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Incidence of *Aerococcus viridans* in Raw Cow Milk in Sohag City, Egypt

Eman M. Shaker[1], Alshimaa A. Hassanien[2], Esraa Y. Abd-Elhamed[1]

[1]Department of Food Hygiene, Faculty of Veterinary Medicine, Sohag University, Egypt; [2]Department of Zoonoses, Faculty of Veterinary Medicine, Sohag University, Egypt.

Abstract | *Aerococcus viridans* considered the second bacterial cause of mastitis in bovine with unclear pathogenic changes role. Therefore, the current study was conducted to investigate incidence of subclinical mastitis and *A. viridans* in 100 raw milk samples collected from different dairy cattle breeding farms in Sohag city, Egypt, its effect on some milk composition and their antibiotic resistance was described. Subclinical mastitis was detected in high incidence rate (92%). A total of eleven *A. viridans* isolates were identified from 92 bovine subclinical mastitis cases. Comparatively with milk of healthy cows, the mean chloride content (%) of infected milk was 0.110 ± 00013, which showed highly significant (P = 0.01) increase, while, the mean lactose (%) decreased significantly. All *A. viridans* isolates were 100% susceptible to Streptomycin, Amikacin and Ciprofloxacin, and followed by (90.91%) to Vancomycin while, all *A. viridans* isolates were highly resistant to Penicillin G, Ampicillin and Cefotaxime. This study concluded that *A. viridans* play an important role in subclinical mastitis infection in bovine in Sohag city, where it exerts an effect on some milk composition and contaminated milk considered as a hazard for human health.

Keywords | Subclinical mastitis, *Aerococcus*, *A. viridans*, Sohag, Egypt.

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***Correspondence** | Eman M Shaker, Department of Food Hygiene, Faculty of Veterinary Medicine, Sohag University, Egypt; **Email**: milk_121970@yahoo.com
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INTRODUCTION

Bovine subclinical mastitis resulted in inflammation of mammary glands causing reduction in milk quality and quantities. It is resulted from infection with microbes which either contagious or environmental pathogens (Bakr et al. 2019). Milking process considered the main cause of infection as this microorganism living in the environment (Aguirre and Collins, 1993).

Aerococci showing identity in biochemical and physiological characters as *pediococci*, *enterococci, lactococci* and *leuconostocs*, and are frequently mistaken with *streptococci* (Facklam et al. 1989).This genus exhibit a weakly reaction with catalase test but do not contain cytochrome**.** *Aerococcus* genus primarily reported as one species named *A. viridans* (Williams et al., 1953). New five species of *Aerococcus* were additionally recognized: *A. urinae*, *A. christensenii*, *A. sanguinicola*, *A. urinaeequi*, and *A. urinaehominis* (Euzeby, 1997).

However, nowadays these organisms have a great importance in human and veterinary medicine (Spakova et al., 2012). *A. viridans* responsible for several human hazards as endocarditis, meningitis and arthritis (Gopalachar et al., 2004; Popescu et al., 2005). *A. viridans* was recently involved in bovine mastitis as it has been isolated from clinical and subclinical cases Spakova et al. (2012); Liu et al. (2015); Saishu et al. (2015); Sun et al. (2017), and described as the causative agent of arthritis, pneumonia and meningitis in cows (Liu et al. 2015). Among the infectious diseases of large ruminants, *A. viridans* still remains one of the threats to rural economy of many countries including Egypt. Few scientific literatures are available regarding the incidences in Egypt. The present study aimed to monitor the role of *A. viridans in* cases of subclinical mastitis in Sohag city, Egypt, its effect on some milk composition and describe their antibiotic resistance.

MATERIAL AND METHODS

Sample Collection

The study was conducted between January and August 2018 on one hundred Holstein dairy cows, apparently healthy and not received any specific treatment before study, from different dairy breeding farms in Sohag city.

Detection of Subclinical Mastitis

Examination of collected milk samples (quarter samples) for diagnosis of subclinical mastitis by strip cup test and California Mastitis Test (CMT) (Bovivet®, Kruuse™, Denmark), with subsequent collecting of individual milk samples (mixed quarters' samples) for bacteriological examination.

Isolation of *Aerococcus Species*

All normal milk samples and which show subclinical mastitis by Strip cup test & CMT were subjected to isolation and identification of *Aerococcus species* (Sun et al., 2017).

Enrichment

One ml of each homogenized sample was aseptically inoculated into a sterile test tubes containing 10 ml of tryptone soya broth (TSB) (M011, HiMedia). The inoculated tubes were incubated at 37°C for 24 hr.

Selective Plating

A loopful of incubated broth cultures were streaked on trypticase soya agar (TSA) (M290-500G, HiMedia) with 5% sheep blood, then incubated aerobically at 37°C for 24 h. Translucent colonies with green alpha haemolytic activity were chosen for further identification according to (Liu et al. 2015).

Identification of *Aerococcus species*

Morphological characters: Films were made from pure cultures and stained with Gram's stain and examined microscopically. The organism appears round Gram-positive cocci 1-2µ in diameter usually staining deeply, arranged in singles, pairs, tetrad and irregular clusters.

Biochemical reactions: All cultures that gave negative catalase reaction considered as suspected *Aerococcus* isolates and retained for identification by API 20 strep system (bioMérieux, SA,Marcyl'Etoile, France), the identification is performed using the database (V 7.0) with the apiweb™ identification software.

Detection of *Aerococcus viridans* by using PCR: Extraction of DNA was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's recommendations. Primers used were purchased from Germany (Table 1), 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer with, 4.5 µl water, and 6 µl DNA template. Using an applied biosystem 2720 thermal cycler. PCR conditions involved denaturation for 5 min at 94°C followed by 35 cycles of annealing at 59°C for 40 sec, extension at 72°C for 45 sec and denaturation for 30 sec at 94°C. There was a final extension at 72°C for 10 min. Samples were hold at 4°C until analyzed by agarose gel electrophoresis.

Table 1: Sequence of oligonucleotide.

Primer	Target gene	Sequence of oligonucleotide	Segmented (bp)	Reference
AC2	16S rRNA	(5`- GTG CTT GCA CTT CTG ACG TTA GC-3`)	450 bp	Martín et al., 2007
AC4		(5`-TGA GCC GTG GGC TTT CAC AT-3`)		

PCR were separated by electrophoresis using 100 bp ladder (Fermentas, Thermofisher) and photographed by a gel documentation system (Alpha Innotech, Biometra).

Effect of *Aerococcus viridans* on milk composition of chloride and lactose: Milk composition of chloride (%) and lactose (%) of *A. viridans* positive samples were determined by using automatic milk analyzer (Lactoscan MCC, Lactoscan milktronic) (Draaiyer et al., 2009) in Dairy science Department in Faculty of Agriculture, Sohag University.

Table 2: Incidence of subclinical mastitis in the examined raw milk samples

Source of samples	No. of samples	Normal samples		Subclinical mastitis samples	
		No.	%	No.	%
Dairy farm (A)	75	4	5.33	71	94.67
Dairy farm (B)	25	4	16	21	84
Total	100	8	8	92	92

Antimicrobial Susceptibility Testing

Antibiotic sensitivity of *A. viridans* isolates against 11 antimicrobial agents was performed utilizing the Kirby-Bauer disk diffusion method following rules of (CLSI, 2013) by applying the antibiotic sensitivity discs containing Penicillin G (10µ), Ampicillin (10µ), Amoxicillin/ Clavulanic acid (AMC) (20/10µ), Cefataxime (30µ), Tetracycline (30µ), Streptomycin (10µ), Amikan (30µ), Erythromycin

Table 3: Incidence of *Aerococcus* spp in the examined raw milk samples by using API

Source of sample	No. of samples	No. of isolates		Suspected isolates		Positive *Aerococcus* spp. from Subclinical mastitis samples by API	
		Sub-clinical mastitis samples	Normal samples	Sub-clinical mastitis samples	Normal samples	%	No.
Dairy farm (A)	75	50	4	29	—	30.67	23
Dairy farm (B)	25	11	4	11	—	—	—
Total	100	61	8	40	—	23	23

(15µ), Clindamycin (2µ), Vancomycin (30µ), Ciprofloxacin (5µ) on Muller-Hinton agar plates (Oxoid, Shanghai, China) and swabbed with the broth culture, and then incubated for 24 h at 37°C in aerobic atmosphere. Results were interpreted according to (CLSI, 2013).

Statistical Analyses

Milk composition of healthy cows and *A. Viridans* infected cows were compared and data statistically analysed using SPSS (SPSS 14 for windows, Inc., USA). Means and standards deviations were measured and data was significant when P value < 0.05.

RESULTS AND DISCUSSION

Incidence of Subclinical Mastitis

Examination by field tests for diagnosis of subclinical mastitis in collected raw milk samples indicated that 92% of the total examined raw milk samples were positive for mastitis represented as 71 samples from dairy farm (A) and 21 samples from dairy farm (B) as shown in Table (2). Lower results (19.4, 41.02 and 53%) were obtained by Abdel-Rady and Sayed (2009); El-kholy et al. (2018) and Bakr et al. (2019), respectively**.** The increased incidence of subclinical mastitis among dairy animals may be attributed mainly to poor hygiene practices, inadequate housing and bedding, malfunctioning milking machines, improper milking procedures and in adequate treatment methods (Philipot, 1984).

By using isolation method from both normal and subclinical mastitis raw milk samples, we obtained 8 isolates from normal raw milk samples and 61 isolates from subclinical mastitis milk samples. With respect to preliminary identification of *Aerococcus spp*, this organism distinct and different from most of other microorganisms, only 40 isolates were identified as suspected *Aerococcus* species which were isolated from subclinical mastitis samples only, and needed more identification, these suspected isolates were obtained as 29 isolates from dairy farm (A) and 11 from dairy farm (B) as shown in Table 3.

Incidence of different *Aerococcus spp.*

By applying API 20 strep system on suspected isolates it was cleared that the incidence of *Aerococcus* species from the total examined raw milk samples was 23% obtained from subclinical mastitis milk samples from dairy farm (A) only (Table 3), and distributed as *A. viridans* (11%), *A. urinae* (10%) and *A. sanguinicola* (2%) as show in Table (4). Higher results (15%) of *A. viridans* by API were obtained by Sukru et al. (2018) and lower results 1% and 2% of *A. viridans* were obtained by Spakova et al. (2012); McDonald et al. (2005), respectively.

Table 4: Incidence of different *Aerococcus spp*. in the examined raw milk samples by using API

Aerococcus species	Number of isolates	
	No./100	%
Aerococcus viridans	11	11
Aerococcus urinae	10	10
Aerococcus sanguinicola	2	2
Total	23	23

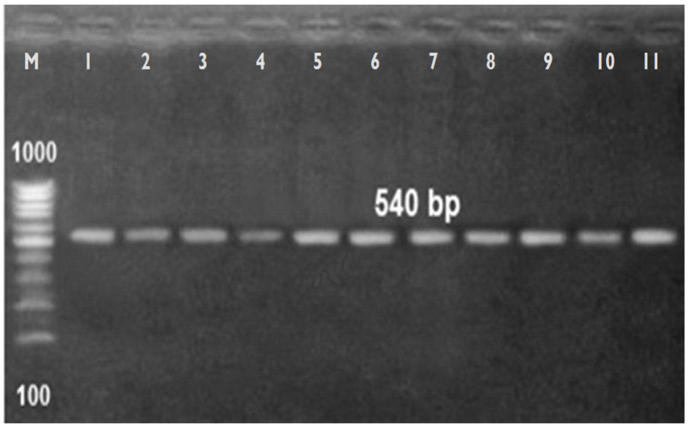

Figure 1: Agarose gel electrophoresis of PCR products obtained
Lane M : ladder marker
Lane 1-11: positive for *A. viridians*

Detection of *Aerococcus viridans* by using PCR

Previous investigations reported a minimal involvement of *A. viridans* in cases of mastitis and this could have been ascribed to an underestimation, resulting from misidentifications as *streptococci* or *staphylococci***.** However, enhancements in identification techniques, particularly the introduction of molecular assays has promoted to confident detection

Table 5: Incidence of *A. viridans* in the examined raw milk samples

Examined samples	Positive *A. viridans* samples			
	API		PCR	
	No./100	%	No./100	%
Normal samples	—	—	—	—
Subclinical mastitis samples	11	11.96	11	11.96
Total	11	11.00	11	11.00

Table 6: Statistical analytical results of CMT in positive *A. viridans* samples

Examined quarter	Score ±		Score +		Score ++		Score +++	
	No./11	%	No./11	%	No./11	%	No./11	%
FL	1	9.091	4	36.363	2	18.182	4	36.363
FR	3	27.273	1	9.091	4	36.363	3	27.273
HL	__	___	3	27.273	3	27.273	5	45.454
HR	___	___	2	18.182	6	54.545	3	27.273
Total/44	4	9.09	10	22.73	15	34.09	15	34.09

FL=front left, FR= front right, HL= hind left, HR= hind right

Table 7: Effect of *A. viridans* on milk composition as compared to healthy cows

Milk contents	Healthy cow (n = 8) (mean ± SD)	Subclinical mastitis cows (n = 11) (infected with *A .viridans*) (mean ± SD)	* *P* value
Chlorid %	0.087 ± 0.005	0.110 ± 0.013	0.01
Lactose %	4.78 ± 0.2	3.06 ± 0.3	0.01

*P value is highly significant at level of 0.01

Table 8: Antibiotic susceptibility profile of *A. viridans* isolates using Kirby-Bauer disk diffusion method

Antimicrobials	Concentration	Breakpoints			ZID[a] (mm)	S (%)		I (%)		R (%)	
		S	I	R		No./11	%	No./11	%	No./11	%
Penicillin G	10 IU	22 ≤	19-21	18 ≤	9-15	----	----	----	----	11	100
Ampicillin	10 µg	17 ≤	---	16 ≤	7-10	----	----	----	----	11	100
Amoxicillin/ Clavulanic acid (AMC)	30 µg	20 ≤	---	19 ≤	12-23	2	18.18	----	----	9	81.82
Cefotaxime	30 µg	28 ≤	26-27	25 ≤	8-15	----	----	----	----	11	100
Tetracycline	30 µg	23 ≤	19-22	18 ≤	15-20	----	----	4	36.36	7	63.63
Streptomycin	10 µg	18 ≤	14-17	13 ≤	19-39	11	100	----	----	----	----
Amikacin	30 µg	17 ≤	15-16	14 ≤	25-38	11	100	----	----	----	----
Erythromycin	15 µg	21 ≤	16-20	15 ≤	17-28	5	45.45	6	54.54	----	----
Clindamycin	2 µg	19 ≤	16-18	15 ≤	13-20	6	54.54	4	36.36	1	9.09
Vancomycin	30 µg	17 ≤	----	----	16-22	10	90.91	1	9.09	----	----
Ciprofloxacin	5 µg	16 ≤	13-15	12 ≤	20-32	11	100	----	----	----	----

S -susceptible, I- intermediate, R – resistant [a]Zone of inhibition range

of *A. viridans*. The eleven *A. viridans* isolates detected by API examination were finally confirmed by 16S ribosomal RNA (*rRNA*) sequencing as *A. viridans* (Table 5 & Figure 1). Notably, recovery of *A. viridans* from milk of cows showing subclinical mastitis referred to its role as an environmental pathogen resulted in bovine subclinical mastitis. Liu et al. (2015) and Saishu et al. (2015) isolated *A. viridans* in pure culture from cows with subclinical mastitis in percentages of 6.1% and 8 %, respectively.

The degree of quarter attack due to *A. viridans* infection was varied from 15 quarters (34.09%) showed degree (+++), 15 (34.09%) showed degree (++), 10 (22.73%) showed degree (+), 4 (9.09%) showed degree (±), as shown in Table 6**.** From previous result we found that the highest degrees of quarter attack were more in hind quarters than in fore quarters which may be due to the morphological structure of the udder and their proximity to the rear of animal which considered as a source of contamination.

Effect of *A. viridans* on Milk Composition of Chloride and Lactose

Table 7 shows describe the changes caused by *A. viridans* on some milk constituents compared to healthy cow milk. Mean chloride percent of subclinical mastitis milk was 0.110±0.013 showing high significant (P<0.01), while, the mean lactose (%) decreased significantly. The reduction in lactose content in milk infected with *A. viridans* was also observed by Sun et al. (2017).

Antimicrobial Susceptibility Profile

A. viridans isolates which isolated from bovine mastitis from different geographical areas Martin et al. (2007); Špaková et al. (2012); Sukru et al. (2018) were highly diverse in their antibiotic resistance patterns. Table 8 Determine the effect of some antibiotics against 11 *A. viridans* isolates. All *A. viridans* isolates were 100% susceptible to Streptomycin, Amikacin and Ciprofloxacin, and highly susceptible (90.91%) to Vancomycin but only 5 (45.45%) and 6 (54.54%) *A. viridans* isolates were susceptible to Erythromycin and Clindamycin respectively. On the other hand all *A. viridans* isolates were highly resistant to Penicillin G, Ampicillin, Cefotaxime and majority of isolates were resistant to Amoxicillin/Clavulanic acid (AMC) (81.82%) and Tetracycline (63.63%).

The same results were reported by Špaková et al. (2012), particularly for the resistance patterns of beta lactamase resistanse while, Martin et al. (2007) showing a different results as he found that all *A.viridans* isolates were susceptible to B-lactamase antibiotics. The resistant of *A. viridans* for some commercial antibiotics which used commonly in different programs of animal and human treatment lower the efficacy of antibiotics against infections and attributed to the hazards for human when transmitted through milk consumption.

CONCLUSIONS

From our results *A. viridans* play an important role as a causative agent of subclinical mastitis in cows and milk considered as a hazard for human infections with *A. viridans* through milk consumption. Hence understanding of epidemiology and risk factors is highly essential in order to formulate appropriate management programs.

CONFLICT OF INTERESTS

None.

AUTHORS CONTRIBUTION

Contribution is equal to all authors

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