Al-Mukhtar Journal of Agricultural, Veterinary and Environmental Science 01 (1): 61-67, 2023
Doi: https://doi.org/10.54172/jqa2ce75

Research Article **Open Access**

The causes of Clinical and Subclinical Pathogenic Mastitis and anti-bacterial Resistance in Milk of Dairy Cows in and around Shahat, Libya

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Received:
20 September 2023

Accepted:
15 December 2023

Publish online:
31 December 2023

Abstract: Overall, 200 milk samples were collected from January 2018 to March 2020, which led to the isolation of 126 pathogen agents (63%). Escherichia coli 46 (36.5%), Streptococcus pyogens 31 (24. 6%), and staphylococcus aureus 19 (15 %) were specified as the larger causative agents of clinical mastitis. While Streptococcus dysgalactiae 21 (16.7%) were mostly implicated in subclinical mastitis. With that, in both types of mastitis, also about 4 (3.2) % positive cases were caused by other bacterial agents that were identified at a low frequency (<4%) and cannot be considered as important pathogens like (*Klebsiella* spp) or non-contagious like (Corynebacterium spp.). The antimicrobial resistance was moderate in the total proportion; exclude penicillin in staphylococci, and tetracycline in streptococci, which are used as antimicrobial drug for humans' treatment in Libya. In addition to collection the date information about mastitis issue in Libya.

Keywords: antibacterial resistance, Shahat, Mastitis, Dairy Cows.

المسببات المرضية لالتهاب الضرع الظاهري والصامت للأبقار الحلوب في مدينة شحات وضواحيها

المستخلص: من إجمالي 200 عينة حليب جمعت في الفترة ما بين يناير 2018 إلى مارس 2020 تم عزل 126 عينة مصابة بالتهاب بكتيري بنسبة (63٪. (مقسمة كالتالي: كأكبر عامل مسبب لالتهاب الضرع السريري وهي :
Escherichia coli 46 (36.5%), Streptococcus pyogens 31 (24. 6%), and positive staphylococcus aureus 19 (15 %). بينما *Streptococcus dysgalactiae* (15.079.
21%) كانت المسبب الأكبر في التهاب الضرع الإكلينيكي. مع ذلك، وفي كلا النوعين من التهاب الضرع، تم أيضًا عزل 4 حالة إيجابية من عوامل بكتيرية أخرى والتي كانت بنسبة منخفضه، وبالتالي لا يمكن اعتبارها مهمة مثل Klebsiella spp أو غير معدية مثل *Corynebacterium spp*.وكانت مقاومة البكتريا للمضادات الميكروبية معتدلة في إجمالي العينات إلا penicillin وكذلك tetracycline في *Streptococci* وفي *Staphylococci* النسبة وإن كانت ليست عالية إلا أنها تهدد الصحة العامة ويرجع أهمية هذه المضادات الحيوية إنها تستخدم في علاج البشر بالإضافة إلى أنه يجب التنويه إلى أن المعلومات حول مشكلة التهاب الضرع في ليبيا ضعيفة.

الكلمات المفتاحية: المسببات المرضية، التهاب الضرع الظاهري والصامت الأبقار، مدينة شحات.

INTRODUCTION

Mastitis, the most common infection or inflammation of the mammary gland in dairy cows, is known to have a negative impact on both the welfare of the animals and the financial success of dairy farms. Since the advent of contemporary dairy farming, farmers have looked for practical

ways to reduce the risk of mastitis in their herds (Ruegg, 2017). Clinical and subclinical mastitis, which is responsible for one of the most common diseases affecting cows worldwide. causes a big economic problem. Many bacterial pathogens induce udder inflammation, which in the case of mastitis results in an increase in leukocytes or somatic cell count in the mammary gland in response to microbes invading the teat canal, multiplying, and producing toxins that harm milk-secreting tissue as well as the mammary gland's various ducts. The procedure yields less milk and different milk composition with a higher concentration of leukocytes or somatic cells (Ahmed et al., 2020).

Before treatment with antibiotic, it is necessary to reduce the somatic cell count (SCC) and the antimicrobial resistant test. Unfortunately, almost all veterinarians don't do that before choosing the antibiotic. One source of the reinforced occurrence of resistant bacteria is the inexpert and excessive use of antibiotics in veterinary medicine. The wrong choice or small dose when useing antibiotics to treat mastitis in dairy cows causes a big problem with antimicrobial resistant bacteria (Ahmed et al., 2020; Li et al., 2016).

Multidrug-resistant isolates of different bacterial species were confirmed via antimicrobial resistant tests as well as the detection of the type and incidence of antimicrobial resistance. Although many of the tests discussed are of value in detecting the presence of cute to sub-acute mastitis, isolation and identification of specific microorganisms from milk is usually. Udder infections with Staphylococcus aureus, Streptococcus dysgalactiae, and Streptococcus uberis are common causes of bovine mastitis (Li et al., 2016).

Over all 126 pathogens have been isolated from milk for bovine mastitis, which are classified as chronic, acute or sub-acute mastitis. Most causes of bacterial mastitis in this study were designed to detect the type, concentration of bacteria, and their antibacterial resistance isolated from milk to clinical or subclinical mastitis cows in this region.

MATERIALS AND METHODS

Sampling collection:

A total of 200 milk samples were divided into two sets of milk from clinical (100) and apparently healthy cow (100), collected and tested using the culturing method to detect the bacterial agents that cause clinical and subclinical mastitis. According to National Mastitis Council guidelines and the Dairy Cattle Herd Selection, milk samples were taken after washing and drying the udder (S. P. Oliver & National Mastitis, 2004). Teat ends were disinfected with cotton swabs soaked in 70% ethanol. The first few streams were discarded. Approximately 10 mL of milk from each udder quarter was put into sterile tubes. Samples were transported to the laboratory in an ice box and stored at 40 C for subsequent bacteriological analyses. Samples were collected according to (Reyher et al., 2011) with some modifications. Different sets of milk from (Ruegg, 2017) cows with clinical mastitis (M1to M3), (Ahmed et al., 2020) apparently healthy and lactating cows were divided into cows before drying-off (BD) and after calving (AC).

All producer-diagnosed clinical mastitis cases were sampled (M1), as shown (Table 1). The M1 samples were taken before antimicrobial treatment, whereas the M2 and M3 samples were taken after antimicrobial treatment. All samples until use were saved at –20°C in deep freezing. Clinical mastitis was defined as an inflammation of the udder leading to the occurrence of flakes, clots, or other gross alterations in milk, whereas subclinical mastitis was defined as SCC >200,000 cells/ mL from a cow without clinical signs of mastitis (S. Oliver & Calvinho, 1995).

Table: (1). Divided of milk sample according to symptoms of mastitis

Sy	Healthy condition	Antimicrobial treatment	s-n
M1	Cows with sever clinical mastitis	Taken before antimicrobial treatment	35
M2	Cows with moderate clinical mastitis	Taken after antimicrobial treatment	32
M3	Cows with chronic clinical mastitis	Taken after antimicrobial treatment	33
AC	Apparently healthy and lactating cows after calving	Taken without antimicrobial treatment	50
BC	Apparently healthy and lactating cows before dry-off	Taken without antimicrobial treatment	50

Laboratory examination:

Somatic cell count (SCC):

SCC ≤150,000 Cells /ml normal>150,000 to ≤200,000 cells/ml suspected and retested. Subclinical mastitis was defined as SCC >200,000 cells/ mL(S. Oliver et al., 1995).

Detection of bacterial agents:

Bacterial culturing and identification of the milk samples were done according to National Mastitis Council guidelines (Hogan et al., 1999). The bacterial agents were isolated on MacConkey agar and blood agar. Thin stained Gram stains were used to Characterize gram-positive cocci (*Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus dysgalactiae, Staph. aureus*), small single gram negative cocci non motile (*Corynebactterium*) and gram negative rods (*Escherichia coli* and *Klebsiella* species).

On blood agar, the identification of *Streptococcus* and *Staphylococcus aureus* showed that all Streptococcus *spp* are urea test positive, mannitol fermentation positive, Coagulase, Catalase negative and between *Streptococcus spp* Clam F, TSI. T, Voges-Proskauer test, Citrate and Sodium hippurate test between *Streptococcus galactiae, Streptococcus dysgalactiae* (Table 2). on MacConkey agar were identified as *E. coli. Klebsiella* species isolates were positive to Voges Proskauer test, non-motile, negative to indole production, produced urease, negative to both the Methyl red and utilized citrate tests (Table 3).

Table: (2). Identification of cocci bacteria

	G. S	Clam F	Coag	TSI	U T	Ca	C	L .F	MT	V R	Sodium hippurate
St. aur	+Cl.co	+	+	+	-	+	+	+	-	+	-
S. Py	+str. co	+	-	+ G	+	-	-	+	+	-	-
S. dys	+str.co	-	-	+	+	-	+	+	+	+	-
S. a	+str. co	-	-	+ G	+	-	-	+	+	-	+
C. b	- co	-	+	+	-	-	-	-	-		-

G S: Gram stains, TSI: a triple-sugar iron agar slant, U: a urea, C: citrate, V.P: Voges Proskauer, M: motile, L F: lactose fermenters, SA: staphylococcus aureus, S. Py: Streptococcus pyogenes, Streptococcus galactiae: Sa, Streptococcus dysgalactiae: Sdys, Klebsiella ssp: K. ssp, Cb: Corynebacterium.

Table: (3). Identification of rod bacteria results by biochemical tests

Rod bacteria	I T	MR T	C T	V-P T
E.coli	+	+	-	-
Klebsiella species	-	-	+	+

Indole production (I.T) test, Methyl red (MR) test, Citrate Utilization (C T) test, Voges-Proskauer (V-P) test.

Antimicrobial resistant Test (ART):

It was conducted on a Mueller Hinton Agar plate contained antimicrobial discs that are commonly used for mastitis prevention and control, and those included: Oxytetracycline, Ampicillin, Amoxicillin, Penicillin, Gentamicin, Tetracycline, Ceftiofur, Cephaloxhine and Sulfadimethoxine

RESULTS AND DISCUSSION

A total of 200 samples were examined by SCC techniques.130 samples were positive, but only 126 (63%) were positive by microbial examination in cases of clinical and subclinical bovine mastitis with a P Value of (0.001-0.02). This difference may be due to birth and lactation, especially since the positive samples were after the lactation period and were not bacterial infections of the udder. While it was positive by microbial examination. only 100 (50%) were positive for Clinical mastitis and 26 (13%) were apparently healthy cow. The most prevalent bacterial mastitis types detected are Escherichia coli, Streptococcus pyogens, Streptococcus dysgalactiae, Stahpylotococcus aureus, Streptococcus agalactiae, Klebsiella spp and thin Corynebacterium spp nearly (see Table 4 and Figure 1). These partly agreed with the data of (Ahmed et al., 2020) who found Staphylococcus are high prevalent bacterial agent than this research.

Table: (4). Bacteriological Findings on the milk sample

Bacterial species	Sub clinical mastitis		Clinical mastitis		Percentage %	P.v
E.coli	8		34		33.333%	0.005
Strep- pyo	3		28		24.603%	0.025
Strep- dys	9		12		16.666%	0.05
Staph- aureus	4		15		15.079%	0,01
Strep- ag	2		7		7.140%	0.01
K-spp	0		2		1.587%	0.005
C b,	0		2		1.587%	0.005
Total ve+	(13%)	26	(50%)	100	(63.0%)	

Results revealed that Positive milk samples according to symptoms of mastitis were found in cows with sever clinical mastitis (M1) (35) Ve+, and the high positive number was E. coli 18, cows with moderate clinical mastitis (M2) (32) Ve+, and Staph. aur was high 14, 25Ve+cows with clinical mastitis (M3) S.py (9) Ve+ ,(12) Ve+ apparently healthy and lactating cows after calving (AC)in S. dys with (10) Ve+ and (22) Ve+ apparently healthy and lactating cows before dry-off (BD) in S.dys with (9) Ve+ (see Table 5) .

E. coli represented the highest presence in the result. It may be that these bacteria are naturally found in feces of animals and therefore a source of infection. Staphylococci are major causative agents of clinical or subclinical bovine mastitis and generate important losses in the dairy industry. Other previous studies have confirmed that Staph. aureus and Str. agalactiae were the most prevalent causative agents of mastitis (Elsayed & Dawoud, 2015; Hamed & Ziatoun, 2014).

Table: (5). Positive milk samples according to symptoms of mastitis

Sy	Ve+	T.S	*S.py*	*S.ag*	*S.dys*	*K*	*E.coli*	*C.b*	*St. aur*	%	P.V
M1	35	35	10	3	2	1	18	1	0	87.5%	0.015
M2	32	32	9	1	0	0	8	0	14	80%	0.01
M3	33	33	7	1	10	0	14	0	1	62.5%	0.02
AC	14	50	4	2	8	0	0	0	0	24%	0.025
BD	12	50	1	2	1	1	2	1	4	44%	0.01

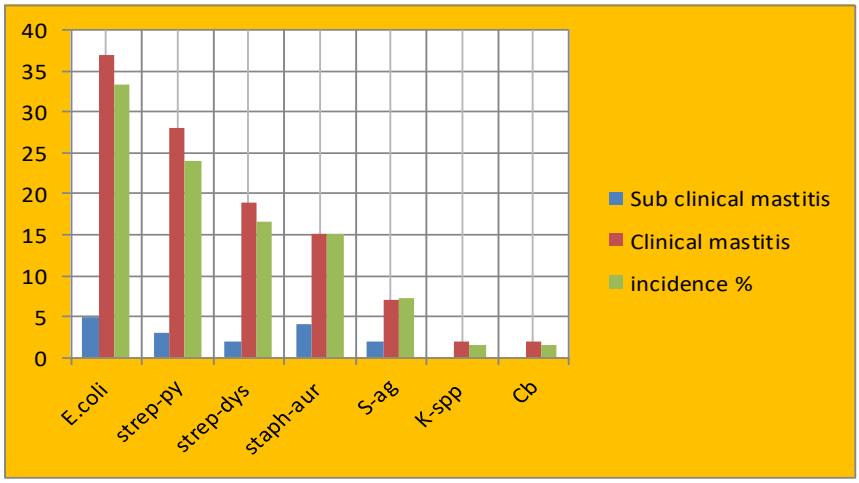

Figure: (1). Bacteriological Findings on the milk sample

The highest percentage of sensitive antibiotic was Ceftiofur (82%), followed by Cephalexin86%, then penicillin (65%), and low prevalent were Tetracycline (37. 3%), ampicillin and Sulfadimethoxine (30%). This finding nearly agreed with the data of (Ahmed et al., 2020), which found high resistance to ampicillin. Depending on the results, when AMR techniques were used to detect antimicrobial resistance, almost all positive mastitis milk samples were resistant. Streptococcus pyogens was the most predominant antimicrobial resistant bacteria, with a resistant rate of 54% followed by Streptococcus dysgalactiae and Streptococcus agalactiae, which had a resustant rate of (47%).

Nonetheless, Escherichia coli exhibited the lowest percentage of resistance to antibiotics (25%); conversely, Escherichia coli was more common than the other bacteria (33.33%). *Streptococcus pyogens* had a resistance rate of 54% to almost all antibiotics. A significant proportion of the non-Streptococcus pyogens isolates (76.6%) and *Streptococcus pyogens* isolates (24.6%) exhibited phenotypic resistance to sulfadimethoxine (70%) compared to ampicillin (63%), tetracycline (63%) and penicillin (35) (refer to Table 6, Figure 2). Egypt was found to have a high prevalence of penicillin-resistant bacteria in penicillin-resistant *staphylococci* (Awad, Ramadan, Nasr, Ateya, & Atwa, 2017), China (Qu et al., 2019) and Brazil (da Costa Krewer et al., 2015). Polish *Streptococcus* isolates produced similar outcomes (Kaczorek, Małaczewska, Wójcik, Rękawek, & Siwicki, 2017). Since amoxicillin is the most often prescribed and utilized antibiotic for the treatment of staphylococcal mastitis, resistance to these antibiotics is crucial. The overuse of antibiotics in the treatment of mastitis can be the cause of the rise in penicillin and amoxicillin resistance

Table: (6). Positive antimicrobial sensitive bacteria

Spp	Tet	Pen	Oxa	Ge	Cef	Cep	Sul	Amp	Am	P.V	Re/Bac
E.coli	17	9	6	6	3	11	18	19	5	0.01	%25
S. pyo	23	18	15	13	7	21	26	21	7	0.01	%54
K	1	1	0	1	1	0	1	1	1	0.02	%39
S. a	7	3	5	3	4	3	4	5	4	0.001	%47
S.dys	15	7	9	2	3	11	18	20	5	0.025	%47
C.b	1	1	0	1	1	0	1	0	1	0.01	%33
St. aur	15	7	1	1	3	10	20	17	3	0.01	%45
Res/N	79	44	36	27	22	56	88	83	26		
Res%	63	35	29	29	18	32	70	66	21		
Sen/N	47	82	90	89	104	70	38	43	100		
Sen%	37	65	71	71	82	68	30	34	79		

AMP: ampicillin, PEN: penicillin, TET: Tetracycline, Cef: Ceftiofur, Cep; Cephaloxhine, Sul: Sulfadimethoxine AMX: amoxicillin, Gen: Gentamicinte, OXA: Oxytetracycline.

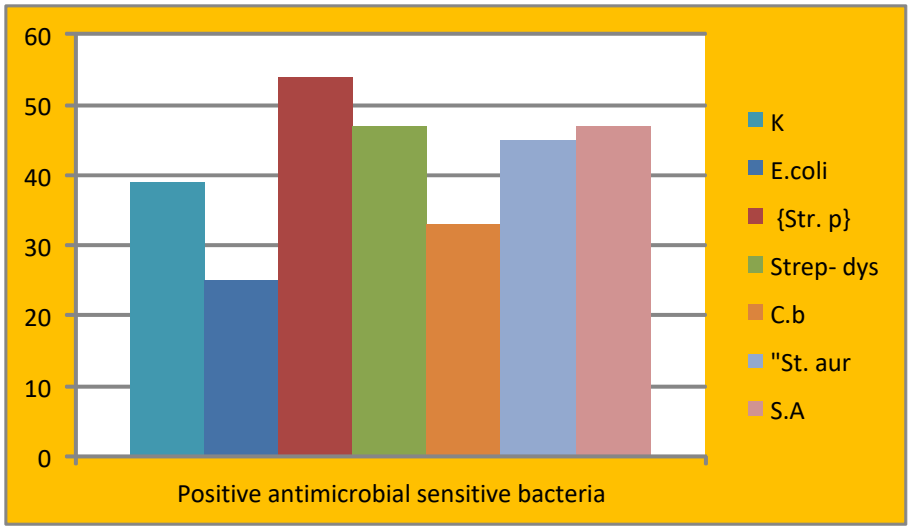

Figure: (2). Positive antimicrobial sensitive bacteria

CONCLUSION

The obtained results demonstrated the importance of studies of pathogenic mastitis in Shahat region, which is associated with weakness, decreased milk production and sometimes loss of calf. This study provides baseline information about the status of the pathogenic udder inflammation in cattle in Shahat, Libya. Also, this study highlighted the importance of awareness of the antimicrobial resistant, especially among veterinary workers when using antimicrobial drugs, the treatment of dairy cows, and their impact on the human population.

Since amoxicillin and penicillin are the most commonly used antimicrobial drugs in human medicine, their controlled use as antimicrobial agents for the treatment of mastitis is crucial. Effective veterinary care and regular epidemiological monitoring regarding antibiotic-resistant bacteria that used to treat dairy cow mastitis are recommended. This calls for a decrease in the use of antibiotics, antimicrobial medication use training for farmers and veterinarians, diagnosis before therapy, and, last but not least, pasteurization of milk. Governmental surveillance instruments can also aid in lowering the use of antibiotics.

Duality of interest: The authors declare that they have no duality of interest associated with this manuscript.

Author contributions : Contribution is equal between authors.

Funding: No specific funding was received for this work.

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