

Original Article

Isolation, identification and virulence determinants of *Streptococcus agalactiae* from bovine subclinical mastitis in Egypt

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Abstract

Introduction: This study aimed to investigate the prevalence of contagious mastitis caused by *Streptococcus agalactiae* (*S. agalactiae*) in cattle from households and small-scale dairy farms in Egypt. Molecular characterization of *S. agalactiae* isolates was described including the genetic determinants of virulence to assess the genetic variation in isolated strains of *S. agalactiae*.

Methodology: Three hundred and sixty milk samples were collected from 90 apparently healthy dairy cows randomly selected from household and small-scale dairy farms were examined by Somatic Cell Count (SCC) as an indicator for subclinical mastitis. *S. agalactiae* isolates were bacteriologically and molecularly identified followed by identification of virulence genes using PCR.

Results: A total of 172 milk samples (47.77%) were positive with SCC > 200×10³/ml. Bacteriological examination of the positive SCC milk samples revealed that 28 (16.28%) of the isolates were *S. agalactiae*. Molecular examination using PCR confirmed only 22 isolates of *S. agalactiae* (12.8%). Moreover, we used the pattern of virulence genes to address the genetic variation of *S. agalactiae* strains isolated from cases of contagious mastitis in cattle in Egypt. Virulence genes *hylB*, *cylE*, *iagA*, and *bac* were determined in 100%, 68.2%, 13.6% and 100% of isolates respectively.

Conclusions: The use of molecular methods for the identification of the causative agent in mastitis confirmed that, in Egypt, *Streptococcus agalactiae* is considered as one of the predominant infectious agents among contagious mastitis causing pathogens. The pattern of virulence genes presented the genetic diversity of highly virulent *S. agalactiae* strains isolated from cases of contagious mastitis in cattle in Egypt.

Key words: *S. agalactiae*; Subclinical mastitis; Virulence; PCR.

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Introduction

Regardless of the effort implemented in the control and prevention of mastitis by the application of udder health plans, mastitis is still the utmost prevalent and costly disease with significant economic impact due to the direct effect on dairy production, and a significant impact on both animal and public-health as well [1,2]. Around 150 species of microbes, typically bacteria, are capable to cause mastitis [3]. Based on the source of infection, these microbes are categorized into contagious and environmental udder pathogens. The main contagious mastitis pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma spp.* They primarily infect the cow's udder and they can spread from cow to cow during the time of milking [4]. The presence of mastitis causing organisms in bulk milk is a strong indicator of the existence of intramammary

infections in the herd [5]. *Streptococcus agalactiae* is a contagious obligate gram-positive bacterium of the bovine mammary gland, and mostly causes subclinical mastitis and elevated cow SCC [6]. In Egypt, *S. agalactiae* has a great importance as a major causative agent of contagious mastitis [7]. The importance of *S. agalactiae* arises from the emergence of multidrug resistant strains and it is also responsible for severe invasive diseases in humans including both adults and neonates [8]. The early, fast and effective identification of bovine mastitis remains of utmost importance that directly influences the speed with which treatment decisions and management are undertaken for disease control. The early identification of mastitis cases increases the cure rate by 60 % and diminishes the time needed to retain normal milk production when joint with suitable antimicrobial treatment [9]. A number of

diagnostic methods are available for the diagnosis of mastitis and for the determination of the causative agents, including California Mastitis Test (CMT), Somatic Cell Count (SCC), bacteriological methods including Christie-Atkins-Munch-Petersen (CAMP) test, and molecular examination. Although bacteriological examination is successful in identifying the microbial pathogens, it is not accurate [10]. Molecular recognition of pathogenic microorganisms based on unique DNA sequences within the 16S or 23S subunit of the ribosomal RNA (rRNA) gene has been proven to be an accurate, reliable, and fast diagnostic method [11,12].

The ability of *S. agalactiae* to colonize and survive the host is determined by their virulence factors. More than 15 virulence genes were identified for *S. agalactiae* including *hylB*, *cylE*, and *iagA* which determine the invasion ability of the pathogen and others such as the *bac* gene which determines the immune evasion ability of the pathogen [13,14].

The aim of the current study was to investigate the prevalence of contagious mastitis caused by *S. agalactiae* in cattle from households and small-scale dairy farms at Giza and Al-Kalubia governorates in Egypt, in addition to the assessment of the efficacy of various diagnostic methods for mastitis especially in subclinical cases. Some of the virulence genes were also determined to assess the genetic variation in isolated strains of *S. agalactiae*.

Methodology

There was no need for ethical approval from the Institutional Animal Ethics Committee as there were no invasive techniques required for the study. All the methods were carried out in accordance with the relevant guidelines and regulations. Permission was obtained from the owners of the animals for the use of the milk samples in the current study.

Animals

A total of 90 lactating, apparently health cows were used for milk sampling. The cows were randomly selected from household and small-scale dairy farms during the period 2017-2018 from different areas in Giza and Al-Kalubia governorates, Egypt.

Sampling

A total of 360 quarter milk samples were collected aseptically by the authors at different stages of lactation for bacterial examination following the guidelines of the National Mastitis Council [15]. Samples were immediately placed on ice and transferred to the Animal Reproduction Research Institute, Giza, Egypt to perform SCC, and then transferred to the Veterinary Division, National Research Center (NRC) for bacteriological and molecular examination.

Somatic cell count (SCC)

Somatic cell Count of milk samples was measured using the automatic somatic cell counter (NucleoCounter SCC-100).

Bacterial isolation and identification

The collected milk samples were incubated for 18-24 hours at 37°C in peptone water, then all samples were cultured on blood agar and Edwards medium (Oxoid). All plates were incubated for 18-24 hours at 37°C and examined for bacterial growth. The colonies were examined for their morphological characters and hemolytic activity. Smears from suspected colonies were examined microscopically then transferred into semisolid slope agar to be identified [16]. Also, CAMP test was carried out [17] to identify *S. agalactiae*.

Molecular identification using Polymerase Chain Reaction (PCR)

The isolated bacteria were additionally cultured in TSB (Difco Laboratories, Detroit, Mich.) at 37°C for about 17 h followed by bacterial DNA extraction using GF-1 DNA extraction kit (Vivantis Co., Malaysia). The

Table 1. Oligonucleotide primer sequences used for PCR.

Primer	Sequence (5'-3')	PCR product	Annealing temp.	Reference
<i>GSag-S</i>	ATTGATAACGACGGTGTACTGTCAT	487bp	55°C	[18]
<i>GSag-AS</i>	AGTAGCGTTCGTAAATGATGT C			
<i>hylB-F</i>	CACCAATCCCCACTCTACTA	503bp	55°C	[19]
<i>hylB-R</i>	TGTGTCAAACCATCTATCAG			
<i>bac-F</i>	AAGCAACTAGAAGAGGAAGC	479bp	58°C	[19]
<i>bac-R</i>	TTCTGCTCTGGTGTTTTAGG			
<i>cylE-F</i>	CATTGCGTAGTCACCTCCC	380bp	58°C	[20]
<i>cylE-R</i>	GGGTTTCCACAGTTGCTTGA			
<i>iagA -F</i>	CGGGATTGATCTAAGTCGCT	459bp	58°C	[20]
<i>iagA-R</i>	CCATCAACATCAGTCGCTAA			

sklA3 gene coding for fibrinogen binding protein was used as the target to detect *S. agalactiae* (Table 1). All PCR reactions were carried out in a final volume of 25 µl containing 0.6 µl (100p mol) primer, 5 µl of Taq PCR Master Mix (5x FIREPol® Master Mix Ready to Load, Cat. no.04-12-00115, Solis Biodyne Co.) and 2 µl of bacterial DNA.

A pre-PCR step at 95°C for 2 min was applied. 35 PCR cycles were run with denaturation at 94°C for 45s, annealing at 55°C for 1min and extension at 72°C for 2min and final extension at 72°C for 7 min.

Amplicons with 50bp ladder plus (Cat.M7115, Biomatik Co., Ontario, Canada) were separated on 1.5% agarose gel by electrophoresis.

Detection of virulence genes

Screening was performed by PCR using specific primers (Table 1) to detect the virulence genes: (*hylB*) encodes hyaluronatylase, (*bac*) encodes beta protein, (*cylE*) encodes b-haemolysin/cytolysin and (*iagA*) encodes invasion-associated gene. All reactions were conducted as above and the reaction conditions as in Table 1.

Results

Three hundred and sixty milk samples collected from 90 apparently healthy dairy cows were examined by SCC as an indicator for subclinical mastitis. A total of 172 milk samples (47.77%) were positive using SCC (> 200×10³) and the given numbers were 110 (63.9%), 56 (32.6%), 6 (3.5%) in (200×10³-500×10³), (500×10³-1500×10³) and (1500×10³-5000×10³) ranges respectively (Table 2).

Bacteriological examination of the positive SCC samples revealed 28 *S. agalactiae* isolates (16.28%) as determined by CAMP. However, molecular examination using PCR confirmed 22 isolates (12.8%) as *S. agalactiae* (103).The PCR gave the expected product for *S. agalactiae* isolates (487bp) as shown (Figure 1).

Virulence genes

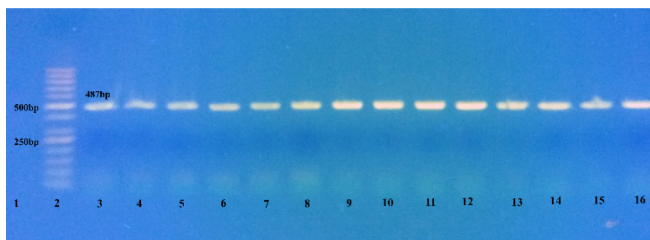
In the present study, screening of virulence genes was performed by PCR using the primers and reaction conditions in Table 1 to detect *hylB*, *cylE*, *iagA*, and *bac* genes (Figure 2). In the 22 strains studied, virulence genes *hylB*, *cylE*, *iagA*, and *bac* were determined in 100%, 68.2%, 13.6% and 100% of isolates respectively

(Table 3). Fifteen isolates showed three or more of the virulence genes studied (3 with 4 genes, 12 with 3 genes and 7 with 2 genes).

Discussion

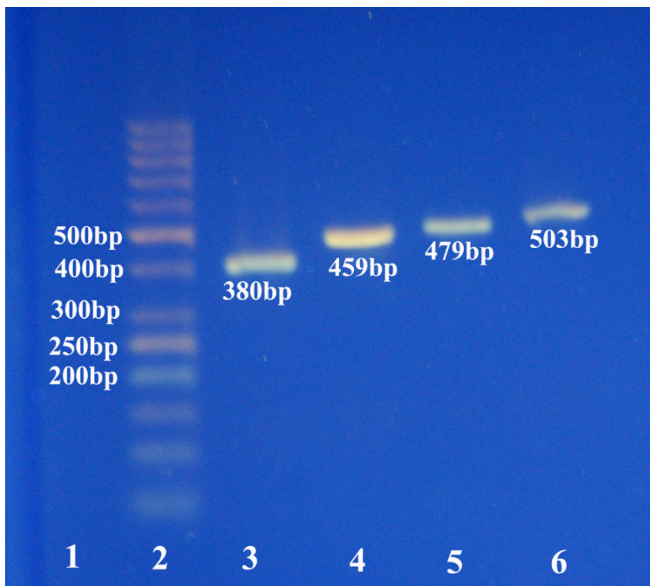
Smallholders who are small-scale farmers own more than 60% of the total livestock population in Egypt. They keep a few animals for the production of milk and dairy products for family consumption and as a source of income [21].

Figure 1. PCR amplified products among the examined *S. agalactiae* isolates.



Lane 1, negative control; Lane 2, DNA Ladder; Lane 3, positive control, Lane 4-16, positive *S. agalactiae* isolates.

Figure 2. Polymerase chain reaction amplified products of *S. agalactiae* virulence genes in an agarose gel.



Lane 1, negative control; Lane 2, DNA ladder; Lane 3, *cylE* (380bp); Lane 4, *iagA* (459bp); Lane 5, *bac* (479bp); Lane 6, *hylB* (503bp).

Table 2. Prevalence of mastitis using SCC test for collected cow milk samples.

SCC value (×10 ³)	200-500	500-1500	1500-5000	Total (%)
Number of positive isolates	110 (63.9%)	56 (32.6%)	6 (3.5%)	172 (47.77%)

Table 3. Results of *S. agalactiae* identification from 172 SCC-positive sample and distribution of virulence genes.

Test	<i>S. agalactiae</i> positive isolates		Prevalence of virulence genes			
	Bacteriological exam.	Molecular exam.	<i>hylB</i>	<i>cylE</i>	<i>iagA</i>	<i>bac</i>
Number (%)	28 (16.28%)	22 (12.8%)	22 (100%)	15 (68.2%)	3 (13.6%)	22 (100%)

Mastitis is one of the most important destructive infectious diseases of dairy cattle industry [22]. Bovine mastitis is an economic burden for farmers especially in its subclinical form due to decreased milk yield and changes in milk composition, premature culling and cost of veterinary treatments [23].

Streptococcus galactiae is considered as the main contagious mastitis causative agent. Contagious mastitis is considered of fairly vital significance to the public health as it is linked with many zoonotic diseases in which milk turns as a vehicle for the infectious agents [24].

Somatic cell count (SCC) is one of the diagnostic techniques and is considered as a superior marker for subclinical mastitis [25]. The use of SCC is critical and essential for the determination of the reduction of the milk yield [26]. SCC in healthy cow's milk is between 50,000 and 100,000 cells/mL, and it is considered unhealthy for consumers when it exceeds 200,000 cells/mL [27].

In the present work, SCC indicated the presence of subclinical mastitis in 172 milk samples (47.77%) with SCC value above 200×10^3 /ml. Comparable findings were recorded by different authors in different places over time including [28-31] with values of 62.08%, 69.4%, 51.6% and 56.3% respectively. Meanwhile, higher prevalence was recorded [32-35] with values of 78%, 75.9%, 71.6 % and 91.48% respectively. These findings declare the high prevalence of subclinical mastitis that could be due to neglecting some of the preventive and control measures for subclinical mastitis including milking hygiene practices as well as the inability of small holders to afford the cost of intervention methods including diagnosis and treatment [36]. In Egypt, *S. agalactiae* has a great importance as a major causative agent of contagious mastitis [7] and its severity emerges from the development of multidrug resistant strains.

Several methods have been reported for the identification of the causative agent of mastitis including the use of bacterial culture for isolation and biochemical identification, which is considered as the gold standard method. But, it is expensive and time consuming. Molecular techniques using PCR has several advantages over conventional methods including the accuracy, rapidity and sensitivity as well

as the ease of analysis and interpretation of data. The use of PCR routinely for early detection of pathogens ensures quick results with increased sensitivity to lower the cost of treatment and improves mastitis management [34,37].

The bacteriological method using CAMP herein confirmed the identification of 28 (16.28%) isolates of *S. agalactiae* from cattle diagnosed with subclinical mastitis, which confirms the poor hygienic measures within the examined cattle. This finding was comparable with the results reported by others [32,35,38-40] that isolated *S. agalactiae* with an incidence of 15.1%, 15.4%, 16%, 12.7%, and 14.7% respectively.

Higher incidence of *S. agalactiae* isolated from mastitic cows were reported by El-Jakee *et al.* [7], Elhaig and Selim [34], Hogan *et al* [41], Kuzma and Malinowski [42] and Khan and Mohammad [43], and with incidence rates of 21.7%, 20% 47%, 41.2% and 30% respectively.

Molecular examination using PCR confirmed the identification of only 22 (12.8%) *S. agalactiae* isolates. The molecular method did not confirm the identification of six isolates, which were identified as *S. agalactiae* using CAMP. These results may be due to lacking of sensitivity of CAMP for the identification of *S. agalactiae* [44]. Therefore, the use of molecular methods for the recognition of the causative agent of infection provides a promising, rapid and more accurate option for the detection in hours, instead of days spent by conventional cultural methods.

The ability of *S. agalactiae* to colonize different tissues and different hosts including both humans and animals is assisted by the contribution of a variety of virulence factors in the process of pathogenicity. These factors support bacterial survival by limiting or escaping host immune response [14]. Moreover, the high frequency of these factors is crucial during the process of infection [45]. In the current study, four virulence genes *hylB*, *cylE*, *iagA*, and *bac* were screened in the isolated 22 strains. Virulence genes *hylB*, *cylE*, *iagA*, and *bac* were determined in 100%, 68.2%, 13.6% and 100% of isolates respectively. Fifteen isolates showed three or more of the virulence genes (3 with 4 genes, 12 with 3 genes and 7 with 2 genes).

The zoonotic importance can be proofed by the comparable results with that of virulence determinants of *B streptococci* isolated from patients in Brazil and Malaysia where most strains under study contained the *hylB*, *cylE* and *bac* gene as reported by Correa *et al.* [19] and Eskandarian *et al.* [46].

Conclusions

The use of molecular techniques for the identification of the causative agent of mastitis in Egypt, confirmed the predominance of *Streptococcus agalactiae* as one of the infectious agents among contagious mastitis causing pathogens. Therefore, molecular methods are reliable and accurate diagnostic tools and should be utilized for the early treatment and prevention of contagious mastitis in cattle. The pattern of virulence genes presented the genetic diversity of *S. agalactiae* strains isolated from cases of contagious mastitis in cattle in Egypt. Further epidemiological studies are needed to investigate the genetic diversity of *S. agalactiae* using a large number of isolates collected from different sources.

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Authors' Contributions

KAA and HAH, designed the study. AAA and EAF performed the bacterial isolation and identification. AMY and KAA finished the molecular characterization. AMA and AMA drafted and reviewed the manuscript. All authors read and approved the final manuscript.

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