and *Klebsiella* mastitis in both forms originating from the environment may be such a big problem that can only be effectively treated by specific protocols (Fuenzalida et al., 2021; Osman et al., 2014). A rapidly growing veterinary public issue is antibiotic resistance problem. Antibiotic use in farm animals can contribute to the emergence of resistant bacteria that can be transferred from the farm animal species to human somehow. However, the hypothesis, *Klebsiella* might be

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transmitted to human via consumption of contaminated milk or meat is still to be verified (Davis & Price, 2016). Therefore, more researches are needed to reveal various aspects of bovine *Klebsiella* mastitis worldwide. In terms of Türkiye, numerous studies on aetiological agents and prevalances of bovine mastitis have been done in the country, so far (Arda & İstanbulluoğlu, 1979; Bozkır, 1985; Öztürk et al., 2019). However, data on prevalance of *Klebsiella* caused bovine mastitis in Konya needs to be cleared. Occurrence of the agent in the same environment along with the infected cows is also worth of examined. This study aimed to preliminarly present biofilm formation and antibiotic resistance profiles of the *Klebsiella* isolates from local dairy farms at different lactation periods.

MATERIALS and METHODS

A total of 483 cows were included in the study for causative isolation and identification. The samples were transferred to the laboratory maintainig the cold chain and inoculated on trypticase soy agar containing 5% sheep blood then incubated for 24-48 h at 37 °C under aerobic conditions. Based on colony morphology and gram staining characteristics, the isolates suspected of coliform were passaged into Mc Conkey Agar and incubated at 37 °C overnight. Biochemical tests were made by using Lassen triple tube method, MR-VP and citrat utilization test (Hogan et al., 1999; Lassen, 1975). Sensitivities of the isolates to various antimicrobials were detected by Kirby-Bauer Disk Diffusion Method (Bauer, 1966) and performed as per the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2012). Each of the bacteria suspensions was adjusted to the turbidity of 0.5 McFarland Standart and a Mueller Hinton Agar plate was inoculated with the test organism by means of streaking a swab. Then, antimicrobial-impregnated commercial disks Ampicillin (AM10) (10 mg), Carbenicillin (PY100) (100 mg), Cephotaxime (CTX30) (30 mg), Chloramphenicol (C30) (30 mg), Erythromycin (E15) (15 mg), Gentamicin (CN10) (10 mg), Neomycin (N30) (30 mg), Oxytetracycline (T30) (30 mg), Sulphamethoxazole/Trimpethoprim (SXT25) (1.25 mg/23.75 mg), Amoxcillin–Clavulanate (AMC30) (30 mg), Imipenem (IPM10) (10 mg), Flumequin (FLM30) (30 mg) (Bioanalyse, Türkiye) were placed on the surface of the agar. Results were read after 18 h incubation as aerobik condition at 37 °C. Inhibition zones around the disks were measured using a ruler according to Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2012). Colistin sensitivities of the test strains were measured using colimycin (Sigma Aldrich, St. Louis, MO, ABD) by broth dilution test based on the standards of European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC values for Colistin that were ≤2 µg/ml were accepted as sensitive while those >2 µg/ml were noted resistant. The double disc synergy method was used for the antibiotic susceptibility tests and the Extended Spectrum β-Lactamase (ESBL) production in concordance with the procedures stated by the (CLSI).

In order to determine the *in vitro* biofilm forming properties of the isolates, cultivation was performed in Congo Red Agar (KKA) (BHI agar containing 37 g/L, 5% sucrose and 0.8 g/L Congo Red dye) to obtain single colonies (Arslan et al., 2005). After the incubation period, black colonies were accepted as biofilm positive.

RESULTS

All udder lobes of 483 cows from 2 dairy farms located around the Konya were individually sampled and thirty-six milk samples positive by California Mastitis Test (CMT) were included study. In addition, 19 udders with clinical mastitis were also sampled from the same farms. Based on results from biochemical characters, 14 out of 37 coliform suspected isolates were identified as *Klebsiella* spp. (Table 1). The prevalance of *K. pneumoniae* in the both farms were 2.9 % (data not shown). Biofilm formation was observed only in 3 of the strains. ESBL production by double disc synergy method showed an inhibition zone and ESBL production between Amoxicillin–Clavulanate and Cephotaxime in 4 samples (Table 3).

DISCUSSION

Mastitis is likely the costliest disease in dairy breeding. Its prevalance varies between countries. Frequency of mastitis in the dairy cow population, Türkiye seems not to be low, either (Arda & İstanbulluoğlu, 1979; Erdoğdu, 2019; Klaas & Zadoks, 2018; Öztürk et al., 2019)

Klebsiella mastitis has become a major concern in USA and in some parts of the Europe, recently (Fuenzalida et al., 2021; Nielsen, 2009; Osman et al., 2014). In Türkiye, 1277 lactating cows in 3 state ruled farms from the provinces Ankara, Eskişehir and Bursa have been reported to be monitored periodically for 3 years and no *Klebsiella* spp. isolation was made (Arda & İstanbulluoğlu, 1979). Of 14 other studies conducted on bovine mastitis all around the Türkiye during 1979-2019, the prevalance of *Klebsiella* spp. was highest (34.3 %; 23 *Klebsiella* spp. isolation from 162 cows' mastitic udders) in Province Aydın (Erdoğdu, 2019), whereas 0.6 % in Marmara and Paşaeli Regions (Batu et al., 1979) or much later in Marmara 0.2 % (Türütoğlu et al., 1995), 6.8 % in Balıkesir (Çokal & Konuş, 2012). 1.49 % in Bursa and 4 other cities (Büyükcangaz et al., 2012), 0 % in Ankara (Ulusoy, 1985), 4.76 % in Afyonkarahisar (Alaçam et al., 1989) 2.1 % (Muz et al., 1992) and 1.37 % (Gülcü & Ertas, 2004) in Elazığ, 3.83 % (Aydın et al., 1995) and 0 % (Şahin & Çolak, 1997) in Kars, 17.88 % in Burdur (Öztürk et al., 2019) , 1.5 % in Şanlıurfa (Tel et al., 2009) and 1.87 % in Diyarbakır (Yeşilmen et al., 2012). Briefly, different frequencies of *Klebsiella* mastitis in dairy cattle occurred in various regions, provinces or dates in Türkiye.

To the best of our knowledge, the first report on isolation of aerobic bacterial agents from mastitic cow milk stated that no *Klebsiella* was isolated from 150 mastitic milk samples of 691 cows sampled (Bozkır, 1985). Another study form Konya at that time has also noted that *Klebsiella* spp. was not found by examining 39 dairy cattles at the end of the lactation period (Tekeli et al., 1985). First report on *Klebsiella* spp. as a causative agent in mastitis has apparently raised from ovine cases in Konya (Erer et al., 1990) In that study, 1198 sheep were screened for the presence of clinical or subcinical mastitis at a state slaughter house. Roughly six percent of (n=119) milk samples were positive for *K. pneumoniae* growth. Later, no *K. pneumoniae* isolation from bovine mastitis cases was noted

Table 1. Number of samples and isolates based on their animal origins

Cows	Number of sample	Gram(-) bacteria	K.pneumonia	%
With subclinical mastitis	36	21	6	16.66
With clinical mastitis	19	16	8	42.10
Total	55	37	14	25.46

Table 2. Antimicrobial susceptibility of Klebsiella isolates

	Susceptibility					
Antimicrobial agents	Susceptibility		Intermediate		Resistance	
	n	%	n	%	n	%
AM10	0	0	3	21.42	11	78.57
PY100	0	0	3	21.42	11	78.57
CTX30	6	42.85	3	21.42	5	35.71
C30	8	57.14	0	0	6	42.85
E15	0	0	0	0	14	100
CN10	8	57.14	5	35.71	1	7.14
N30	6	42.85	7	50	1	7.14
T30	7	50	0	0	7	50
SXT25	11	78.57	1	7.14	2	14.28
AMC30	11	78.57	3	21.4	0	0
IPM10	13	92.85	1	7.14	0	0
FLM30	14	100	0	0	0	0
Kolistin	14	100	0	0	0	0

AM10: Ampicillin (10mg), PY100: Carbenicillin (100mg), CTX30: Cephataxime (30mg), C30: Chloramphenicol (30 mg), E15: Erythomycin (15mg), CN10: Gentamicin (10mg), N30 Neomycin (30mg), T30 : Oxytetracycline (30mg), SXT25: Sulphamethoxazole/ Tripethoprim (1.25/23.75mg), AMC30: Amoxcillin- Clavulanate (30mg), IPM10: Imipenem (10mg), FLM: Flumequin (30mg)

Table 3. In vitro slime forming properties and Extended Spectrum β-Lactamase production (ESBL)

Mastitis caused by *K.pneumonia*	Number of samples examined	ESBL	Slime forming (In vitro)	%ESBL	%Slime
Subclinical	6	3	0	50	0
Clinical	8	1	3	12.5	37.5
Total	14	4	3	28.57	21.4

from the same region (Ateş et al., 1991; Dinç et al., 1991) have examined 82 lactating cows from a state farm in Konya and found 43 subclinically mastitic udders, giving 2.32 % *K. pneumoniae*, 4.65 % *Klebsiella* spp and 2.32 % *K. pneumonia* mixed with yeast. In order to early diagnose mastitis in subclinical bovine cases, milk samples from healthy and mastitic udders from 40 number of cows (aged between 4-5) have been sampled and examined bacteriologically in Konya (Nizamlıoğlu et al., 1992). The authors noted that *K. pneumoniae* isolated from a healthy milk sample only (6.25 % of the healthy udders). Later on, using limited samples, an isolation of *Klebsiella* spp has also been reported from cow mastitic udders in Konya (Semacan et al., 2012). In our study, *K. pneumoniae* were isolated 16.7 % and 42.1 % from subclinical and clinical cases, respectively. By comparison with the *Klebsiella* mastitis occurrence in Konya for years, *Klebsiella* mastitis have increased dramatically in the past 3 decades although low numbers of mastitic samples studied mostly.

To update information on antimicrobial resistance pattern of *Klebsiella* isolates from bovine mastitis cases is important since decision on effective treatments almost solely depend on.

The antibiotic resistance of *Klebsiella* from milk with either clinical or subclinical forms mastitic udders was also tested against twelve antimicrobials commonly used by present study. Chloramphenicol showed complete resistance to *K. pneumoniae*. Both flumequin and colistin showed sensitivity (Table 2). Restricted data exists on the issue of antimicrobial resistance patterns of *Klebsiella* from mastitic cows in Konya. (Dinç et al., 1991) have reported that all the isolates they had were sensitive to enroflaxacin at different levels. At that time, this can be an expected outcome since enroflaxacin had been in use for a few years for udder health. At present study, this antimicrobial was not examined since it has not been a first preference for treatment of mastitis for some recent years. Some other researchers from Konya have found that sensitivity of *Klebsiella* spp. isolates to chloramphenicol was 100 %. More than 3 decades later in a neighbouring province sensitivity of the local isolates to the same antimicrobial was nil, likely pointing out a growing resistance in this geography (Alaçam et al., 1989).

A study carried out more recently has highlighted 162 cattle with 23 cases of *Klebsiella* mastitis in Aydın (Erdoğdu, 2019). They stated that resistance to ampicillin by 23 isolates was full (100 %). At present study the isolates were found resistant at 78.57 % against ampicillin. By gaining more data on this issue would clear figures of ampicillin resistance in the Country.

Members of *Enterobacteriaceae* generally contain extanded spectrum beta lactamase (ESBL) enzymes. This class of enzymes confers resistance to penicillins, one to third generation cephalosporins such as cephotaxime. Present study also evaluates the occurrence of ESBL-producing *Klebsiella* spp. in some dairy farms. We found a degree of ESBL (28.57 %), not to a low extent (Table 3). Fifty percent of *Klebsiella* strains isolated from subclinical cases showed ESBL activity is of importance since occurrence of ESBL producing *Klebsiella* spp. from subclinical cases of bovine mastitis in Konya is first reported and supports the observation that ESBL producing bacteria are increasingly being appeared from the livestock and environments (Sivaraman et al., 2021).

Understanding recurrent infections in bovine mastitis is still under investigation. One of the underlying mechanisms is attributed to biofilms formed by different causatives (Hillerton and Kliem 2002, Çökülgen and Uçan 2022). In case of *Klebsiella* spp. mastitis cases, biofilm formation has also been reported by several studies (Schönborn et al., 2017, Rudenko et al., 2021). *Klebsiella* spp. biofilms has been noted to possess a high degree of adhesiveness and strenghtness (Lenchenko et al.,2020). Our study evidences that the occurrence of *Klebsiella* spp. isolates positive for biofim from Konya are not quite high (37,5 %) whereas reports from World that state figures from 60 % to 84 % (Schönborn et al., 2017, Rudenko et al., 2021)

A list of pathogens published by WHO in the context of a global action plan on combating antimicrobial resistance considers various mastitis pathogens such as *E.coli, Klebsiella* and *Staphylococcus aureus* with high priority (Becker et al., 2016; Prigitano et al., 2018; Tacconelli, 2017).

In conclusion, to treat *Klebsiella* mastitis with effective antimicrobials in bovine dairy farms located in Konya appears to be mostly limited by Amoxicilin/Clavulunat or Sulfametaxazole/Trimetoprim combination and to a lesser extent by Gentamicin or Cephotaxim, at present. Moreover imipenem, flumequin and colistin showed complete sensitivity to *Klebsiella* of bovine origin. More data from a bigger sample size needs to be undertaken.

DECLARATIONS

Ethics Approval

For this study, it was unanimously decided that the principles of Selcuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee (SÜVDAMEK) Directive were complied with and that it was "appropriate" in terms of research ethics (18.09.2020- 2020/85).

Conflict of Interest

The authors declare that they have no conflict of interests.

Author contribution

Idea, concept and design: MA, USU

Data collection and analysis: MA

Drafting of the manuscript: USU

Critical review: MA, USU

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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