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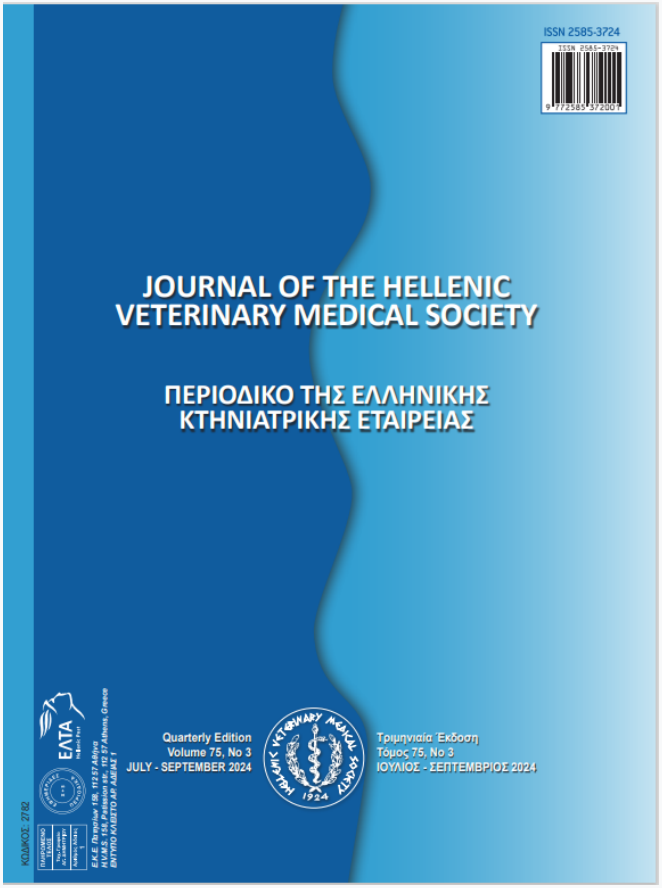

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Distribution of bacterial pathogens and antimicrobial resistance in cows with clinical mastitis in a dairy farm, Türkiye

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ABSTRACT: This study aimed to identify the pathogens causing clinical mastitis (CM)and their resistance levels to six common antimicrobials in a dairy farm in Türkiye. A total of 973 CM milk samples were cultured and a Kirby-Bauer disc diffusion method was performed for antimicrobial susceptibility. While 64.0% (623/973) of CM samples were culture-positive, 36.0% (350/973) of CM samples yielded no growth. *Escherichia coli* was the most frequently isolated pathogen (36.3%), followed by coagulase-negative staphylococci (8.3%), *Streptococcusdysgalactiae* (7.3%), *Staphylococcus aureus* (3.1%), *Streptococcus uberis* (1.5%), *Enterococcus*spp. (1.4%), *Mycoplasma* spp. (1.4%), *Streptococcus agalactiae* (0.7%), and *Corynebacterium* spp. (0.4%). Antimicrobial resistance was higher (P >0.01) to amoxicillin/clavulanic acid (AMC, 32.3%) than that to enrofloxacin (ENR, 23.4%), cefoperazone (CFP, 17.9%), cefquinome (CEQ, 17.7%), penicillin G (P, 15.2%), and gentamicin (CN, 3.6%) in culture-positive 642 isolates. For *E. coli* isolates, percentage of resistance to AMC, ENR, CFP, CEQ, P, and CN was 37.7, 30.6, 24.4, 23.2, 5.9, and 1.1%, respectively. Resistance to AMC (31.2%) and P (46.3%) was higher in CNS than *Strep. dysgalactiae* isolates (1.5% and 12.7%), respectively. Multidrug resistance was detected in 34 *E. coli* isolates (9.6%), 7 CNS isolates (8.6%), and 2 *Strep. dysgalactiae* (2.8%). In conclusion, the higher identification of *E. coli* demonstrated the higher risk of environmental microorganisms for CM in this study. Higher resistance to commonly used five of six antimicrobials showed the requirement of frequent bacteriological and antimicrobial susceptibility tests for CM. Thus, proper hygienic programs may help to reduce the clinical mastitis caused by environmental pathogens in high-yielding cows. The determination of mastitis pathogens and antimicrobial resistance in cows may help to improve the treatment efficacy and welfare of dairy cows with clinical mastitis as well as the production of safe milk for consumers.

***Keywords*:** Antibiotic resistance; Bovine mastitis; Pathogens; Multidrug

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INTRODUCTION

Mastitis is a common herd health problem worldwide and the clinical form of mastitis (CM) is considered to be one of the most costly diseases in dairy cows (Ruegg, 2017; Jamali et al., 2018). The incidence rate of CM ranged from 15 to 40 cases per 100 cow-years depending on the countries and regions (Santman-Berends et al., 2015; Levison et al., 2016; Zigo et al., 2019; Krishnamoorthy et al., 2021; Singha et al., 2021; Alanis et al., 2022). The dairy industry exposes to significant financial losses due to reduced milk production, higher discharged milk, increased culling rate and increased treatment costs (Jamali et al., 2018). Furthermore, 4 to 8.7% of CM cases resulted in mortality in dairy cows (Hertl et al., 2011).

Recent studies showed that among the 135 pathogens, *Staphylococcus aureus* (*Staph. aureus*), Coagulase-negative staphylococci (CNS), *Streptococcus uberis* (*Strep. uberis*), and *Escherichia coli* (*E. coli*) account for almost 80% of CM in dairy cows (Abdi et al., 2021; Brennecke et al., 2021; Singha et al., 2021; Dyson et al., 2022; Alanis et al., 2022). Milk yield, climate, control strategies for contagious pathogens, and management of the environment may reveal the differences in dominant pathogens between countries and dairies (Duse et al., 2021; Miles and Huson, 2021). Environmental pathogens, mostly *E. coli*, were detected more prevalent than contagious pathogens by the development of modern milking practices in cows with CM (Lago et al., 2011; Oliveira et al., 2013; Alanis et al., 2022). Furthermore, it was reported that vaccination with gram-negative vaccines agents had no positive-large effect on decreasing new CM and importance of mastitis caused by environmental pathogens was emphasized in the 100-year review (Ruegg, 2017).

Antibiotics have been the mainstay of mastitis treatment for many years (de Jong et al., 2018; Doehring and Sundrum, 2019). It was reported that 85.4% of farms used at least one antibiotic to treat cows with CM (Oliver and Murinda, 2012) and 67% of cows received antibiotics for the prevention and treatment of bovine mastitis in a large-scale study (Doehring and Sundrum, 2019). It was reported that some herds used antimicrobials for mastitis treatment without microbiological and susceptibility test (Oliveira et al., 2013). Improper-treated cows may also transmit pathogens to humans through contaminated dairy products and the increased risk of antibiotic residues due to misuse and overuse of antibiotics leads to great concern not only in veterinary medicine but also in human medicine (Ben et al., 2019; Miles and Huson, 2021). Improving of the treatment efficacy and protection of animal/human health depends on an understanding of antibiotic resistance and reducing antimicrobial consumption in each region or country (Alvarez-Uria et al., 2018; Ben et al., 2019; Doehring and Sundrum, 2019; Yu et al., 2020; Zigo et al., 2021). More research related to incidence and antibiotic resistance in CM samples is required due to different epidemiological features and control methods in dairy herds (Bradley et al., 2007). Therefore, this study aims to determine the CM pathogens and their resistance to six common antimicrobials in a modern dairy herd in Türkiye.

MATERIAL AND METHODS

Animals and collection of milk samples

This study was conducted on a commercial dairy farm in Çanakkale, Türkiye (39°53’39’’N, 26°12’12’’E). Cows were housed in free-stall barns and the average herd size ranged from 960 to 1150 for five years. All cows were equipped with a neck collar comprised of an electronic identification tag (Dataflow II, Allflex, Israel). The rumination-activity monitoring system were used to monitor health status of cows at 2-hour intervals during the postpartum period. Following NRC (2001) recommendations, cows were grouped in free-stall barns and fed a total mixed ration according to milk yield.

The average milk production was 42 kg per cow/day (ranging from 40 to 43.5 kg per cow/day) during the study. Cows were milked three times each day using a 2 × 25 milking unit in this study per year. Fore milk and udder were evaluated at each milking by the milkers. The cows with clinical suspicion of mastitis were examined by the veterinarian and clinical mastitis was diagnosed with symptoms such as abnormal milk and abnormal udder with inflammation symptoms. Cow identification number, season, and CM at the quarter level were recorded.

A clinical udder examination was routinely performed on all cows during pre-milking in this herd. A total of 1000 Holstein-Friesian cows with CM were examined by a farm veterinarian after detection of mastitis symptoms during fore-milking examination. However, 17 cases were excluded from the analysis due to recurrent cases within 14 days in the same quarter (Alanis et al., 2022) and a total of 973 milk samples were collected by a farm veterinarian in Hol-

stein-Friesian cows with CM for 21 months.

Milk samples were aseptically collected from affected quarters before milking according to guidelines described by National Mastitis Council (NMC, 2017). Teats were pre-dipped into an antiseptic solution and were dried with paper towel. Then, teat orifices were scrubbed with wet wipes with 70% ethanol. The first 2-3 squirts were stripped out to remove contaminant bacteria from the teat canal. Milk samples (50 mL for each) were aseptically collected in a sterile tube from the quarter. If more than one quarter was affected in the same cow, one vial sample from the affected quarters was collected. Collected samples were transported in coolers with ice packs to the herd laboratory in an icebox within a few minutes. Samples were collected from the cows that did not receive any antibiotics at least 7 days ago.

The months between April and September were determined as the hot season and the months between October and March as the cool season (Gao et al., 2017).The new CM rate was calculated by the number of cows diagnosed with clinical mastitis, divided by the number of 100 cows per cow per month.

Isolation and identification of pathogens

Microbiological analysis including identification and isolation were performed by a microbiologist in this commercial dairy farm where a private laboratory. Milk samples were spread onto a Columbia agar with 5% sheep blood (Biomerieux M1013), MacConkey agar (Merck 1.05465, Germany), Sabouraud Dextrose Agar with chloramphenicol (Biomerieux 46979, France), Mycoplasma agar (Oxoid CM0401, UK) with Mycoplasma supplement G (Oxoid SR0059, UK) and Mycoplasma broth (Oxoid CM0403, UK) with Mycoplasma supplement G (Oxoid SR0059, UK). Columbia agar with 5% sheep blood and MacConkey agar were incubated at 37°C for 24 to 48 hours in aerobic condition (NUVE, Türkiye). Mycoplasma agar and broth were incubated at 5-10% CO2 and at 37°C for 7 days (NUVE, Türkiye). A sample was defined as no growth if no colonies were observed on the agar plate after 48 h of incubation (Alanis et al., 2022). Bacteria were identified based on colony morphology, Gram staining (Euromex, Holland), and biochemical tests. The gram-positive cocci grown on blood agar were distinguished into *Streptococcus* spp. (catalase negative) and *Staphylococcus* spp. (catalase-positive) by performing the catalase test. A coagulase test was performed to classify catalase-positive staphylococci (CPS). Catalase-positive staphylococci isolates showing positive coagulase, mannitol, DNase, and α- and β-hemolysis were determined as *Staph. aureus*. Coagulase-negative staphylococci were not identified. *Streptococcus* spp. were transferred to Enterococcosel agar and incubated at 37 °C for 24 hours. Colonies that grew in the brown-black zone on Enterococcosel agar were inoculated on 6.5% NaCl broth, and *Enterococcus* spp. was identified by growing in broth. Gram-positive cocci, catalase-negative, that were grown at non-zone on Enterococcosel agar were classified as *Strep. uberis.* Non-growth at Enterococcosel agar colonies were differentiated as *Strep. dysgalactiae* (negative β-hemolysis on blood agar) and *Strep. agalactiae* (positive β-hemolysis on blood agar) for colony morphology. In addition to colony morphology (pinpoint colonies and β-hemolysis) and gram-positive pleomorphic, catalase-negative to identify *Trueperella pyogenes*, they were cultured in 5% CO2. *Bacillus* spp., and *Corynebacterium* spp. were confirmed by means of colony morphology, catalase, and Gram staining. Colonies that grew on MacConkey agar with pink colony morphology were inoculated on Eosin Methylen-blue Lactose Sucrose (EMB) agar and a green metallic sheen was identified as *E. coli*. Gram-negative bacilli that non-growth green metallic sheen on EMB agar was inoculated suspension medium and cultured at gram-negative identification system (Microgen GN-ID A and B). Gram-negative bacilli were identified according to gram negative identification system software. Apart from *E. coli* bacteria such as *Serratia* spp., *Enterobacter* spp., *Klebsiella* spp., and *Yersinia* spp. were defined as the other Coliform group. Samples were cultured at 37 °C for 7 days in a microaerobic condition to isolate *Mycoplasma* spp. After the pre-enrichment step in Mycoplasma broth medium, liquid cultures were transferred to Mycoplasma agar and cultured at 37 °C for two weeks under the same circumstances. The colonies on Mycoplasma agar were inspected under a microscope (Olympus SZ61, Japan) to see if they had the usual fried egg-shaped form necessary for *Mycoplasma* spp. identification. Yeasts were visually confirmed by analyzing colony morphology and 40 × magnification microscopic analysis. Sabouraud Dextrose Agar with chloramphenicol was used to isolate fungi and the plates were cultured aerobically at 25 °C for 5 to 7 days. Colonies were initially examined macroscopically, and after that, microscopic analysis was performed using lactophenol cotton blue stain.

Antimicrobial susceptibility test

Growing colonies for analysis were seeded in Brain Hearth Infusion Broth at 0.5 McFarland turbidity and switched to Mueller-Hinton agar. The same concentration of antibiotic discs for each pathogen was used. After insertion of the discs, they were incubated at 37°C for 24 hours. At the end of the incubation, the diameters of the non-growth zones around the antibiotic discs were measured and recorded. In the study, amoxicillin/clavulanic acid (AMC, 20/10 µg), penicillin G (P, 10 IU), cefoperazone (CFP, 75 µg), cefquinome (CEQ, 30 µg), enrofloxacin (ENR, 5 µg) and gentamicin (CN, 10 µg) antibiotic discs were used. Antimicrobial susceptibility was performed on each isolate (except for Mycoplasma spp., yeast, and other pathogens) using the Kirby-Bauer disc diffusion method according to methods described in the Clinical and Laboratory Standards Institute (CLSI) performance standards (CLSI, 2018).

Penicillin G was not used for antimicrobial susceptibility in *E.coli* isolates. Isolates were categorized into susceptible, resistant and multidrug resistance (MDR) was assumed when the isolate was resistant to three or more antimicrobials classes (Lopes et al., 2022).

Statistical analysis

The SPSS 23.0 software (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses. The incidence of bovine clinical mastitis and antibiotic resistance was expressed in percentage. The results of quarter level (front and rear), seasonal effect (hot and cool), antibiotic resistance, and MDR were analyzed by using chi-square test.

RESULTS

The mean cumulative incidence of CM per 100 cows/month was 4.21% and ranging from 7.90 to 1.09% (Table 1). There was no difference ($P > 0.05$) in the mean incidence of CM between cool (3.72%, 450 cases in 11 months) and hot seasons (4.65%, 523 cases in 10 months). Clinical mastitis considerably more ($P < 0.05$) affected one quarter (88.3%, 860/973) than multiple quarters (11.6%, 113/973). There was a significant difference ($P < 0.05$) in the incidence of CM for one quarter between the front (34.7%, 338/973) and rear quarters (53.6%, 522/973). The distribution of affected multiple quarters was 100 cases in two, 11 cases in three, and 2 cases in four quarters. An average of 2.1 quarters were influenced in cows with CM in multiple quarters.

Among all enrolled cows, 64.0% (623/973) of

Table 1. Percentage of cows with clinical mastitis (CM) per month and distribution of positive cows with clinical mastitis for 21 months

Months	Percentage of cows with CM	Number of cows with CM
May	7.27	80
June	7.90	87
July	6.82	75
August	3.00	33
September	4.55	50
October	4.73	52
November	4.09	45
December	3.27	36
January	2.00	22
February	1.82	20
March	1.09	12
April	3.64	40
May	4.18	44
June	2.73	30
July	3.00	33
August	1.09	12
September	7.18	79
October	5.09	56
November	6.73	74
December	5.55	61
January	2.91	32
Total 21 months	**4.21%**	**46.3**

CM samples were culture-positive whereas 36.0% (350/973) of CM samples yielded no growth after microbiological diagnosis. 642 pathogens were isolated and the majority of CM (97.0%, 623/642) samples yielded a single culture. Mixed pathogens 19 of 623 (3%) samples consisted of *E. coli* + CNS (n = 1), *E. coli* + *Mycoplasma* spp. (n = 11), *E. coli* + *Microsporum* spp. (n = 2), *Mycoplasma* spp. + *Strep. dysgalactiae* (n = 3), Yeast + *Pseudomonas* spp. (n = 1), and Yeast + other coliforms (n = 1). The culture-positive samples were 61.7%, 64.23, and 73.3% in the affected front quarter, rear quarter, and mix quarters, respectively (P >0.05).

Escherichiacoli (36.3%, 353/973) was the most frequent pathogen, followed by CNS (8.3%), *Strep. dysgalactiae* (7.3%), *Staph. aureus* (3.1%), *Strep. uberis* (1.5%), *Enterococcus*spp. (1.4%), *Mycoplasma* spp. (1.4%), *Strep. agalactiae* (0.7%), and *Corynebacterium* spp. (0.4%, Table 2). Environmental pathogens accounted for 48.5% (472/973) of CM samples and this incidence was 73.4% (472/643) among the total culture-positive samples. The distribution of other bacterial pathogens (5.0%), fungal pathogens (0.3%), and yeast (0.2%) was also shown in Table 2. Considering the incidence of most isolated bacteria as *E. coli* was not different (P >0.05) between the hot (35.3%) and cool seasons (37.6%). The incidence of culture-negative samples was also similar between the hot (35.9%) and cool seasons (36.1%).

In the antimicrobial susceptibility profile of positive 624 isolates, antimicrobial resistance was higher (P >0.01) to AMC (31.9%) than that to ENR (23.1%), CFP (17.6%), CEQ (17.3%), P 11.8%), and CN (3.6%). For *E. coli* isolates, the percentage of resistance to AMC, ENR, CFP, CEQ, and CN was 37.7, 30.6%, 24.4, 23.2, and 1.1%, respectively. Higher resistance was also found for AMC (31.2%) and P (46.3%) in CNS isolates. However, lower resistance to AMC (1.5%) was determined in *Strep. dysgalactiae* compared to the two highest isolates. *Staph. aureus* isolates were completely susceptible to CFP, CEQ, and CN. The percentage of susceptibility, and resistance to antimicrobials of all isolates were shown in Table 3. Furthermore, overall 55 isolates demonstrated resistance to two different antimicrobials and 52 isolates had resistant to three or more antimicrobials (multidrug resistance). Considering three higher isolates, MDR was detected in 34 *E. coli* isolates (9.6%), 7 CNS isolates (8.6%), and 2 *Strep. dysgalactiae* (2.8%). *Staphylococcus aureus*, *Enterococcus* spp., and *Trupere alla pyogenes* isolates did not show MDR (Table 4).

Table 2. Distribution of pathogens isolated from 973 cows with clinical mastitis

Gram-positive	n	%
Coagulase-negative staphylococci	81	8.3
Streptococcus dysgalactiae	71	7.3
Staphylococcus aureus	30	3.1
Streptococcus uberis	15	1.5
Enterococcus spp.	14	1.4
Bacillus spp.	12	1.2
Trueperella pyogenes	11	1.1
Streptococcus agalactiae	7	0.7
Corynebacterium spp.	4	0.4
Gram-negative		
Escherichia coli	353	36.3
Other Coliform bacteria	19	1.9
Pseudomonas spp.	6	0.6
Acinetobacter baumannii	1	0.1
Other pathogens		
Mycoplasma spp.	14	1.4
Microsporum spp.	2	0.2
Yeast	2	0.2
No growth	350	36.0

Table 3. Antimicrobial resistance profile of 638 bacterial pathogens isolated from 973 cows with clinical mastitis

Isolates		AMC		ENR		CFP		CEQ		P		CN	
Gram-positive	**n**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
Coagulase-negative Staphylococci	81	55	26	73	8	77	4	75	6	43	38	79	2
Streptococcus dysgalactiae	71	68	3	61	10	68	3	69	2	62	9	63	8
Staphylococcus aureus	30	22	8	28	2	30		30		21	9	30	
Streptococcus uberis	15	11	4	10	5	9	6	13	2	10	5	12	3
Enterococcus spp.	14	10	4	14		14		14		9	5	12	2
Bacillus spp.	12	9	3	9	3	10	2	10	2	6	6	11	1
Trueperella pyogenes	11	9	2	10	1	11		8	3	10	1	10	1
Streptococcus agalactiae	7	7		6	1	6	1	6	1	6	1	6	1
Corynebacterium spp.	4	4		3	1	4		4		4		4	
Gram-negative													
Escherichia coli	353	220	133	245	108	267	86	271	82	NT	NT	349	4
Other Coliform bacteria	19	9	10	15	4	12	7	10	9	19		18	1
Pseudomonas spp.	6	0	6	5	1	5	1	5	1	6		6	
Acinetobacter baumannii	1	1		1		1		1		1		1	
OVERALL	624	425	199	480	144	514	110	516	108	197	74	601	23
Frequency of susceptibility (%)		68.1		76.9		82.4		82.7		31.6		96.3	
Frequency of resistance* (%)			31.9		23.1		17.6		17.3		11.8		3.6

CNS; Coagulase-negative staphylococci, S; Susceptible, R; Resistance, NT; Not tested.
AMC; amoxicillin/clavulanic acid, P; Penicillin G, CFP; Cefoperazone, CEQ; Cefquinome, ENR; Enrofloxacin, CN; Gentamicin.
*Intermediate isolates were combined with resistant isolates to form frequency of an antimicrobial resistance.

Table 4. Multidrug resistance (MDR) pattern of 52 bacterial pathogen isolated from cows with clinical mastitis

MDR patterns	Number of pathogens
AMC, CEQ, ENR	*E. coli* (4), Other coliforms (1)
AMC, CFP, CEQ	*E. coli* (3), Other coliforms (1)
AMC, CFP, ENR	*E. coli* (3)
AMC, ENR, CN	*E. coli* (1)
AMC, ENR, P	CNS (2)
CFP, CEQ, ENR	*E. coli* (1)
CEQ, ENR, P	*Strep. uberis (2)*
CFP, ENR, P	*Strep. dysgalactiae* (1)
AMC, CFP, CEQ, ENR	*E. coli* (21), *Pseudomonas* spp. (1)
AMC, CFP,CEQ, CN	Other coliforms (1)
AMC, CFP, CEQ, P	CNS (2), *Bacillus* spp. (1)
AMC,CEQ, ENR, P	CNS (2)
CFP, CEQ, ENR, CN	*E. coli* (1)
CFP, CEQ, ENR, P	CNS (1)
CFP,CEQ, ENR, CN, P	*Strep. dysgalactiae* (1), *Strep. agalactiae* (1), *Bacillus* spp. (1)

CNS; Coagulase-negative staphylococci,
AMC; Amoxicillin/clavulanic acid, P; Penicillin G, CFP; Cefoperazone, CEQ; Cefquinome, ENR; Enrofloxacin, CN; Gentamicin.

DISCUSSION

The incidence rate (4.21%) of new CM per 100 cows/month was higher in this study than that in previous reports (Santman-Berends et al., 2015; Levison et al., 2016; Zigo et al., 2019; Krishnamoorthy et al., 2021 Singha et al., 2021; Alanis et al., 2022). Numerous calculation methods such as case number per 365 cow-days, case number per 100 cows-year, and case number per 1000 cows-month were used to estimate the risk of CM in previous studies(Ruegg et al., 2017; Alanis et al., 2022). Calculation methods during specific time intervals affect the incidence of CM. When prediction of CM risk was performed over a period of time such as a one-month interval in this study, mastitis at a different quarter of the same animal might have caused the overestimation of incidence (Alanis et al., 2022). Furthermore, higher milk production could be a possible reason for increased incidence of

CM in this study (Jamali et al., 2018).

In agreement with our findings, it was noteworthy that almost 80% of CM caused by five bacterial pathogens such as *Staph. aureus*, CNS, *Strep. uberis*, and *E. coli* in dairy herds (Brennecke et al., 2021; Singha et al., 2021; Abdi et al., 2021; Alanis et al., 2022; Dyson et al., 2022). The predominant pathogen was *E. coli* (36.3%) in this study. Similar to our findings, Bradley and Green (2001) reported that the mean annual incidence was 41.6% and isolation of *E. coli* incidence was 34.7% of CM cases in England (Bradley and Green, 2001). De Jong et al. (2018) identified the *E. coli* isolates as the highest number (22.1%) of CM samples collected from nine European Countries. However, our results regarding the incidence of *E. coli* was higher than those reported in Netherlands 26.7% (Steeneveld et al., 2008), 22.5% in the USA (Oliveira et al., 2013), and 14.4% in China (Gao et al., 2017). Coagulase-negative staphylocci (8.3%) *and Strep. dysgalactiae* (7.3%) emerged as the major pathogen associated with CM in this study. Although the CNS has traditionally been the minor pathogen for bovine mastitis, it has been identified as the most frequently isolated bacteria in CM samples in previous studies (Levison et al., 2006; Zigo et al., 2019).On the other hand, the frequency of *Strep. dysgalactiae* was higher than *Strep. uberis* in this study. However, *Strep. dysgalactiae,* ranged from 2.8% to 5.5% of all CM samples, was reported as the second Streptecoccus species in bovine CM, following the *Strep. uberis*(Wente and Kromker, 2020).

Environmental pathogens identified almost half (48.5%) of CM cases in this study. Similar to other countries, environmental microorganisms were also isolated as the predominant pathogens following data collection from the individual cases in Türkiye (Öztürk et al., 2019). However, predominant pathogens can be highly different based on data collection methods between small-scale and large-scale modern herds that conduct different mastitis control strategies (Zhang et al., 2016; Ozbey et al., 2022). Therefore, it was hypothesized to determine the prevalent pathogens with controlled data collection from large-scale a dairy farm in Türkiye. Consistent with our results, environmental microorganisms were the most frequently isolated pathogens that those accounting for 43.3% of CM samples in England (Bradley et al., 2007), 37.9% of CM samples in China (Gao et al., 2017), and 33.7% of CM samples inBelgium (Verbeke et al., 2014).

Although CM recurrent rate was low (6%) and the efficacy of treatment was unclear in this study, a high recurrent rate was reported following environmental pathogens affected CM in dairy herds (Jamali et al., 2018). Reduced diameter and stretch ability of teat canal cause pathogenic bacteria invade udder by penetrating the teat canal (Cheng et al., 2020; Zigo et al., 2022). In general, high-yielding dairy cows are more prone to CM mastitis than those with low-yielding cows (Heikkilä et al., 2018). The incidence of CM was higher in the front quarters than rear quarters in this study. Similar to our results, quarter risk for CM was different due to contamination with bedding material and rear quarters are more susceptible to environmental pathogens than front quarters (Steeneveld et al., 2008; Zigo et al., 2021). In agreement with the previous studies, partial open teat canal could possibly contribute to the greater exposure to environmental pathogens through feces and higher isolation of environmental pathogens in the rear quarters than in the front quarters. Environmental pathogens were more commonly isolated in the rear quarter than the front quarter in high-yielding cows with CM (Zhang et al., 2016).

Seasonal trends of prevalent mastitis pathogens varied (Riekerink et al., 2007), and increased temperature and humidity index in summer is favorable for environmental pathogens in bedding (Zhang et al., 2016). Some studies reported that environmental pathogens were commonly isolated in summer compared to winter (Riekerink et al., 2007; Zhang et al., 2016; Gao et al., 2017). Conversely, Yu et al. (2020) stated that *E. coli* isolates are more common in autumn than in spring, as rainy weather can enhance the growth of coliform bacteria. Also, Osteras et al. (2006) isolated *E. coli* and *Strep. dysgalactiae* more frequently in winter compared to summer. However, in this study, there was no seasonal effect on the incidence of CM or predominant bacteria.

Major contagious mastitis pathogens including *Staph. aureus*, *Strep. agalactiae*, and *Mycoplasma bovis*had lower frequency than environmental pathogens in this study. Contagious mastitis agents are especially transmitted from cow to cow during the milking (Barlow, 2011; Zigo et al., 2021). Contagious pathogens mostly cause chronic mastitis cases and the collection of samples from new CM cases could be a reason for the low incidence of contagious pathogens in this study (Awandkar et al., 2022). As a major reason for low frequency, it was thought that the culling of

cows with chronic mastitis (especially *Staph. aureus*) within the scope of the mastitis management program could cause a decrease in the incidence of contagious mastitis (Levison et al., 2016; Singha et al., 2021). However, *Staph. aureus* remains a challenge for some farms that have not effectively implemented mastitis control practices (Duse et al., 2021). Therefore, we also assumed that the widespread adoption of well-known mastitis control practices and milking procedures could contribute the low frequency of contagious pathogens in this study (Oliveira et al., 2013; Ruegg, 2017). Furthermore, low frequency of *Pseudomonas* spp. and *Acinetobacter baumannii* were isolated. It is noteworthy that the frequency of these bacteria, which have serious risk of public health, indicate geographical dissemination and health status of herd (Awandkar et al., 2022).

The percentage of no growth (36% of CM samples) in this study was in the range of previous results. Similarly, it was reported that the incidence of culture-negative samples varied from 10% to 40% in cows with CM.The rate of culture-negative was detected as 41.3% in Slovakia (Zigo et al., 2019), 29.1% in Australia (Dyson et al., 2022), 29.6% in the USA (Alanis et al., 2022), 24.3% in Germany (Brennecke et al., 2021), and 19.9% of CM samples in Belgium. The presence of inadequate bacteria below the detectable threshold for culture and freezing CM samples could lead to increase culture-negative rate (Kuehn et al., 2013; Levison et al., 2016). In addition, it was demonstrated that a higher incidence of culture-negative cases was caused by *E. coli* and a strong correlation was reported for incidence between culture-negative cases and *E. coli*. Spontaneous bacterial cure (common in isolation of *E. coli* and CNS) by successful inflammatory response due to the presence of inhibitory substances in milk affects the incidence of culture-negative (Barlow, 2011; Levison et al., 2016).

Although broad-spectrum antibiotics are frequently used to treat CM, there has been no definitive evidence of antimicrobial efficacy without findings related to antimicrobial resistance (Saini et al., 2013). The use of antibiotics clearly affects the treatment efficacy depending on antimicrobial susceptibility(Ruegg, 2017). In this study, each isolates demonstrated antimicrobial resistance and approximately one-third of all isolates were resistant to AMC. *E. coli* isolates demonstrated high resistance to five antimicrobials (mostly to AMC) except for gentamicin. Similar to our results, Saini et al. (2013) reported the resistance to AMC was 31.5% in Canada. Ardicli et al. (2022) found that 56% of *E.coli* isolates showed resistance to AMC in Türkiye. Our study indicated a lower prevalence of AMC in *E. coli* isolates than previous results (81% to 92.7%) in the other countries (Supré et al., 2014; Cheng et al., 2019).

Moreover, about 10% of *E. coli* isolates were demonstrated MDR in this study. Similar to our findings, Yu et al. (2020) indicated that resistance to at least one antimicrobial ranged from 20% to 33% of *E. coli* isolates and 20% of the isolates had MDR. Higher antimicrobial resistance and MDR to *E. coli* is progressively increasing serious concern around the world (Guerra et al., 2020; Yu et al., 2020).

One of the most important findings of this study was that all isolates were highly susceptible (>95%) to gentamycin in this study. Consistent with our findings, previous studies reported the low resistance to aminoglycosides in different countries (Lehtolainen et al., 2003; Saini et al., 2013; Yu et al., 2020). However, isolates showed higher resistance to ß-lactams, three/four generation cephalosporins, and fluoroquinolones in this study. In agreement with the recent studies in Brazil (Lopes et al., 2022) and Türkiye (Ardicli et al., 2022), higher resistance these antimicrobials were plausible due to the widespread use of these antimicrobials for the treatment of CM. A recent study demonstrated that *E. coli* isolates had higher resistance (ranged from 83% to 100%) to cephalosporins (Ardicli et al., 2022) in CM cases than that in our study. Alvarez-Uria et al. (2018) suggested that cephalosporins would likely to be ineffective for treating *E. coli* infections in most countries by 2030. Moreover, it was reported that cefoperazone and cefquinome achieved a total market share of 39% in Germany in 2020 (Bolte et al., 2020). One of the most important findings of this study is the detection of high resistance to cefoperazone and cefquinome, which have been extensively used for treatment in veterinary and human medicine. Within sixteen MDR patterns, at least one cephalosporin was in fourteen of these variations.

CONCLUSION

Isolation of *E coli* as the most common pathogen from clinical mastitis samples in this study showed the importance of environmental microorganisms in increasing the risk of mastitis in high-yield dairy herds. The observation of higher resistance to five common antimicrobials which are extensively used in veteri-

nary and human medicine also showed the requirement for bacteriological and susceptibility tests and the increasing threat to public health. Thus, the implementation of proper hygienic programs may help to reduce the clinical mastitis caused by environmental pathogens in high-yielding cows. The determination of mastitis pathogens and antimicrobial resistance in cows may help to improve the treatment efficacy and welfare of dairy cows with clinical mastitis as well as the production of safe milk for consumers.

CONFLICT OF INTEREST

The authors have declared no conflict of interests.

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