Bacterial And Fungal Causes Of Bovine Mastitis In Menoufiea Governorate

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ABSTRACT

Incidence of bacterial and fungal causes in both clinical and sub-clinical cases of bovine mastitis were detected. Isolation ,biotyping and serotyping of causative agents were analyzed in addition to confirmation by PCR.

Clinical mastitis was more prevalent in cows , meanwhile subclinical mastitis was more prevalent in buffaloes. Infections by one or two causative agents were observed. *Staph. Aureus, Strept. agalactia , Strept. dysgalactia , Staph. epidermidis , E. coli , P. aeruginosa , K. pneumonia , Proteus vulgaris, Candid* spp., *Aspergillus* spp., *Penicillium* spp. and *Alternaria* spp. were isolated from all clinical and subclinical cases of mastitis,there was incidence variation in isolation of microorganisms from cows and buffaloes.

Serogrouping revealed 6 types in E.coli and 5 types in *P. aeruginosa.* Antibiogram revealed that norfloxacin and gentamycin are the most effective antibacterial , Meanwhile amoxicillin was less effective than any antimicrobial. *Staph. Aureus* coagulase gene were detected by PCR using specific primers.

INTRODUCTION

Mastitis is a worldwide disease affecting animals, causing a lot of economic losses in milk quality and quantity ***(1).*** It still remains one of the most costly disease to animal agriculture throughout much of the world ***(2).*** Most cases of bovine mastitis result from infection by microorganisms that invade the udder through the teats, however the udder may also become infected by organism already present in the animal's systems.

Mastitis is considered the most important disease affecting dairy herds and individual lactating animal. It means inflammation of mammary gland associated with changes in tissues and produced milk and may take subacute , acute or chronic form which are only detected by careful manipulation of the udder. From public health a consumption of affected milk(apparently normal) associated with many zoonotic diseases in which milk act as a source of infection to human. Subclinical form is considered the most serious one as infected quarter showed no symptoms either in udder or in milk for long time and the causative organism act as invisible source for spreading infection in the herd and to humans drinking this milk ***(3,4).***

Bacteria causing mastitis can be divide into two main groups contagious and environmental . The contagious bacteria transmitted from infected quarters to other quarters primarily during milking as *Strept. agalactiae, Staph. aureus* and *Corynebacterium bovis.* While, the environmental bacteria as *E. coli, Klebsiella ,Citrobacter, Pseudomonas aeruginosa, Proteus Vulgaris, Strept. dysgalactiae* and *Strept. uberis* . Animals mostly infected from locations in the bedding and reach the teat end from environmental then mammary gland and cause mastitis ***(5 - 8).*** Mastitis may occur by single or mixed bacteria cooperated with other to produce diseases. Bacterial culture is the standard method for identification of intramammary infection ***(9, 10).***

Staph. aureus is most prevalent contagious mammary pathogens associated with clinical and subclinical bovine mastitis with serious economic loss and herd management problems, which rapidly and easily transmitted ,as well as it is a zoonotic disease which transmitted to human being, it produces enterotoxines which cause food poisoning ***(11 , 12).***

Coliforms represents one of the most important environmental pathogens causing mastitis, *E. coli* is the predominant coliform

species reported as causing intra mammary infections in most studies *(13). E.coli* causes inflammation of the mammary gland in dairy animals around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms. This disease affects mainly high producing animals in dairy herds and may cause several cases of death per year in the most severe cases *(8).*

Strept. agalactia and *Strept. dysgalactiae* , were the most predominant microorganisms which causes subclinical bovine mastitis *(14, 15).*

Infection of the mammary gland by yeasts should be suspected, when there was a history of unsuccessful antibiotic treatment which might aggravate fungal mastitis such as infection with Candida spp. which are penicillin and tetracycline utilizers as a source of nitrogen *(16 ,17)* . Mycotic infections of mammary glands usually occur as sporadic cases affecting a small percentage , or as outbreaks affecting the majority of animals. The serious of infection depends on the number of organisms present in the glands and the species of yeast involved *(18,19).* All of the fungal isolates were yeasts of the genera *Candida* spp. *Rhodotorula* spp. *Cryptococcus* spp. and *Trichosporon.* Moulds classified in the following genera *Aspergillus* spp., *Alternaria* spp. and *Penicillium* spp. *(20 ,21).*

Yeasts are microorganisms which present in the surrounding nature and normal inhabitants of the skin of the udder and teats. They can invade mammary glands where they are opportunistic, producing disease when normal defense mechanisms are lowered or when entrance in large numbers and cause clinical and subclinical mastitis, especially *Candida krusei, Candida albicans,* and *Cryptococcus neoformans (22 - 24).*

Molecular biology techniques have become integrated into the practice of infection disease epidemiology. In particular Polymerase chain reaction is an *in vitro* amplification technique for enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in target DNA *(25).*

Identification of *Staph. aureus* and *Candida albicans* by isolation was time consuming and the cultures need to be handled with care because of the zoonotic potential. So we are using PCR assay as an alternative method in routine diagnosis for rapid, sensitive, and specific simultaneous detection of *Staph. aureus* and *Candida albicans* in milk samples *(26 - 28).*

The aim of the present study was isolation and identification of bacteria and fungi causing mastitis in apparently healthy and mastitic buffaloes and cows. Besides, serological identification of isolated *E. coli* their susceptibility to chemotherapeutic agents as an aid to overcome this problem and reduce losses. Also, using polymerase chain reaction (PCR) test to substitute the conventional cultural methods and rapid diagnosis of *Staph. aureus.*

MATERIAL AND METHODS

Samples

A total of 720 milk samples were collected from each quarter of 180 animals including 95 buffalloe and 85 cows from apparently healthy and clinically infected animals from various private farms, in Menoufiea Governorate during period from August 2008 till December 2009. All samples were examined for investigation of bacterial and mycotic causes of subclinical and clinical bovine mastitis .

Collection of milk samples

The udder region , teat orifice and hands of milkers were washed, then disinfected with 70% ethyl alcohol before the milk samples were taken, the first jet of milk was discarded, then 25 ml of milk sample were collected from each quarter separately in sterile screw capped bottles and transported as quickly as possible to laboratory in ice box with minimum of delay (Animal Health Research Institute–shebin El-Kom). The milk samples were subjected to California mastitis test (CMT) in the farm to detect the subclinical mastitis. Out of these the positive samples in CMT were subjected to bacteriological and mycological examination *(29).*

Bacteriological examination of milk samples *(30)*

Milk samples were incubated at 37°C for 2hours then centrifuged at 3000.r.p.m. for 20 minutes. The cream supernatant fluid were discarded. A loopfull from the sediment was streaked directly onto nutrient agar, sheep blood agar 5%, MacConkey agar, Eosin Methyline Blue agar, mannitol salt agar media for Staph. aureus and crystal violet blood agar plates, modified Edward's medium for *Strept. agalactiae* and medium contain cetrimid for *P. aeruginosa.* The inoculated plates were incubated aerobically at 37 °C for 24-48 hours and examined for bacterial growth. Suspected colonies were subcultured, purified and preserved in semisolid nutrient agar for further identification .

Suspected colonies appearing on different media were identified by studying the characters of the colonies as well as Gram's stain, then identified morphologically and biochemically *(31 - 34).*

Serological identification

1- Serological identification of *E. coli* isolates for detection of different sero groups of somatic antigens "O" using slide agglutination test *(35).*

2- Serological identification of *P. aeruginosa* for detection of different sero groups of somatic antigens "O" using *P. aeruginosa* antisera was carried out *(36).* Antisrea of both *P. Aeruginosa* and *E. Coli* were obtained from Denka Seiken Co. Ltd, Tokyo, Japan.

Mycological examination

The milk samples were inoculated onto the surface of Sabouraud's dexrose agar (SDA) containing 0.05% chloramphenicol, and Candida agar (CA), the spot inoculation method was followed to culture fungi . Plates were inoculated at 25°C for a minimum period of 7 successive days . The inoculated plates were examined and cultures identified .Mould isolates *(32,38)* and yeast isolates were identified *(37 , 39).*

Susceptability of isolates to chemotheraputic agents

Antibiotic sensitivity discs were obtained from Oxoid. Antibiogram was applied on the most predominant isolated strains using disc diffusion technique *(34,40)* with Mueller agar. The results were interpretated according to the manual supplied by Oxoid Company.

The susceptibility of some strains of fungal isolates to antifungal discs were assayed by disc diffusion method . Disks from each drug were put on the plate and incubated at 25 for 18 hours. The results were interpretated according to the manufactures instructions *(41).*

Extraction of *Staph. aureus* DNA *(42)*

Isolated *Staph. aureus* strains were incubated overnight in 10 ml brain heart infusion broth (Oxoid), centrifuged at (5000 rpm, for 15 min) and resuspended in 0.5 ml TE buffer (10 mM Tris, 1 mM EDTA - pH 8).

Total cellular DNA was extracted using Qiagen DNA extraction kit (Qiagen, Germany) according to manufacturer's protocol for gram-positive bacteria. The extracted DNA from milk samples was dissolved in 25 µl sterile distilled water and stored at –20°C until further use.

Multiplex PCR was performed on the extracted DNA from milk samples (part C) to detect coagulase (coa) and 16SrRNA genes *(42, 43).*

Primers for *Staph. aureus* coagulase (coa) and 16SrRNA genes:

Specific oligonuclotide multiplex primer assay (synthesized by MWG-Biotech AG, Holle & Huttner GmbH, Germany) , for rapid diagnosis of *Staph. aureus* coagulase (coa) and 16SrRNA genes. The forward primer for coagulase (coa) was 5'-ATAGAGATGCTGGT -3' , while the reverse primer was 5`-GCTTCCGATTGTTCG -3` *(43).* While the forward primer for 16SrRNA gene was 5'-GTAGGTGGCAAGCG -3', while the reverse primer was 5`-CGCACATCAGCGTC-3` *(42).*

***Staph.aureus* DNA amplification by PCR**

The PCR was performed *(42 , 43),* in a touchdown thermocycler in a total reaction volume of 30 ul containing 2.5 µl of extracted DNA, 1 µl of each primer (10 pmol/µl), 0.6 µl of deoxynucleoside triphosphate (10 mmol/L), 3 µl of 10 X thermophilic buffer (Promega), 1.8 µl of

MgCl2 (25 mmol/L), 0.1 µl of Taq DNA polymerase (5 U/µl), and complete the reaction volume using distilled water in 0.2-ml reaction tube. The presence of PCR products was determined by electrophoresis of 10 µl of the DNA product in a 1.5 % agarose gel with 1 X TAE buffer (40 mM Tris-HCl, 1 mM EDTA/L, 1.14 ml/L glacial acetic acid, pH 7.8) at a voltage of 4 volts /cm and stained with 0.5 mg/ml ethidium bromide and the Fluorescent bands were visualized with a UV transilluminator and photographed. A 100-bp DNA ladder (Gibco BRL) was used as a molecular marker. Amplification was obtained with 35 cycles. Each cycle involved initial denaturation at 93 °C for 3 minutes, denaturation at 92 °C for 1 minutes, annealing at 52 °C for 1 minutes , and extension at 72 °C for 1 minutes. The final extension was performed at 72°C for 7 minutes .

The presence of PCR products was determined by electrophoresis of 10 µl of the DNA product in a 1.5 % agarose gel with 1 X TAE buffer (40 mM Tris-HCl, 1 mM EDTA/L, 1.14 ml/L glacial acetic acid, pH 7.8) at a voltage of 4 volts /cm and stained with 0.5 mg/ml ethidium bromide and the Fluorescent bands were visualized with a UV transilluminator and photographed. A 100-bp DNA ladder (Gibco BRL) was used as a molecular marker.

RESULTS

Table 1 showed that the prevalence of total bacteria and fungi causes mastitis in buffaloes and cows were 67.4% from the total examined samples (720) with total incidence of 60.5% and 75% in buffaloes and cows respectively. Table (1), also show the prevalence of subclinical mastitis in buffaloes was 39.2%, while in cows was 33.2%, The incidences of single and mixed subclinical infection were (51.7% & 48.3%) in buffaloes and (43.4% &56.6%) in cows.

On the other hand , Table 2 showed that, the prevalence of clinical mastitis in buffaloes and cows were 21.3% and 41.8% respectively, while the single and mixed infection were (43.2% and 47.9%) in buffaloes while it was (56.8% and 52.1%) in cows.

Table 3 showed the isolation of different types of bacteria and fungi from mastitic buffaloes with various incidences varying according to type of mastitis. In subclinical mastitis of buffaloes the total bacteria isolated were *Staph. aureus* (22.1%), *Strept. agalactia* (10.1%), *Strept. dysgalactia* (10.1%) , *Staph. epidermidis* (5.4%), *E. coli* (11.4%), *P. aeruginosa* (17.4%), *K. pneumonia* (3.4%) and *Proteus vulgaris* (2%).

While the total isolated fungi in subclinically mastitic buffaloes were *Candida krusei* (4.7%), *Candida* tropicalis (2.7%), *Candida albicans* (2%), *Cryptococcus neoformans* (4.7%), *Aspergillus* spp. (2%), *Penicillium* spp. (1.3%) and *Alternaria* spp.(0.7%) .

Meanwhile the total bacteria isolated in clinically mastitic buffaloes were *Staph. aureus* (19.8%), *Strept. agalactia* (12.3%) , *Strept. dysgalactia* (7.4%) , *Staph. epidermidis* (4.9%), *E. coli* (13.6%), *P. aeruginosa* (21%), *K. pneumonia* (6.2%) and *Proteus vulgaris*(3.7%).

While the total isolated fungi in clinically mastitic buffaloes were Candida krusei (2.5%), Candida tropicalis (2.5%), Candida albicans (3.7%) Cryptococcus neoformans (1.2%), Aspergillus species (1.2%), Penicillium species (0%) and Alternaria species(0%).

Table 4 showed the isolation of different types of bacteria and fungi from mastitic cows with various incidences varying according to type of mastitis. In subclinical mastitis of the cows total bacteria isolated were *Staph. aureus* (23.9%), *Strept. agalactia* (10.6%), *Strept. dysgalactia* (8%), *Staph. epidermidis* (5.2%), *E. coli* (8%), *P. aeruginosa* (13.3%), *K. pneumonia* (3.5%) and *Proteus vulgaris* (0.9%).

While the total isolated fungi in subclinical mastitic cows were Candida krusei (7.9%), Candida tropicalis (3.5%), Candida albicans (6.2%), Cryptococcus neoformans (4.7%), Aspergillus species (3.1%), Penicillium species (1.6%) and Alternaria species(1.6%).

Meanwhile the total bacteria isolated in clinically mastitic cows were *Staph. aureus* (21.8%), *Strept. agalactia* (10.6%), *Strept.*

dysgalactia (7.8%), *Staph. epidermidis* (3.5%), *E. coli* (6.3%), *P. aeruginosa* (18.3%), *K. pneumonia* (1.4%) and *Proteus vulgaris*(1.4%).

While the total isolated fungi in clinical mastitic cows were *Candida krusei* (9.9%), *Candida tropicalis* (4.2%), *Candida albicans* (5.6%), *Cryptococcus neoformans* (4.9%), *Aspergillus* spp.(1.4%), *Penicillium* spp. (1.4%) and *Alternaria* spp.(1.4%).

Table 5 showed the serogrouping of isolated *E. coli* revealed different O serogroups. The most prevalent serogroups were O149, O126, O86, O 128 , O119 and 08 with the incidence of (19.6%, 17.3%, 15.2%, 15.2% 13.1% 10.9% and 8.7%) respectively. While the serogrouping of *P. aeruginosa* isolates revealed different serogroups. The most prevalent serogroups were K, H, M, A, and G with the incidence of (21.4%, 19.05%, 19.05%, 17.9% 11.9% and 10.7%) respectively.

Table 6 showed the in vitro sensitivity of the most prevalent bacteria isolated from mastitic buffaloes and caws were done against (14) chemotherapeutic agents. Most tested strains of *Staph. aureus, Strept. agalactia, Strept. dysagalactia, Staph. epidermidis, E. coli, P. aeruginosa* and *K. pneumoniae* were highly sensitive to enrofloxacin, norfloxacin, ciprofloxacin and kanamycin. Most of these strains were highly resistant to erythromycin and amoxicillin .

The results of four tested antifungal agents, are summarized in Table (7), in which the most of tested strains of *Candida* spp. were highly sensitive to nystatin and ketoconazole in vitro.

Fig. 1 showed four milk samples representative for positive *Staph. aureus* isolates, were selected and subjected to PCR analysis. The specificity of the oligonucleotide primer was confrimed by the positive amplification of 228bp fragments for *Staph. aureus* coagulase (coa) and variable fragments for 16SrRNA genes from the extracted DNA of *Staph. aureus*.

Table 1. Frequency of subclinical mastitis in quarter milk samples from buffaloes and cows

Animal species	No. of examined animals	No. of examined quarters	Total no. of positive quarters	%	Total no.of Negative quarters	%	No. of positive quarters	%	Single		Mixed	
									No.	%	No.	%
Buffalloes	95	380	230	60.5	150	39.5	149	39.2	77	51.7	72	48.3
Cows	85	340	255	75	85	25	113	33.2	49	43.4	64	56.6
Total	180	720	485	67.4	235	32.6	262	36.4	126	48.1	136	51.9

Table 2. Frequency of clinical mastitis in quarter milk samples from buffaloes and cows

Animal species	No.of examined animals	No.of examined quarters	No.of positive quarters	%	Single		Mixed	
					No.	%	No.	%
Buffalloes	95	380	81	21.3	35	43.2	46	56.8
Cows	85	340	142	41.8	68	47.9	74	52.1
Total	180	720	223	30.97	103	46.2	120	53.8

Table 3. Incidence of bacteria and fungi causing subclinical and clinical mastitis in buffaloes

Microorganisms	Subclinical						Clinical					
A-Bacterial isolates	Single		Mixed		Total		Single		Mixed		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Staph. aureus	16	20.8	17	23.6	33	22.1	7	20	9	19.6	16	19.8
Strept.agalactia	10	12.9	5	6.9	15	10.1	5	14.3	5	10.9	10	12.3
Strept.dysgalactia	7	9.1	8	11.1	15	10.1	2	5.7	4	8.7	6	7.4
Saph. Epidermidis	4	5.2	4	5.6	8	5.4	1	2.9	3	6.5	4	4.9
E. coli	9	11.7	8	11.1	17	11.4	4	11.4	7	15.2	11	13.6
P. aeruginosa	13	16.9	13	18.1	26	17.4	9	25.7	8	17.4	17	21
K.pneumoniae	2	2.6	3	4.1	5	3.4	2	5.7	3	6.5	5	6.2
Proteus vulgaris	1	1.3	2	2.8	3	2	1	2.9	2	4.3	3	3.7
Total bacterial isolates	62	80.5	60	83.3	122	81.9	31	88.6	41	89.1	72	88.9
B-Fungus isolates												
Candida krusei	4	5.2	3	4.2	7	4.7	2	5.6	0	0	2	2.5
Candida tropicalis	3	3.9	1	1.4	4	2.7	1	2.9	1	2.2	2	2.5
Candida albicans	2	2.6	1	1.4	3	2	1	2.9	2	4.3	3	3.7
Cryptococcus neoformans	3	3.9	4	5.6	7	4.7	0	0	1	2.2	1	1.2
Aspergillus species	1	1.3	2	2.8	3	2	0	0	1	2.2	1	1.2
Penicillium species	1	1.3	1	1.4	2	1.3	0	0	0	0	0	0
Alternaria species	1	1.3	0	0	1	0.7	0	0	0	0	0	0
Total fungal isolates	15	19.5	12	16.7	27	18.1	4	11.4	5	10.9	9	11.1

Table 4. Incidence of bacteria and fungi causing subclinical and clinical mastitis in cows

Microorganisms	Subclinical						Clinical					
A-Bacterial isolates	Single		Mixed		Total		Single		Mixed		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Staph. Aureus	12	24.5	15	23.4	27	23.9	13	19.1	18	24.3	31	21.8
Strept.agalactia	6	12.2	6	9.4	12	10.6	7	10.3	8	10.8	15	10.6
Strept.dysgalactia	5	10.2	4	6.3	9	8	6	8.8	5	6.8	11	7.8
Staph.epidermidis	3	6.1	3	4.7	6	5.3	3	4.4	2	2.7	5	3.5
E. coli	4	8.2	5	7.8	9	8	5	7.4	4	5.4	9	6.3
P. aeruginosa	5	10.2	10	15.6	15	13.3	15	22.1	11	14.9	26	18.3
K.pneumoniae	2	4.1	2	3.1	4	3.5	0	0	2	2.7	2	1.4
Proteus vulgaris	0	0	1	1.5	1	0.9	0	0	2	2.7	2	1.4
Total bacterial isolates	37	5.5	46	71.8	83	73.5	49	72.1	52	70.3	101	71.1
B-Fungus isolates												
Candida krusei	4	8.2	5	7.8	9	7.8	6	8.8	8	10.8	14	9.9
Candida tropicalis	2	4.1	2	3.1	4	3.5	3	4.4	3	4.05	6	4.2
Candida albicans	3	6.1	4	6.2	7	6.2	4	5.8	4	5.4	8	5.6
Cryptococcus neoformans	2	4.1	3	4.7	5	4.4	3	4.4	4	5.4	7	4.9
Aspergillus species	1	2	2	3.1	3	2.7	1	1.5	1	1.35	2	1.4
Penicillium species	0	0	1	1.6	1	0.9	1	1.5	1	1.35	2	1.4
Alternaria species	0	0	1	1.6	1	0.9	1	1.5	1	1.35	2	1.4
Total fungal isolates	12	24.5	18	28.1	30	26.5	19	27.9	22	29.7	41	28.8

Table 5. Serogrouping *E. coli* and *P. aeruginosa* isolated from mastitic buffaloes and cows

Microorganism	No. of isolates	Serogroups	No. of serogroups	%
E. coli	46	O149	9	19.6
		O126	8	17.3
		O86	7	15.2
		O128	7	15.2
		O119	6	13.1
		O8	5	10.9
		Untyped E. coli	4	8.7
P. aeruginosa	84	K	18	21.4
		H	16	19.05
		M	16	19.05
		A	15	17.9
		G	10	11.9
		Untyped P. aeruginosa	9	10.7

No. of isolates from all examined samples of buffaloes and cows.
% was calculated according to total number of each isolates.

Table 6. Results of antibiogram pattern of the most prevalent bacteria isolated from mastitic buffaloes and caws

Antibacterial agents	*Staph. aureus*		*Strept. agalactia*		*Strept.dys galactia*		*Staph. epidermidis*		*E.coli*		*P.aeruginosa*		*K .pneumoniae*	
	S.	%	S.	%	S.	%	S.	%	S.	%	S.	%	S.	%
Amoxycillin (25ug)	6/15	40	5/15	33.3	0/15	0	6/15	40	0/15	0	0/15	0	1/15	6.7
Chloramphenicol (30ug)	11/15	73.3	1/15	5.7	1/15	6.7	11/15	73.3	0/15	0	1/15	6.7	2/15	13.3
Ciprofloxacin (5ug)	14/15	93.3	13/15	86.7	14/15	93.3	13/15	86.7	14/15	93.3	15/15	100	13/15	86.7
Erythromycin (10ug)	1/15	6.7	1/15	6.7	5/15	33.3	3/15	20	2/15	13.3	0/15	0	3/15	20
Flumequine (30ug)	11/15	73.3	8/15	53.3	14/15	93.3	11/15	73.3	1/15	6.7	0/15	0	14/15	93.3
Gentamicin (10ug)	10/15	66.7	15/15	100	14/15	93.3	14/15	93.3	13/15	86.7	12/15	80	13/15	86.7
Norfloxacin (10ug)	15/15	100	13/15	86.7	12/15	80	15/15	100	15/15	100	8/15	53.3	9/15	60
Polymyxin (10ug)	9/15	60	12/15	80	6/15	40	8/15	53.3	1/15	6.7	0/15	0	6/15	40
Streptomycin (10ug)	12/15	80	10/15	66.7	8/15	53.3	5/15	33.3	14/15	93.3	10/15	66.7	5/15	33.3
Penicillin G (10ug)	0/15	0	13/15	86.7	7/15	46.7	9/15	60	1/15	6.7	0/15	0	5/15	33.3
Kanamycin (30ug)	13/15	86.7	12/15	80	13/15	86.7	13/15	86.7	15/15	100	15/15	100	15/15	100

S: Sensitive.
% : Percentage of sensitive isolates in relation to total isolates.

Table 7. Results of antibiogram pattern of the most prevalent yeast strains isolated from mastitic buffaloes and caws

Antimycotic agents	*Candida krusei*		*Candida tropicalis*		*Candida albicans*		*Cryptococcus neoformans*	
	S.	%	S.	%	S.	%	S.	%
Nystatin (100 µ/d)	8/10	80	7/10	70	8/10	80	10/10	100
Ketoconazole (10 µ/d)	7/10	70	5/10	50	7/10	70	6/10	60
Clotrimazole (10 µ/d)	6/10	60	5/10	50	8/10	80	3/10	30
Floconazole(10µ/d)	3/10	30	4/10	40	5/10	50	4/10	0

S: Sensitive.
% : Percentage of sensitive isolates in relation to total isolates.

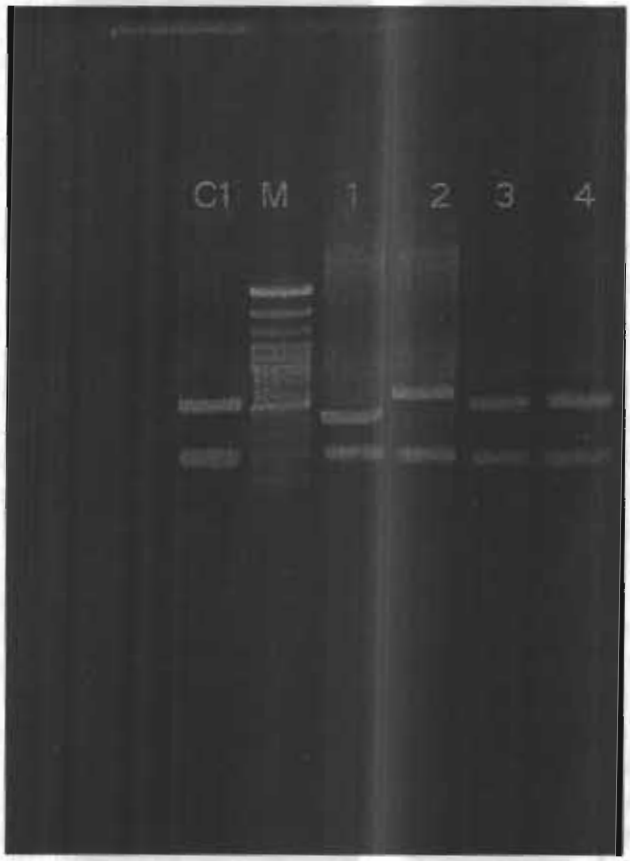

Fig. 1. Electrophoresis analysis of PCR product of amplified *Staph. aureus* coagulase (coa) and 16SrRNA genes

M : 100bp marker.
Lane1,2,3 and 4 indicate a positive amplification *Staph. aureus* coagulase (coa) at the 228bp and variable for 16SrRNA genes:
C_1: Control positive for *Staph. aureus* coagulase (coa) and 16SrRNA genes:

DISCUSSION

Mastitis is a general term which refers to inflammation of the mammary gland, regardless of cause . It is characterized by physical, chemical, bacteriological and mycological changes in the milk , and by pathological changes in the udder. Early recognition and prompt treatment are important for limiting tissue damage and production losses *(44)*.Clinical signs of mastitis appeared 7 to 14 days with a peak of high fever, severe anemia, and the complete cessation of milk production from all quarters *(45)*.

In the present study, the examination of 720 lactating buffaloes and cows (Table,1) , show that the prevalence of total bacteria and fungi causes mastitis in buffaloes and cows were 67.4% from the total examined samples, with total incidence of 60.5% in buffaloes, which is lower than that of cows (75%), this may be attributed to the fact that buffaloes appear more resistant animal. On the other hand, it was clear that the prevalence of subclinical mastitis in buffaloes was higher (39.2%) than in cows (33.2%). The incidences of single and mixed subclinical infection were (51.7% & 48.3%) in buffaloes and (43.4% &56.6%) in cows. Meanwhile, the incidence of clinical mastitis in buffaloes and cows were 21.3% and 41.8% respectively, while the single and mixed infection were (43.2% and 47.9%) in buffaloes and (56.8% and 52.1%) in cows. The incidence of subclinical mastitis in buffaloes was 43.7% *(46)*. The incidences of clinical mastitis in the Sweden, Norway, Denmark and Finland were (21, 30, 56, and 32 cases per 100 cows) respectively *(47)*. The prevalence of subclinical mastitis in dairy herds in Urnguay was (32.5%) on a cow basis and (26.7%) 0n an udder quarter basis *(48)*. The heard prevalence of bovine mastitis was 30.6% *(49)*.The mastitis pathogens were isolated from 26.4% of milk samples in dairy cows in Brandenburg, Germany *(50)*.

The results in table (3 and 4), revealed isolation of different types of bacteria and fungi from mastitic buffaloes with various incidences varying according to type of mastitis. The most predominant cases of subclinical mastitis in buffaloes were due to *Staph. Aureus* *(46)* . Meanwhile *Staph. aureus, Strept. Dysgalactia , Strept.agalactia* and *Enterobacteriaceae* (*E. coli, Klebsiella* spp., *Proteus* spp. and other *coliforms*) (35.3%, 8.1%, 4.9% and 3.3%) respectively, were isolated in 0f all cases of subclinical mastitis in Germany *(51)*. 23.6% of *E. coli* caused mastitis in bovine *(52)*. Bovine mastitis caused by *Strept.agalactia* was mainly subclinical *(15)*. *Staph. aureus* (62.8%), *Strept.agalactia* (11.3%), *Strept. dysgalactia* (1.8%), and *E. coli* (1.5%) were isolated from cases of subclinical mastitis, While the most prevalent isolated pathogens in clinical cases were *Staph. aureus* (37.5%) and *E. coli* (12.5%) in dairy herds in Uruguay *(48)*. The major mastitis pathogens *Streptococcus* spp., *Coliforms, Bacillus Cereus, P. aeruginosa* and *Staph. aureus* at prevalence of (64, 47, 33, 17 and 10%) respectively were isolated from 85 Friesian cows with subclinical mastitis *(53)*.

Meanwhile the total bacteria isolated in clinically mastitic buffaloes were Staph aureus (19.8%) , *Strept .agalactia* (12.3%) , *Strept. dysgalactia* (7.4%) , *Strept. epidermidis* (4.9%), *E. coli* (13.6%), *P. aeruginosa* (21%), *K. pneumonia* (6.2%) and *Proteus vulgaris*(3.7%). While the total bacteria isolated in clinically mastitic cows were *Staph aureus* (21.8%), *Strept. agalactia* (10.6%), *Strept. dysgalactia* (7.8%), *Strept. epidermidis* (3.5%), *E. coli* (6.3%), *P. aeruginosa* (18.3%), *K. pneumonia* (1.4%) and *Proteus vulgaris*(1.4%). It is worthy to mention that there were variations between subclinical and clinical mastitis and between buffaloes and cows. The high prevalence of clinical and subclinical mastitis was mainly caused by *Staph. aureus, Strept. agalactia* and *E. coli* in southern Ethiopia *(54)*. On the other hand ,*E. coli* (12.5%), *C.bovis* (15%), *Staph aureus* (5%)and *Streptococcus* spp.(10%) were isolated from bovine mastitis in Sharkia Governorate *(55)*.

E. coli (41.02%), *Staph. aureus*, (21.09%), *Strept. dysgalactia* (10.16%), *Strept. epidermidis* (9.38%), *Strept. agalactia* (9.38%), *Strept. intermedius* (7.42%), and *Strept. hyicus* (2.34%), respectively were detected from mastitic buffaloes *(56)*. *E. coli* was identified with an

incidence of (25.3%) in mastitis in buffaloes *(57)*. *K. pneumoniae* (9.6%), and (3.75%) *K. oxytoca* (1.6%) and (6.25%) were isolated from apparently healthy and mastitic cows while *K. pneumoniae* (5.36%) and *K. oxytoca* (0.89%) were detected from apparently healthy buffaloes *(58)*. The most commonly isolated bacteria from 56 examined cows with toxic mastitis were *E. coli* from 26 cows and *Staph. aureus* from 11 cows *(59)*.The etiological agents of dairy cows mastitis in Poland were *Streptococcus* spp. (15.7%), coagulase negative *Staphylococci.* (14.6%), *Staph. aureus*, (8.6%), Gram- negative bacilli (4%) and *Corynebacterium* spp. (3.8%). *E. coli* (52.3%), dominated among Gram-negative bacilli followed by *K. pneumoniae* (4.1%), *P. aeruginosa* (3.6%), *Enterobacter cloacae* (3.6%), *P. multocida* (3.1%) and 26 other bacteria species. *Strept. dysgalactia* (19.7%) and *Enterococcus faecalis* (5.3%) were mostly among CAMP –negative *Streptococci (60)*.

While the total isolated fungi in subclinical mastitic buffaloes were Candida krusei (4.7%), Candida tropicalis (2.7%), Candida albicans (2%), Cryptococcus neoformans (4.7%), Aspergillus species (2%), Penicillium species (1.3%) and Alternaria species(0.7%). But, the total isolated fungi in subclinical mastitic cows were Candida krusei (7.9%), Candida tropicalis (3.5%), Candida albicans (6.2%),Cryptococcus neoformans (4.7%), Aspergillus species (3.1%), Penicillium species (1.6%) and Alternaria species (1.6%).

While the total isolated fungi in clinical mastitic buffaloes were Candida krusei(2.5%), Candida tropicalis(2.5%), Candida albicans (3.7%),Cryptococcus neoformans (1.2%), Aspergillus species (1.2%), Penicillium species (0%) and Alternaria species(0%). But, the total isolated fungi in clinical mastitic cows were Candida krusei (9.9%), Candida tropicalis (4.2%), Candida albicans (5.6%), Cryptococcus neoformans (4.9%), Aspergillus species (1.4%), Penicillium species (1.4%) and Alternaria species(1.4%). *Cryptoccus neoformans* and *Candida albicans* were isolated from mastitic Friesian herd *(61)*. *Yeast, Proteus* spp. and *Klebsiella* spp. were detected from mastitic cow's milk samples *(62)*. *Proteus* spp. and *Candida* spp. were isolated from mastitic heifers *(63)*. On the other hand, 12.07% fungi were detected from clinical and subclinical mastitic cows milk (82.86% of these samples were yeasts and 11.95% were moulds) *(20)*. The yeast isolated were, *Cryptococcus* spp.(71 strains), *Rhodotorula* spp. (40), *Candida* spp. (68), *Trichosporon cutaneum* (21) and *Aureobasidium pullulans* (7). Also moulds isolated were *Aspergillus* (3), *Penicillium* (3) and *Alternaria* (3). *Candida* species were isolated from cow milk samples *(64 , 65)*. 9.6% of examined cow milk were positive for fungi, all isolated fungi were yestes of the genera *Candida, Rhodotorula* and *Trichosporon (21)*. On the other hand *Candida krusei, Candida rugosa , Candidaglabrata, Candida albicans* and *Cryptococcus neoformans* were isolated from clinically and subclinically mastitic cows *(24)*.

Yeast mastitis produced almost the same clinical manifestations as other cause of mastitis, so it is difficult to set a diagnosis according to this information. The routine tests, like CMT, are not enough for making accurate diagnosis. If microbiological examination was not carried out for mastitic milk samples, the yeast mastitis is suspected when antibiotic therapy failed *(66)*. This variation in incidence may due to country, management and milking practices or difference in immunity between cows and buffaloes or the lack of an udder health program or associated with factors such as locations, climate and breed.

The results of serogrouping of *E. coli* and *P. aeruginosa* as recorded in table (5), . *E. coli* was isolated from mastitic buffaloes belonged to O114, O125, O44, O126 and O86 *(56)*. While, different sero groups of *E. coli* detected from buffaloes mastitic milk including O111, O119, O86 and O 126 *(57)*.

The result presented in Table 6, indicated that, there was marked difference between the sensitivity to antibiotics and different bacterial isolates. Norfloxacin, enrofloxacin, kananmycin and gentamicin were considered the prevalent antimicrobial agents of choice for treatments of bovine mastitis. 52.1% 0f Staph. aureus isolated from bovine mastitis in Finland were resistant to pencillin G *(49)*. Klebsiella spp. were susceptible

to flumequine, gentamicin, kanamycin and nitrofurantion *(58)*. These variations in sensitivity may be due to nature and distribution of bacteria or regional differences or wrong dose , duration of drugs and plasmid helping in the formation of resistant neural strains .

The results in Table 7. Ketoconazole and Nystatin were the most active antifungals against yeasts isolated from the mammary gland *(67)*.The high susceptibility of yeast strains isolated from clinical cases of animal diseases to Ticonazole and Ketoconazole *(41)*. Nystatin introduced into the teat or Amphotericin B given intravenously is recommended for treatment *(68)*.

The use of polymerase chain reaction (PCR), as shown in Fig. (1), revealed positive amplification of *Staph. aureus* on 228bp fragments for *Staph. aureus* coagulase (coa) and variable fragments for 16SrRNA genes from the extracted DNA of *Staph. aureus* on lane 1-4. These results suggest that the PCR assay could be used as an alternative method in routine diagnosis for rapid, sensitive, and specific simultaneous detection of *Staph. aureus* in milk samples. Also, these results are agreement with the results of *(26 - 28)*.

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الملخص العربي

المسببات البكتيرية والفطرية لالتهاب الضرع في الأبقار في محافظة المنوفية

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يعتبر التهاب الضرع من أهم المشاكل التي تصيب الأبقار والجاموس لما يسببه من خسائر اقتصادية كبيرة. وقد أجريت هذه الدراسة على (٧٢٠) عينة من ألبان الأبقار والجاموس الحلوب والسليمة ظاهريا والمصابة بالتهاب الضرع وذلك لمعرفة أهم المسببات البكتيرية والفطرية للالتهاب الضرع الإكلينيكي وتحت الإكلينيكي بمحافظة المنوفية. (وتشمل العينات علي٣٨٠ من الجاموس و ٣٤٠ من الأبقار).

وبفحص العينات وجد أن نسبة الإصابة بالتهاب الضرع التحت الإكلينيكي كانت (٣٩,٢% و ٣٣,٢%) في الجاموس والأبقار علي الترتيب ، بينما الإصابة بالتهاب الضرع الإكلينيكي كانت (٢١,٣% و ٤١,٨%) في الجاموس والأبقار علي الترتيب .

وبالفحص البكتريولوجي لعينات لبن الجاموس المصاب بالتهاب الضرع التحت الإكلينيكي تم عزل الميكروب العنقودى الذهبي والميكروب السبحي الأجلاكتيا والميكروب السبحي الديس أجلاكتي و الميكروب العنقودى ابيدرمس والأشيرشيا كولاى والسيدومونس اريجينوزا والكليبسيلا نيموني والبروتيس فيلجارس بنسب (٢٢,١% و١٠,١% و١٠,١% و٥,٤ % و ١١,٤% و١٧,٤% و ٣,٤% و٢% على الترتيب). بينما في حالة التهاب الضرع الإكلينيكي تم عزل الميكروب العنقودى الذهبي والميكروب السبحي الأجلاكتيا والميكروب السبحي الديس أجلاكتي و الميكروب العنقودى ابيدرمس والأشيرشيا كولاى والسيدومونس اريجينوزا والكليبسيلا نيموني والبروتيس فيلجارس بنسب (١٩,٨% و١٢,٣% و ٧,٤% و ٤,٩ % و ١٣,٦% و٢١% و ٦,٢% و٣,٧% على الترتيب). وبالفحص للفطريات لعينات لبن الجاموس المصاب بالتهاب الضرع التحت الإكلينيكي تم عزل الكانديدا كروسي و الكانديدا تروبيكالز و الكانديدا البيكانس و الكربتوكوكس نيوفورمانز و الأسبيروجيلس والبنسيليوم والألتيرناريا بنسب (٤,٧% و ٢,٧% و ٢% و٤,٧% و ٢% و ١,٣% و ٠,٧% على الترتيب). ولكن حالة التهاب الضرع الإكلينيكي تم عزل الكانديدا كروسي و الكانديدا تروبيكالز و الكانديدا البيكانس و الكربتوكوكس نيوفورمانز و الأسبيروجيلس والبنسيليوم والألتيرناريا بنسب (٢,٥% و ٢,٥% و ٣,٧% و١,٢% و ١,٢% و ٠% و ٠,٠% على الترتيب).

بينما الفحص البكتريولوجي لعينات لبن الأبقار المصاب بالتهاب الضرع التحت الإكلينيكي تم عزل الميكروب العنقودى الذهبي والميكروب السبحي الأجلاكتيا والميكروب السبحي الديس أجلاكتي و الميكروب العنقودى ابيدرمس والأشيرشيا كولاى والسيدومونس اريجينوزا والكليبسيلا نيموني والبروتيس فيلجارس بنسب (٢٣,٩% و١٠,٦% و ٨% و٥,٢ % و ٨% و١٣,٣% و ٣,٥% و٠,٩% على الترتيب). بينما في حالة التهاب الضرع الإكلينيكي تم عزل الميكروب العنقودى الذهبي والميكروب السبحي الأجلاكتيا والميكروب السبحي الديس أجلاكتي و الميكروب العنقودى ابيدرمس والأشيرشيا كولاى والسيدومونس اريجينوزا والكليبسيلا نيموني والبروتيس فيلجارس بنسب (و٢١,٨% و ١٠,٦% و٧,٨ % و ٣,٥% و٦,٣% و ١٨,٣% و١,٤% و ١,٤% على الترتيب). وبالفحص للفطريات لعينات لبن الأبقار المصاب بالتهاب الضرع التحت الإكلينيكي تم عزل الكانديدا كروسي و الكانديدا تروبيكالز و الكانديدا البيكانس و الكربتوكوكس نيوفورمانز و الأسبيروجيلس والبنسيليوم والألتيرناريا بنسب (٧,٩% و ٣,٥% و ٦,٢% و٤,٧% و ٣,١% و ١,٦% و ١,٦% على الترتيب). ولكن حالة التهاب الضرع الإكلينيكي تم عزل الكانديدا كروسي و الكانديدا تروبيكالز و الكانديدا البيكانس و الكربتوكوكس نيوفورمانز و الأسبيروجيلس والبنسيليوم والألتيرناريا بنسب (٩,٩% و ٤,٢% و ٥,٦% و٤,٩% و ١,٤% و ١،٤% و ١,٤% على الترتيب).

وتم أيضا دراسة مدى حساسية العترات البكتيرية المعزولة للمضادات الحيوية فكانت معظم العترات المعزولة حساسة للأنروفلوكساسين والسبروفلوكساسين والكانامايسين والنورفلوكساسين والجنتاميسين. وكانت معظم الفطريات المعزولة أكثر حساسية للنيستاتين والكيتوكونازول .

وقد تم استخدام اختبار تفاعل البلمرة المتسلسل كطريقة حديثة وسريعة لتشخيص الميكروب العنقودي الذهبي باستخدام البريمر المخصص له . وقد أثبتت النتائج سرعة ودقة اختبار تفاعل البلمرة المتسلسل في التشخيص المعملي السريع لالتهاب الضرع من عينات اللبن مباشرة .