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Studies on Coliform Mastitis in River Nile State, Sudan

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Abstract

The term coliform mastitis is used here to include the mastitides in catttle caused by E. coli, Klebsiella spp and Enterobacter aero genes. A total of 100 bovine milk samples positive for California Mastitis Test (CMT) were collected from different River Nile State localities and submitted for bacteriological examination. 155 bacteria isolated from mastitic milk samples. Gram positive bacteria represented 64% of the total isolates. Coliform bacteria represented 28.4% of the bacteria isolated. The incidence of coliform mastitis was 28.4%. Coliform bacteria were subjected for further confirmation by using Api 20E and VITEK2 identification systems. Isolates scored high probability percentages by using rapid Api20E and automated VITEK2 identification systems. Coliform bacteria isolated from mastitic milk samples were Escherichia coli (12.4%), Klebsiella pneumoniae (8.4%) and Enterobacter aero genes (7.6%). Twenty E. coli isolates were examined for enterotoxins production by using suckling mouse test. Fifteen isolates (90.0%) gave positive results with SMT test.

Introduction

The term coliform mastitis is used here to include the mastitides in catttle caused by *E. coli*, Klebsiella spp. and *Enterobacter aerogenes*. Each of these can cause a peracutemastitis, sometimes in outbreak form, resulting in large economic losses. The prevalence of coliform mastitis has increased considerably in recent years and is a cause for concern in the dairy industry and among dairy practitioners [1]. *Escherichia coli* was defined as the most common Gram– negative bacillus associated with clinical and subclinical mastitis [2]. *E. coli* might cause acute and peracute form of clinical mastitis [1]. *Klebsiella spp.* are the second most common Gram–negative rods isolated from cattle milk infected with mastitis [3]. Enterobacter spp. were found to cause bovine mastitis [4]. Other Gram-negative bacteria frequently isolated from intramammary infections include species of *Serratia*, *Pseudomonas*, and *Proteus.*Gram-negative bacteria are considered environmental mastitis pathogens [5]. Transfer of Gram-negative bacteria from the mammary glands of infected cows to uninfected cows appears minimal compared with the constant environmental exposure.

Coliform bacteria occupy many habitats in the cow's environment. *Escherichia coli* are normal inhabitants of the gastrointestinal tract of warm blooded animals. Both *Klebsiella* spp. and *Enterobacter* spp. populate soils, grains, water, and intestinal tracts of animals. *Serratia marcesens* share many environmental sources with *Klebsiella* spp. and *Enterobacter* spp. *Pseudomonas* spp. and *Proteus* spp. commonly contaminate drop hoses used to wash udders before milking. Gram negative bacteria may be isolated from virtually any surface area of the cow or her surrounding and cause a host of diseases other than mastitis. Coliform bacteria are among the aetiological agents commonly responsible for infectious respiratory and urogenital diseases in dairy cows. However, the spread of Gram-negative bacteria from other regions of the body to the mammary gland via the vascular or lymphatic systems appears minimal. Intramammary infections caused by Gram-negative bacteria typically result from the bacteria traversing the teat canal and multiplying in the gland. Although the mammary gland is not considered a natural habitat for coliform bacteria, many strains are capable of surviving and multiplying in the mammary gland [1]. Adherence of *E. coli* and *K. pneumoniae* to epithelial tissue does not play a major role in the pathogenesis of bovine mastitis [6, 7]. Coliform bacteria do not appear to colonize inside the mammary gland, but multiply in the secretion without attachment to epithelial surfaces. Secretions from fully involuted mammary glands do not readily support growth and multiplication of coliform bacteria [8]. Lactoferrin is an iron binding protein that increases in mammary secretion during involution and remains elevated until colostrogenesis [9]. The ability of *E. coli* and *K. pneumoniae* to cause mastitis is related to the ability of these isolates to overcome the inhibitory properties of lactoferrin . *Klebsiella pneumoniae* are more capable than most strains of *E. coli* to overcome the inhibitory effects of lactoferrin and infect involuted mammary glands [10]. Coliform bacteria that can multiply in the secretion of involuted glands probably overcome the effects of lactoferrin by utilization of a high affinity iron acquisition systems.

The objective of this research work was to investigate coliform bacteria implicated in bovine mastitis in River Nile State using conventional, rapid and automated systems for bacterial isolation. And to study the pathogenicity of *E. coli* isolated from matitic milk samples.

Materials and Methods

Area of the Study

A total of 100 bovine milk samples positive for California Mastitis Test (CMT), were collected from farms of the three localities in River Nile State (Atbara, El Damar and Barber) in River Nile State in Sudan. In the three localities samples were collected from special dairy cattle farms markets and veterinary hospitals.

Sampling Procedure

Before collection of milk samples from the tested cows, the udder was thoroughly cleaned with soap and water, rubbed dry, and the teat area was rubbed thereafter with a piece of cotton soaked in 70% alcohol . The first stream of milk was discarded. The California Mastitis Test was directly applied for quarter's milk and samples were collected from positively reacted milk into sterile bottles. Swabs were taken from hands of milkers before milking the cows. The collection of samples was at (2-5) pm. The collected samples were put in ice box containing ice and transported to the laboratory. In most cases the time between collection and arrival to the laboratory was 1-2 hrs. In the laboratory mastitic milk samples were kept in a deep-freezer and swabs

were soaked in tubes containing nutrient broth and incubated at 37C. All samples were examined on the next day. On the next day mastitic milk samples were removed from the deep–freezer and left on the bench to thaw. Swabs were removed from the incubator. Samples were then cultured.

Isolation, Identification and Characterization of Bacterial Isolates

All media (Oxoid media) were prepared and sterilized according to the manufacturer instructions. For the primary isolation of bacteria, a loop full milk sample was streaked onto blood agar, McConkey's agar, and nutrient agar using sterile wire loop. The cultures were incubated aerobically at 37oC for 18-24 hours. Cultures on semi-solid media were examined grossly for colonial morphology and haemolysis on blood agar. Whereas, broth media were checked for turbidity, change in colour, accumulation of gases in CHO media and for sediment formation. One half colony from each plate was used for performing gram staining. Colonies which showed Gram negative bacilli were sub-cultured on nutrient agar. Purification was based on the characteristics of colonial morphology and smear. This was obtained by sub culturing of a typical discrete colony on blood agar plate. Pure cultures were preserved on slants of blood agar and egg media at 4^{o}C.

Biological and Biochemical Identification

The purified isolates were identified as previously described [11] and [12]. The identification include: Gram's reaction, presence or absence of spores, shape of organism, motility, colonial characteristics on different media, aerobic and anaerobic growth, sugars fermentation ability and biochemical tests (staining of smear, catalase test, oxidase test, coaggulase test, oxidation fermentation test, motility test, glucose breakdown test, fermentation of carbohydrates, urease activity, citrate utilization, gelatin hydrolysis test, nitrate reduction test).

API 20E (BIOMERIEUX, France)

According to [13], API 20E is a standardized identification system for Enterobacteriaceae and other Gram-negative rods which uses 23 miniaturized biochemical tests and a data base. The API 20E strip consists of 20 microtubes containing dehydrated substrates. These microtubes are inoculated with a bacterial suspension, prepared in API 20E medium that reconstitutes the tests. During incubation metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The reactions were read according to the reading table and the identification is obtained by referring to the analytical profile index or using the identification soft-ware. Tests included in API 20E strips were Ortho-nitro-phenyle-galactoside (ONPG), Arginine (ADH), Lysine (LDC), Ornithine (ODC), Sodium citrate (CIT), Sodium thiosulphate (H_2S), Urea (URE), Tryptophane (TDA), Indole (IND), Sodium pyruvate (VP), Kohn's gelatin (GEL), Glucose (GLU), Mannitol (MAN), Inositol (INO), Sorbitol (SOR), Rhamnose (RHA), Sucrose (SAC), D-melibiose (MEL), Amygdalin (AMY), Arabinose (ARA), Oxidase (OX), Nitrate (NO3-NO2), Motility (MOB), MacConkey growth (MAC) and Oxidation-fermentation test (OF).

The incubation boxes (trays and lids) were prepared and 5ml of distilled water was distributed into the honey–combed wells of the trays to create a humid atmosphere. The strains references were recorded on the elongated flab of the trays. The strips were removed from their packaging and placed in the incubation boxes. The organisms were sub cultured onto blood agar and incubated at 36^{o}C ± 2 for 18–24 hours. They

were checked to be belonging to *Enterobacteriaceae* Family (morphology, Gram stain, catalase, oxidase, etc.) and they were pure cultures. Ampoles of API 20E medium were opened and homogeneous bacterial suspension prepared. These suspensions were used immediately after preparation. Both tubes and cubules were filled with the inoculated API 20E media by using a pipette. Anaerobiosis was ensured in the ADH, LDC, ODC, URE and H_2Stests by filling the cupules with mineral oil to form a convex meniscus. The incubation boxes were closed and incubated at $36^oC \pm 2$ for 18–24 hours. After the incubation period, the reaction was developed by adding one drop of each of the following reagents and then results were read by referring to the reading tables.

VITEK 2 (BIOMERIEUX, France) [14]

The identification card for Gr –ve bacilli (ID-GNB card) for the vitek 2 system is a 64-well plastic card containing 41 fluorescent biochemical tests, including 18 enzymes tests for aminopeptidases and aminosidases. Substrates used for detection ofaminopeptidases are usually coupled with 7-amino-methylcoumarin (7AMC); substrates for detection of aminosidases are usually coupled with 4-methylumbelliferone (4MU). The 18 tests substrates are as follows: 4MU-α-arabino-pyranoside, 4-MU-α-D-galactoside, α-L-glutamic acid-7AMC, 4MU-β-D-cellobiopyranoside,4MU-β-D-galactoside, 4MU-β-D-glucoside, 4MU-β-D-glucuronide, 4MU-β-D-Mannopyranoside, 4MU-N-acetyl-β-D-glucosaminide, 4MU-N-acetyl-β-D- galactosaminide, 4MU-β-D-xyloside, glutaryl- glycyl-arginine-7AMC, γ-L-glutamic acid-7AMC, 4MU-phosphate, L-proline-7AMC, L-pyroglutamic acid-7AMC, L-lysine-7AMC and Z-arginine-7AMC.Furthermore, the ID-GNB card includes 18 fermentation tests (adonitol, L-arabinose, D-cellobiose, D-galacturonate, D-glucose, glucose-1-phosphate, D-glucuronate, inositol, 5-kito-gluconate, D-maltose, D-mannitol, D-melibiose, palatinose, D-raffinose, L-rhamanose, sucrose, D-sorbitol and D-trehalose, 2 decaboxylase tests (Ornithine and lysine), and miscellaneous tests (urease and utilization of malonate and tryptophane deaminase). A sterile plastic stick applicator was used to take pure colonies from culture media and transfer a sufficient number of them to plastic test tubes. Test tubes contained about 3.0 ml of sterile saline to suspend the microorganism in. After mixing by shaker in order to produce a homogenous suspension of bacteria, the turbidity of suspension was adjusted to (0.50-0.63 MacFarland) by adding proper amounts of saline or bacteria. The density (turbidity) of the suspension was checked by using ATB 1550 densiometer (biomerieux). The time between preparation of the suspension and card filling was less than 30 minutes. GN cards were loaded (inoculated) with bacterial suspensions using a vacuum chamber in machine. Test tubes containing the samples were placed into a cassette (a special test tube rack) and the identification card was placed in the neighboring place while inserting the transfer tube into the corresponding suspension tube. The cassette could accommodate up to 10 test tubes. The filled cassette was placed into a vacuum chamber station inside the vitek 2 analyzer machine. The vacuum was applied then the air was recharged into the station, the bacterial suspension was forced through the transfer tube into micro-channels that filled all the test wells. Inoculated cards were passed by a mechanism, which cut off the transfer tube and sealed the card prior to loading into the circular incubator. The incubator could accommodate up to 60 cards. The card was automatically filled by a vaccum device and automatically sealed. It was manually inserted in the viteck 2 reader-incubater module (incubator T^o , 35.5^oC). and every card automatically subjected to a kinetic fluorescence measurement every

15 minutes. All used cards were automatically discarded in waste container. The results were interpreted by the ID-GNB database after the incubation period.

Detection of STa Enterotoxin by Suckling Mouse Test (SMT):

This biological method described by [15] and standardized by [16]. The principal of the test is that, the injection of STa preparation into the stomach of 2-4 days old suckling mouse causes fluid accumulation in the intestine. Volumes of 100ml of Brain Heart Infusion Broth in 100ml conical flasks were inoculated with a loop swept lightly a cross several colonies of *E. coli* grown on blood agar plates. The flasks were incubated in a water path shaker (100rpm) at 37^{o}C for 24 hours. The broth cultures were coldly (4^{o}C) centrifuged at 4000rpm for 15 minutes and the supernatant was collected. This supernatant constituted the STa which was aseptically stored at 4^{o}C before use. Two days after birth infant mice were inoculated with 0.1ml STa into the stomach using a special canula. After inoculation the mice were kept at room temperature for 4 hours and then decapitated or killed. The abdomen was opened (after killing of mice); the small intestines were examined for distension and then removed by forceps. The intestines were then weighted using a sensitive balance and the ratio of gut weight to the body weight was calculated. Ratios of less than 0.070 were considered negative. Those in range of 0.070-0.090.were considered doubtful positive and those over 0.090 were
positive.

STATISTICAL ANALYSIS

Statistical analysis was done through Microsoft office Excel 2007.

Results and Discussion

A total of 100 CMT positive mastitic milk were collected from different localities of River Nile State.

Isolated Bacteria from Mastitic Milk Samples

According to the cultural characteristics, bacterial morphology, biochemical reactions results, API 20E and VITEK2 systems results, a total of 155 microorganisms were isolated from mastitic milk samples. Gram negative bacteria represented 36% of the total isolates (figure 1). Coliform bacteria included *Escherichia coli* (12.4%), *Klebsiella pneumoniae* (8.4%) and *Enterobacter aerogenes* (7.6%)(figure 2).

API 20E Strips

E. coli, *Klebsiella pneumoniae* and *Enterobacter aerogenes* isolates were selected randomly, inoculated into API 20E strips and incubated aerobically at 37C for 24hrs. There results are shown in table (2) with different identification percentages. Their tests were read according to the interpretation table.

VITEK 2 Identification System

E. coli, *Klebsiella pneumoniae* And *Enterobacter aerogenes* isolates were selected randomly and inserted into VITEK2 automated identification system. According to interpretation results isolates scored high probability percentages (Table 3).

Suckling Mouse Test Results

Twenty *E. coli* isolates were selected randomly and examined for their production of STa. Weight of intestines, weight of residual carcasses and calculated mean ratios for tested isolates were shown in table (4). A mean ratio of 0.09 or over was considered positive

and ratios less than 0.09 were negative results. Fifteen isolates (90.0%) gave positive results with SMT test.

Table 1 Number of Milk Samples Collected From Different Farm

Farms	No. of Samples
Omer Amir	10
Barbar	12
El Damer	20
El Damar market	10
Abdelrahman	10
Alaa Eldin	10
Atbara hospital	10
Research center	8
Akram	10
Total	100

Table 2 API 20E Identification System Results of the Isolated Coliform Bacteria

Tests	***E. coli***	***Klebsiella pneumoniae***	***Enterobacter aerogenes***
Ortho-Nitro-Phenyle-Galactoside (ONPG)	-	+	+
Arginine (ADH)	-	-	-
Lysine (LDC)	+	+	+
Ornithine (ODC)	+	-	-
Na Citrate (CIT)	-	+	+
Na Thiosulfate (H2S)	-	-	-
Urease (URE)	-	+	-
Tryptophane (TDA)	-	-	-
Indol (IND)	+	-	-
Voges-Proskauer (VP)	-	+	+
Gelatinase (GE)	-	-	-
Glucose (GLU)	+	+	+
Mannitol (MAN)	+	+	+
Inositol (INO)	-	+	+
Sorbitol (SOR)	+	+	+
Rhamanose (RHA)	+	+	+
Sucrose (SAC)	+	-	+
Melibiose (MEL)	+	+	+
Amygdalin (AMY)	-	-	+
Arabinose (ARA)	+	+	+
Nitrate reduction (NIT)	-	-	+
Identification (%)	**99.0**	**98.7**	**98.3**

Table 3 VITEK2 System Results of the Isolated Coliform Bacteria

Tests	*E. coli*	*Klebsiella pneumoniae*	*Enterobacter aerogenes*
2 APPA	-	-	-
10 H_2S	-	+	-
17 BGLU	-	+	+
23 ProA	-	-	-
33 SAC	+	+	+
40 lLATK	+	+	+
46 GlyA	-	-	-
58 O129R	+	-	+
3 ADO	-	-	-
11 BNAG	-	-	-
18 dMAL	+	+	+
26 LIP	-	-	-
43 dTAG	-	-	-
41 AGLU	-	+	+
47 ODC	+	-	-
59 GGAA	-	-	-
4- PyrA	-	+	+
12 AGLTp	-	-	-
19 dMAN	+	+	+
27 PLE	-	-	-
35 dTRE	+	+	+
42 SUCT	+	+	+
48 LDC	+	-	-
61 lMLTa	-	-	-
5 lARL	-	-	-
13 dGLU	+	+	+

Table 3 (continued)

Tests	***E. coli***	***Klebsiella pneumoniae***	***Enterobacter aerogenes***
20 dMNE	+	+	+
29 TyrA	-	-	-
36 CIT	-	+	+
43 NAGA	-	-	-
53 lHISa	-	-	-
62 ELLM	+	+	+
7 dCEL	-	+	-
14 GGT	-	-	-
21 BXYL	-	-	+
31 URE	-	+	-
37 MNT	-	-	-
44 AGAL	+	+	+
56 CMT	+	+	+
64 lLATa	-	-	-
9 BGAL	+	+	+
15 OFF	+	+	+
22 Balap	-	-	-
32 dSOR	+	+	+
39 5KG	+	+	+
45 PHOS	-	-	-
57 BGUR	+	+	+
Probability (%)	**99.0**	**98.3**	**97.0**

Table 4 Detection of STa Enterotoxin Produced by *E. coli* Isolates Using Suckling Mouse Test (SMT)

***E. coli* isolates**	**Intestinal weight**	**Carcass weight**	**Ratio**
1	0.096	1.000	0.096
2	0.216	1.304	0.165
3	0.322	1.322	0.244
4	0.212	1.468	0.212
5	0.146	1.000	0.146
6	0.332	1.692	0.196
7	0.091	1.192	0.076
8	0.242	1.416	0.170
9	0.174	1.124	0.154
10	0.027	1.184	0.022
11	0.292	1.486	0.197
12	0.218	1.276	0.171
13	0.356	1.532	0.232
14	0.101	1.224	0.083
15	0.264	2.080	0.127
16	0.099	1.225	0.080
17	0.660	1.312	0.503
18	0.082	1.620	0.078
19	0.146	1.016	0.144
20	0.184	1.04	0.176

Figure 1 Gram Positive and Gram Negative Bacteria Isolated From Mastitic Milk Samples

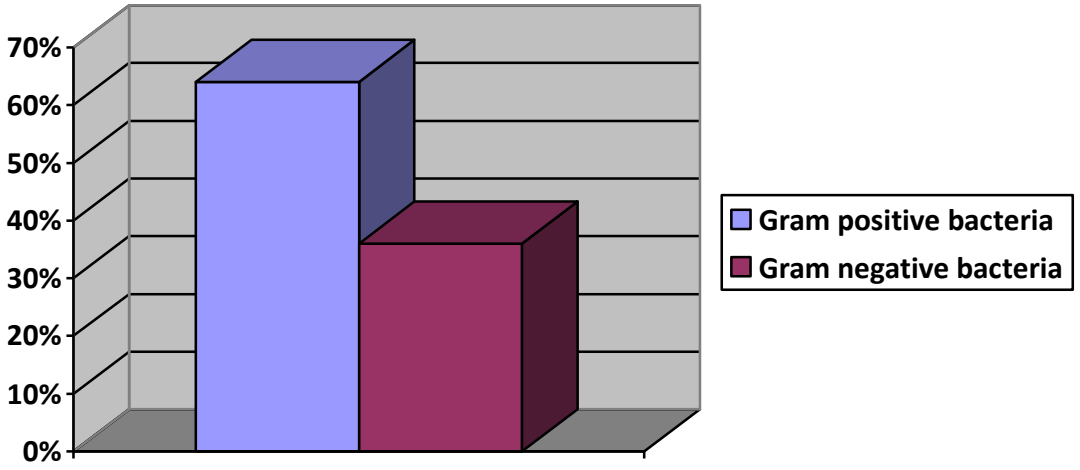
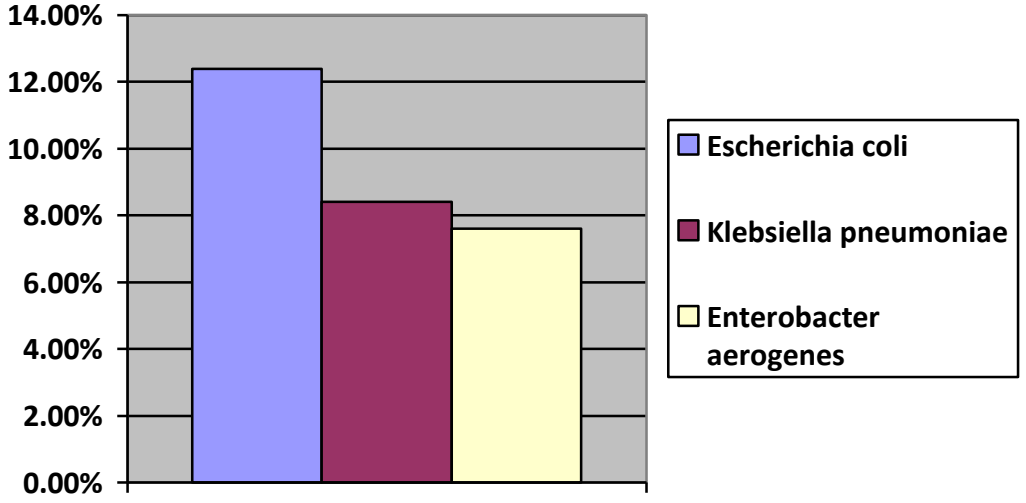


Figure 2 Coliform Bacteria Isolated From Mastitic Milk Samples



Coliform pathogens generally account for the majority of peracute cases of clinical mastitis in a herd. Specifically, *E. coli* and *K. pneumoniae* are the coliform species most commonly isolated from intramammary infections and clinical mastitis [17]. A total of 100 bovine milk samples positive for CMT, were collected from farms of the three localities in River Nile State in River Nile State. 155 bacteria isolates were isolated from the samples. Gram positive bacteria represented 64% of the isolated bacteria and gram negative represented 36%. [18] found that, coliform bacteria are responsible for 30-40% of subclinical cases of bovine mastitis. In thisColiform bacteria included *Escherichia coli* (12.4%), *Klebsiella pneumoniae* (8.4%) and *Enterobacter aerogenes* (7.6%). According to [1] who mentioned that *Escherichia coli* was defined as the most common Gram– negative bacillus associated with peracute, clinical and subclinical forms of mastitis. *Klebsiella pneumoniae* was the second coliform bacteria isolated in this study and that was found by [19]. *Enterobacter aerogenes* came in third place of the isolated coliform bacteria. [4] found that *Enterobacter aerogenes* causes bovine mastitis. For the identification of the isolates API 20E strips were used. They showed different identification percentages ranging from 99.8% to 89.8%. Slight variations in the biochemical behavior of the microorganism may be attributed to variations in the genetic constituents of different stains resulting in different phenotypic characteristics. These genetic variations may be of chromosomal or plasmid origin [20, 21]. In both circumstances, transfer of genetic material between strains of the same species does occur through different mechanisms. These findings generally fulfilled the API 20E requirements for identification as *E. coli* [22, 23]. Isolates were selected randomly and inserted into VITEK2 automated identification system. They scored high probability percentages and there were slight variations in the results of their biochemical tests. Although the results of biochemical reactions revealed many differences between isolates strains, they fulfilled the requirements for identification by VITEK2 system [24, 25]. The present findings showed that VITEK2 system as an automated system provided very good and trustable accuracy and reproducible results as shown in repeated samples of same source. Economic studies estimate a good future for automated systems in microbiology lab [26- 27]. Isolation of *E. coli* doesn't necessarily means the presence of the disease unless, virulence factors are identified i.e. toxins and/or fimbrial antigen [28, 29-30]. In this study 20 randomly selected *E. coli* isolates were tested by the suckling mouse test (SMT) for production of heat-stable (STa) enterotoxin. The result of SMT in this report is in accordance with [31] who found that 85.7% of isolated *E. coli* produced STa enterotoxin. The reliability of the suckling mouse was also confirmed by [32]. Nevertheless this disagrees with [33] who stated that the infant mouse test is unsatisfactory as a method for detection of STa enterotoxins.

Conclusion

This study clearly revealed the high incidence rate of coliform mastitis (28.4%). The study also revealed that *E. coli* is the predominant fungal spp. isolated from bovine mastitic milk samples in River Nile State. Moreover, *E. coli* is involved in bovine mastitis. Other coliform bacteria found in mastitic milk samples in this study included *Klebsiella pneumoniae* and *Enterobacter aerogenes*, which are responsible for bovine mastitis. Rapid API20E and automated Vitek2 systems provided very good and trustable accuracy and

reproducibility.

Further studies should be carried out to investigate the predisposing factors related to the incidence of bovine coliform mastitis and to identify different causes of bovine mastitis. Further studies should include a survey of more animals in different farms and an extensive study of the significance of different microorganisms in bovine mastitis. Moreover the serotyping of isolates obtained from different areas should be given more attention.

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