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Phenotypic and Molecular Identification of *Klebsiella* and *Salmonella* Species Isolated from Subclinical Mastitis Milk of Egyptian Buffalo

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Abstract: A total of 100 dairy buffaloes were included in a field survey to identify subclinical mastitis (SCM) using California Mastitis Test (CMT). Subsequently, samples were screened for the presence of *Klebsiella* and *Salmonella* species (spp.) using classical bacteriological and molecular methods. It was found that 60% (60/100) and 43% (172/ 400) of the examined animals had SCM on the animal and the quarter levels, respectively. Out of the 172 SCM samples, 59 (34. 3%) were contaminated with *Klebsiella* spp.; 28 (16.3%) *K. pneumoniae*, 14 (8.1%) *K. oxytoca* and 17 (9.9%) other unclassified *Klebsiella* spp. Moreover, *Salmonella* spp. were detected in 18 (10.47%) of the examined milk samples. However, molecular identification using PCR revealed that only 36 out of the 59 isolates (20.9%) were identified as *Klebsiella* spp. [20 *K. pneumoniae* (11.6%, n=172) and 16 other *Klebsiella spp.* (9.3%, n=172)]. Also, only 13 out of the 18 *Salmonella* strains (7.6%, n=172) were confirmed as *S. typhimurium*. We concludes that the conventional identification of these two Gram negative bacteria should be confirmed by molecular identification due to great similarity between these two bacteria and other bacteria and this is very necessary to avoid misdiagnosis.

Key words: Subclinical Mastitis · Salmonella · Klebsiella · PCR

INTRODUCTION

Buffaloes occupy a prominent place in the social, economic and cultural life of rural communities in many countries. They are recognized as the world second most important milk producing species [1]. Subclinical mastitis (SCM) is considered as the most important cause of economic losses that results in a marked reduction in quality and quantity of milk as well as it leads to lactation termination and involuntary culling of dairy buffaloes [2]. SCM is also associated with many zoonotic diseases in which milk acts as a vehicle for pathogens. The majority of milk borne diseases such as tuberculosis, brucellosis and typhoid fever occur from consumption of milk from animals that harbor organisms potentially pathogenic for humans [3].

Mastitis caused by Gram-negative bacteria is of increasing importance in modern and well-managed dairy farms. Coliforms are the major environmental pathogens in the etiology of bovine mammary infections [4]. They contaminate milk through feces, water and soil. Among these coliforms, the opportunistic pathogens, *Klebsiella* spp. cause the severe mastitis cases. Mastitis due to *Klebsiella* infection results in higher milk losses than

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those due to *E. coli* and can lead to animal death [5]. *Klebsiella* spp. are defined as germs that easily produce enzymes like extended-spectrum β-lactamases (ESBL) which give them the ability to survive longer than other Gram-negative rods in the environment and on the skin [6]. *K. pneumoniae* and *K. oxytoca* are frequently isolated from domestic livestock [7]. *Salmonella* has been widely reported in buffaloes [8, 9]. Infected animals may shed the organism in their feces without showing any clinical signs of disease. Presence of *Salmonella* in milk is due to direct contact with contaminated sources in the dairy farm environment and excretion from the udder of an infected animal [10].

The currently used standard biochemical/phenotypic tests lack the sensitivity required to accurately discriminate among the Gram negative bacteria. For example, some phenotypically isolated Gram negative bacteria were identified as being *K. pneumoniae*, but actually they were other bacterial spp. (Raoultella, *E. cloacae* and Providencia) as detected by PCR [11, 12]. Therefore, the present study aimed to screen hand milking buffalo affected by SCM for *Klebsiella* and *Salmonella* spp. by microbiological and biochemical methods as well as PCR based-molecular techniques.

MATERIALS AND METHODS

Sample Collection: A total of 400 milk samples were aseptically collected from 100 apparently healthy hand milking buffaloes raised on small holder farms in Kafr EL-Sheikh Governorate, Egypt. No systemic or local antimicrobial drugs were given to these animals for at least 1 month before collection of milk. For initial confirmation of SCM status of the animals, milk samples originated from apparently healthy quarters of the udder were subjected to CMT. Before sample collection, the examined udders and teats were thoroughly washed, dried and disinfected with 70% ethanol. The first few strips were discarded and a milk sample from each quarter was tested by CMT and the results were interpreted as described by Schalm *et al*. [13]. Twenty ml of milk samples from each CMT positive quarter were collected in a sterile container and kept at 4°C for further lab diagnosis.

Standard Phenotypic/Biochemical Testing for Pathogens Identification: For isolation and phenotypic identification of SCM associated bacteria, milk samples were streaked on blood agar with 7% sheep blood and MacConkey agar after their pre-incubation at 37°C for 8-12h. Each milk sample was also inoculated in a tube of Rappaport-Vassiliadis medium with soya (RVS) broth then incubated at 41.5 °C ± 1 °C for 24h ± 3h for *Salmonella* enrichment before streaking onto xylose-lysine deoxycholate agar (XLD) and Salmonella-Shigella (SS) agar as recommended by ISO [14]. The plates were incubated aerobically at 37°C for 24-48h. Suspected colonies were described for their morphological characteristic appearance and hemolytic activity, followed by Gram staining and motility tests before being transferred into semisolid or slope agar to be subjected for further identification according to Quinn *et al.* [15]. The IMViC tests were performed to distinguish between members of Enterobacteriaceae. The methyl red test differentiates *Klebsiella spp.* from *Enterobacter cloacae*; and the indole test differentiates between *K. pneumoniae* and *K. oxytoca*. Urease hydrolysis and triple sugar iron tests were also applied. Later on, the isolates of *Klebsiella* and *Salmonella* spp. were harvested on nutrient broth and submitted to PCR identification.

Molecular Identification by PCR: Chromosomal DNA was extracted from the 59 isolates of *Klebsiella* spp. and the 18 isolates of *Salmonella* spp. using a rapid boiling procedure according to Darwish and Asfour [16]. Briefly, 1ml broth per each isolate was taken from the nutrient broth and centrifuged at 5000 rpm to sediment the bacterial pellet. The latter was washed twice using Tris EDTA buffer and finally suspended in 200 µl of lysis buffer [1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. After boiling for 10 min, the suspension was centrifuged for 5 min to sediment bacterial debris. The supernatant was aspirated and from which 5 µl was used directly for PCR amplification.

Two separate multiplex PCR (The first one for *Klebsiella* genus and *K. pneumoniae* and the second one for *S. typhimurium* and *S. enteritidis*) and a separate individual PCR for *Salmonella* genus were used. A reaction mixture of 20 µl containing 5µl DNA template, 20 pmol of each primer (Table 1) [17-21] and 1X of PCR Master mix (Dream Taq Green PCR Master Mix, Fermentas) was applied into Nexus gradient Master cycler (Eppendorf, Germany). Reaction conditions were optimized to be 95°C for 4 min as initial denaturation, followed by 35 cycles of 95°C for 1 min, annealing at 55, 57°C (Table 1) for 1 min and 72 °C for 1 min, with a final extension step at 72°C for 10 min. PCR products were

electrophoresed in 1.5% agarose gel containing 0.5X TBE at 100 volts for 40 min. Gels were stained by ethidium bromide and visualized by UV trans-illuminator.

RESULTS

CMT results revealed SCM prevalence rate of 60% (60/100) per animal and 43% (172/400) per quarter. The bacteriological identification revealed the prevalence for *Klebsiella* spp. was 34.3% (59/172) (Table 2). The isolated 59 *Klebsiella* isolates were conventionally classified using biochemical tests into; 28 *K. pneumoniae* (47.5% & 16.3% of *Klebsiella* isolates and the total 172 examined milk samples, respectively), 14 *K. oxytoca* (23.7% & 8.1% of *Klebsiella* isolates and the total 172 examined milk samples, respectively) and 17 other unclassified *Klebsiella* spp. (28.8% & 9.9%, of *Klebsiella* isolates and the total 172 examined milk samples, respectively) (Table 2).

Molecular identification using a multiplex PCR assay showed that only 36 out of the total 59 isolated strains were identified as *Klebsiella* spp. with a percentage of 61.02% (about 20.9% of the total 172 examined milk samples). The 36 molecularly identified Klebsiella isolates were classified into 20 *K. pneumoniae* (55.6%, about 11.6% of the total 172 examined milk samples); and 16 unclassified *Klebsiella* spp. (44.4%, 9.3% of the total 172 examined milk samples) (Table 2 and Fig. 1).

Salmonella spp. were detected in 18 (10.47%) of the examined samples by conventional phenotypic method. However, molecular identification of these *Salmonella* isolates revealed presence of only 13 (7.6%) all of them were *S. typhimurium* (Table 2 and Fig. 2).

DISCUSSION

The annual increase rate in buffalo milk production is about 3.8%, which is the highest rate among the other livestock species producing milk in Egypt [22]. Therefore, further advanced research about SCM problem is urgently needed for the dairy buffaloes, also a wider respect for the neglected causative microorganisms of public health hazard for the milk consumers is required. This prompts us to conduct this study.

Although buffaloes have been reported to be less susceptible to mastitis than cattle [23] it was found in the present study that 60% of the examined buffaloes had SCM per animal and 43% per quarter. In consistence,

Table 1: Primer sequences, annealing temperature (Ta), size, species specific and target gene.

Target gene	Primer name	Sequence 5'-3' (Reference)	Ta	Size (bp)	Spp specific	Ref
gyr A	Kleb. F	CGCGTACTATACGCCATGAAC GTA	57	441	*Klebsiella spp*	[18]
	Kleb. R	ACCGTTGATCACTTCGGTCAGG				
rpoB	KP F	CAA CGG TGT GGT TAC TGA CG	57	108	*Klebsiella pneumonia*	[17]
	KP R	TCT ACG AAG TGG CCG TTT TC				
invA	invA-F	GCCATGGTATGGATTTGTCC	55	118	*Salmonella spp.*	[19]
	invA-R	GTCACGATAAAACCGGCACT				
Sdf	Sdf-F	TGTGTTTTATCTGATGCAAGAGG	55	333	*Salmonella enteritidis*	[20]
	Sdf-R	CGTTCTTCTGGTACTTACGATGAC				
mdh	MDH F	TGCCAACGGAAGTTGAAGTG	55	261	*Salmonella typhimurium*	[21]
	MDH R	CGCATTCCACCACGCCCTTC				

Table 2: Phenotypic and molecular identification of *Klebsiella* and *Salmonella spp.*

	Phenotypic identification			Molecular identification		
Isolated species	No.	%*	%**	No.	%*	%**
Klebsiella spp.	59	34. 3	100	36	20.9	61.02
K. pneumoniae	28	16.3	47.5	20	11.6	55.6
K. oxytoca	14	8.1	23.7			
Other unclassified *Klebsiella* spp.	17	9.9	28.8	16	9.3	44.4
Salmonella spp.	18	10.47	100	13	7.6	72.22
S. typhimurium	18	10.47	100	13	7.6	100
S. enteritidis	-	-	-	-	-	-

* The % was calculated according to the total number (n=172) of the tested milk samples.

** The % was calculated according to the total number of the phenotypic isolated strains of each bacterial species (59 for *Klebsiella* and 18 for *Salmonella* spp.)

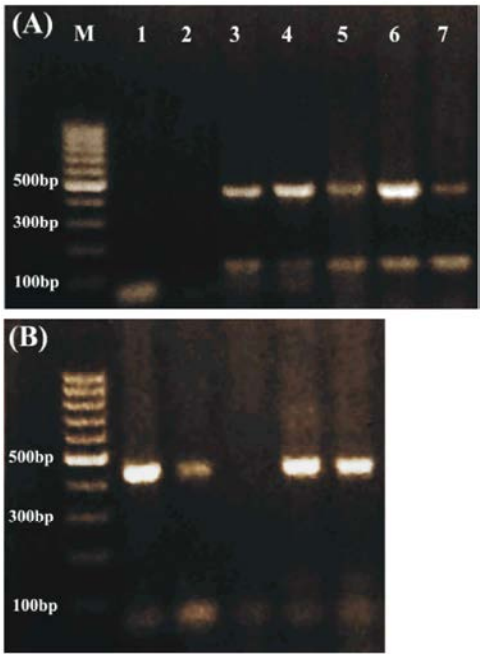

Fig. 1: Gel electrophoresis shows *Klebsiella* multiplex PCR. A) Negative control (Lane 1); Klebsiella negative strain (Lane 2); *K. pneumoniae* strains (441 and 108 bp, lanes 3-7); 100 bp ladder (M). B) Klebsiella spp. other than K. pneumoniae (441 bp, lanes 1, 2, 4& 5); Klebsiella negative strain (Lane 3)

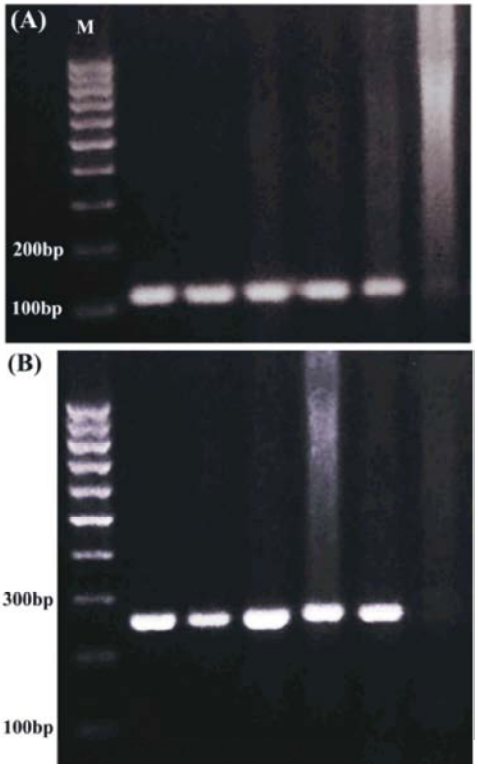

Fig. 2: A) Gel electrophoresis shows *Salmonella* genus PCR products (118 bp) positive strains (First 5 lanes); negative control (Last lane); 100 bp ladder (M). B) Gel electrophoresis shows Salmonella multiplex PCR; *S. typhimurium* (261 bp, first 5 lanes); negative control (Last lane)

47.3% SCM was detected in dairy buffalo farms on quarter level in the same Governorate [24]. In contrast, a lower prevalence (12.9%) was recorded in other districts of Egypt [7]. In other countries, the prevalence was variable; 41% in Iraq and 70% in India [25, 26]. This difference in the prevalence of SCM according to geographical regions may be attributed to national differences in veterinary legislation, laboratory services and management practices. Developing countries, with a less developed dairy sector, have usually the highest prevalence of mastitis and SCM due to lack of udder hygiene. The high level of SCM reported in our study may be attributed to the bad hygienic measures accompanied with absence of sanitary condition of hand milking beside direct contact with milkers during milking process. The causal agents involved in bovine mastitis were conventionally classified into contagious and environmental pathogens. Fungi, algae and coliforms are the most important environmental pathogens. Most of coliforms especially *Klebsiella* were reported to cause severe clinical mastitis and sometimes outbreaks of mastitis in bovine dairy farms [27, 28].

In the current study, the bacteriological identification showed 34.3% prevalence for *Klebsiella* spp. (16.3% for *K. pneumoniae*, 8.1% for *K. oxytoca* and 9.9% for other Klebsiella strains) in milk of SCM buffaloes. The presence of Klebsiella spp. in SCM highlights the significant role of this Enterobacteriaceae in SCM similar to other reports [29-31]. This high prevalence is close to that obtained by other global surveys on the prevalence of Klebsiella intra-mammary infections in America, Europe and Asia which have reported values ranging between 33.5 and 45.0 % [32-35]. A higher prevalence was reported in France (92%) [36] and in other districts of Egypt (51.67%) [24]. A lower prevalence in Africa (15–16%) [29, 31] and a very lower prevalence in Canada were also reported [35]. In contrast, a recent study revealed a very low prevalence (4.12%) for *K. pneumoniae* in clinical mastitic Egyptian buffalo, but failed to detect *K. pneumoniae* in subclinical mastitic animals (n= 23) [37]. This variation may be due to different milking system [hand milking (this study) vs machine milking] and hygienic measures which are low in hand milking. The lower prevalence for *K. oxytoca* (in our study) is consistent with other reports on bovine mastitis (6.1-6.3%)[2, 28]. However, higher incidence (14.3%) was detected by Saidi *et al*. [38].

Klebsiella spp. in general and *K. pneumoniae* and *K. oxytoca* in particular, are associated with urinary and respiratory tract infections and mastitis [39]. An oral-fecal transmission cycle has been suggested for *K. pneumoniae* in dairy herds, with fecal shedding resulting in the contamination of feed and water and subsequent re-ingestion of the organism, resulting in renewed fecal shedding [40]. The mammary infections are frequently associated with wood or sawdust contamination used in the environment of the animals. *K. pneumoniae* has been reported in serious outbreaks or in isolated cases of per-acute or acute bovine mastitis, predominantly in the first two weeks of lactation [41]. For that, it is better to detect it in the early SCM stage before being converted to clinical form.

Phenotypic identification of *K. pneumonia* and *K. oxytoca* based on bacteriological and biochemical assays is not reliable and is time consuming and laborious [17]. To understand the epidemiology of these bacteria and to control their spread, molecular method such as PCR assay had been evaluated for their rapid and easy identification. Our molecular results revealed 20.9% prevalence for *Klebsiella* spp. (11.6% for *K. pneumoniae* and 9.3% for other *Klebsiella* spp.). *K. oxytoca* was not identified using molecular method. Nearly similar prevalence for *K. pneumoniae* (11%) in SCM buffalo's milk samples were reported by Memon *et al*. [42]. However, other reports found lower prevalence rates (3.6, 4.3 and 0.81%) [7, 37, 43] respectively.

The genus Salmonella belongs to the family Enterobacteriaceae and many strains of *Salmonella* are the principal pathogens implicated in human food-borne illnesses [44, 45]. The prevalence for *S. typhimurium* was 10.47% by conventional biochemical method versus only 7.6% by molecular method. In accordance, *Salmonella* spp. were detected in 6% of raw buffalo's milk [4]. Higher prevalence of *S. typhimurium,* 23.33- 28% [46], 11.11% in raw buffalo milk and 14.28 - 20% in pasteurized milk were recorded in India [47]. A lower prevalence of *S. typhimurium* (4%) was detected in bovine milk in EL-Sharkia Governorate, Egypt [48]. Presence of *Salmonella* spp. in milk indicates poor handling practices, sewage contamination or the animal might be infected with mastitis [49].

The variation in results obtained by bacteriological/biochemical and molecular identification might be attributed to poor sensitivity of the former. In support, the conventional methods resulted in incorrect identification of 13% of *Klebsiella* strains [28, 50]. Furthermore, the currently used standard biochemical/phenotypic assays lack the sensitivity required to accurately discriminate among some Gram negative bacteria when compared to molecular identification using specific gene sequencing [51]. Over a one year study, *K. variicola*, *Raoultella* spp. and

Enterobacter cloacae were biochemically misidentified as *K. pneumoniae* in a small number of clinical mastitis cases [11, 12, 28, 50]. In general, multiplex PCR is considered as an alternative method for rapid identification of Klebsiella and Salmonella causing SCM in the dairy animals.

CONCLUSIONS

This study indicated high prevalence for *Klebsiella* and *Salmonella* causing SCM in hand milking buffaloes of Kafr EL-Sheikh governorate, Egypt. This study also offers the basis for further phenotypic and molecular characterization of *Klebsiella* and *Salmonella* in raw milk of SCM animal that help in putting a suitable plan to control them before their progress to be clinical or occurred in an outbreak form and to guarantee safe consumption of raw milk and milk products. The early detection of a contaminated source may prevent other animals from being infected and the dairy farms from sustaining major economic losses.

Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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