Haemolysin and hyaluronidase genes of *Streptococcus agalactiae* recovered from mastitic cows milk

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Abstract: Streptococcal mastitis causes great economic losses in dairy industries all over the world; therefore the aim of the research is to investigate the prevalence of *Streptococci* in mastitic cows, detection of titre of haemolysin as well as identification of two virulence genes in *S. agalactiae*) including *β*- hemolysin/ cytolysin (*cylE*) and *hyl* (hyaluronidase) genes (. About 110 (64.7%) out of 170 milk samples from cows were mastitic either clinical (48.2%) or subclinical mastitis (51.8%) with *Streptococci* positive. Identification of *S. agalactiae (*50, 45.45%), *S. uberis* (46, 41.82%) and *S. dysgalactiae* (14, 12.73%) were screened by biochemical methods. Six of 10 isolates of *S. agalactiae* produced haemolysin titre ranged from 1:16 to 1:64. By PCR amplification, 6 (60%) of 10 phenotypically beta (*β*) haemolysis on modified Edward's media and sheep blood agar were cylE gene positive and 3 (30%) of 10 isolates were *hyl* gene positive. The genotype of *β*-hemolysin of *S.agalactiae* seemed to be having correlation with the expression of their phenotypes and also correlating well with the result of titres of haemolysin. The high percentage of *S. agalactia*e cylE gene and hyl gene in the present study help in understanding of the distribution of S. *agalactiae* and contribute to the establishment of preventive approaches to reduce the spread of infection.

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Introduction

Mastitis is a multifactorial disease caused by several species of gram-negative and gram-positive bacteria, mycoplasmas, fungi, and algae (***Zadoks et al., 2011***). Mastitis leads to economic losses including reduction in milk yield or milk quality and early culling of severely affected animals. It leads to expensive antibiotic treatment, veterinary services and losses of the young ones ***(Sordiell et al., 2000; and Leitner et al., 2001)***. *Streptococcus* is isolated frequently from bovine mammary glands ***(Facklam. 2002; and Fortin et al., 2003)***. *Streptococcus agalactiae*, *S. dysgalatiae* and *S. uberis* have been reported as the three most common causative agents of mastitis ***(Leigh, 1999; and Khan et al., 2003)***.

Streptococcus agalactiae (group B Streptococcus, GBS) has been widely reported as an important pathogen of both animals and man ***(Keefe, 1997; Mosabi et al, 1997; and Ko et al., 2001)***. In cattle, it causes bovine clinical mastitis and subclinical mastitis, and in humans, it is associated with infections among neonates and adults ***(Pinto et al., 2013)***. Man may be a source of infection for cattle ***(Zadoks et al., 2011).***

GBS exhibits cytolytic toxin, the beta (***β***) haemolysin. GBS ***β***-haemolysin is primarily a broad-spectrum cytolysin capable of destroying many eukaryotic cells ***(Tapsall and Phillips, 1991; and Nizetet al., 1996)***. It is therefore referred to as the GBS *β*- hemolysin/ cytolysin ***(Doran et al., 2002)***. The first report of the GBS *β*- hemolysin/ cytolysin provided by ***Todd (1934)*** described an extracellular molecule that is oxygen stable, acid and heat labile, and non-immunogenic, only *cylE* was essential for *β*-hemolysin/ cytolysin expression ***(Pritzlaff et al., 2001).***

S. agalactiae hyl encodes hyauronate lyase (hyaluronidase), a putative virulence factor facilitates the spreading of bacteria in host tissues ***(Akhtar and Bhakuni, 2004).*** The *hyluronidase* activity in *S. agalactiae* is associated with host specificity ***(Lin et al., 1994).***

Little data is available on the role of *S. agalactiae* in disease exacerbation through the production of *β*-haemolysin toxin and hyaluronidase. Therefore, the aim of this study was to determine the role of *S. agalactiae* in bovine mastitis in Egypt, determine the titre of haemolysin and detect *β*-haemolysin gene as well as hyaluronidase gene by PCR.

Materials and Methods

Samples collection

A total of 170 milk samples were collected from cows with clinical (n=70) or sub clinical (n=100) mastitis from 3 bovine dairy farms in *Mansoura* City, Egypt from May to September 2016. All milk samples were used for microbiological analysis.

Microbiological analysis

The milk samples were inoculated on modified Edwards media **(Oxoid)** as a selective media for isolation of *Streptococci* and incubated aerobically at 37°C for 24-72hr. The suspected colonies yielding gram-positive cocci with catalase-negative were subcultured on 7% sheep blood agar. Colonies yielding *β*-haemolysis on blood agar were subjected to CAMP test and aesculin hydrolysis test as previously described (***Cruikshank et al., 1975; and Barrow and Feltham, 1993)***.

Determination of titre of β-hemolysin

Ten randomly selected isolates were inoculated in brain heart infusion (BHI) broth and incubated at 37°C under 20-25% Co_2 tension for 24hrs. The BHI broth was centrifuged and filtrated. The supernatant was collected to get a high yield of β-haemolysin. Two-fold serial dilutions were made in saline. One ml of saline was pipetted into dilution tubes. One ml of 1% sheep RBCs suspension was pipetted into all tubes. So, the final dilutions became 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. The tubes were incubated at 37°C for 30 min., and then over night at 4°C. The greatest dilution of the sample resulting in 50% hemolysis of 1 ml of sheep erythrocyte suspension was defined as 1 hemolytic unit ***(Marchlewics and Duncan, 1980)***.

Molecular detection of cylE and hyl genes of S. agalactiae

The polymerase chain reaction (PCR) was applied for the determination of cylE and hyl genes that were encoding β-haemolysin and hyaluronidase enzymes of *S. agalactiae.* DNA was extracted from *S.agalactiae* using QIAamp DNA Mini Kit **(Qiagen, Germany, GmbH)**. The DNA amplifications were performed using certain primers and under specific profiles, as shown in **Table (1) and (2)**. The amplified DNA products were separated by electrophoresis on 1.5% agarose gel **(Applichem, Germany, Gmbh)**. Gel pilot 100bp DNA ladder **(Qiagen, Germany, gmbh)** was used to determine the fragment sizes. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Results and discussion

Prevalence of streptococcal species

Bovine mastitis is a global problem responsible for many losses in growing dairy industry ***(Zeryehun and Abera, 2017)***. In the present study the overall prevalence of mastitis was 64.7% (110/ 170) where 48.2% (53/110) and 51.8% (57/110) cows with clinical and subclinical mastitis, respectively **(Table 3)**. This result was closely in agreement with the finding of 64.9% in AL-Diwanyia ***(Al-kuzaay and Kshash, 2013)*** and 64.3% in Eastern Ethiopia ***(Zeryehun and Abera, 2017)***. The present study also showed the prevalence of 48.2% for clinical mastitis that was much higher than the findings of 10.7% in Ethiopia ***(Zeryehun and Abera, 2017)*** and 15.9% in AL-Diwanyia ***(Al-kuzaay and Kshash, 2013)***. In addition, the research revealed subclinical mastitis prevalence of 51.8% which was in agreement with the finding of 51.8% in Ethiopia ***(Zeryehun and Abera, 2017)***. The high prevalence of mastitis has been reported to be due to deficient dry cow therapy, unhygienic milking practices, poor udder hygiene, high yielders, and no grazing ***(Abrahamsen et al., 2014)***.

Streptococcus is a common pathogen of clinical or sub-clinical mastitis resulting in great economic losses in dairy farms in Egypt ***(Benić et al., 2012)***. In the present study, a total of 110 (64.7%) *Streptococci* recovered from milk samples were identified by biochemical methods as *S. agalactia*e (50, 45.45%), *S. uberis* (46, 41.82%) and *S. dysgalactiae* (14, 12.73%) **(Table 3)**. The organisms were isolated from cases of mastitis by other investigators ***(Kerro-Dego et al., 2003; and Seyoum et al., 2003)***. The biochemical characteristics of recovered *S. agalactiae* were catalase negative, *β*-haemolysis on sheep blood agar **(photo. 1)**, CAMP test positive, lactose positive and aesculin hydrolysis negative (***Watt, 1988)***. Our results revealed that *S. agalactiae* is the predominant species of *Streptococci*. This result is consistent with previous researchers ***(Prabhu et al., 2012)***. Also, another study recorded the prevalence of infection with group B streptococci (GBS) up to 44% in infected herds (**Keefe, 1997). *Ekin and Gurturk (2006)*** isolated 44.7% *S. agalactiae* from bovine mammary glands. Recently, other researchers ***(Kia et al., 2014; and Ding et al., 2015)*** recovered 52.95% and 70.4% *S. agalactiae* from mastitic cows milk. However, the result in this study was higher than those obtained by ***Elhaig et al. (2014)*** who recorded 20% 0f bacterial isolates were *S. agalactiae* in Egypt.

Phenotypic characterization of β-haemolysin of S. agalactiae

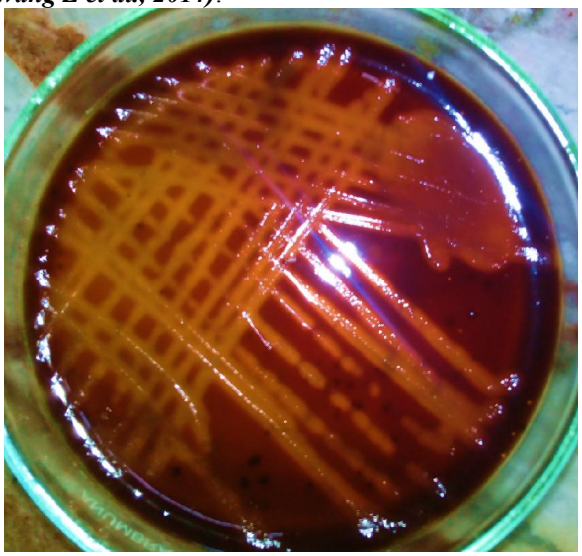
Most GBS strains as *S. agalactiae* produce a surface-associated beta haemolysin/cytolysin (β-h/c), which plays a key role in GBS pathogenesis. It can target a wide spectrum of cells, and hyper production of this haemolysin is associated with fulminant disease in clinical GBS cases as well as severe cases of infection in animal models ***(Rosa- Fraile et al., 2014)***. *S. agalactiae* invasion and disease pathogenesis is a complex process that is achieved through numerous virulence factors. The *S. agalactiae* β-hemolysin is considered as one of the most important virulence factors. Invasive *S. agalactiae* infections are almost

exclusively caused by β-hemolytic strains and absence of *S. agalactiae* β-hemolysin prevents the bacteria to survive inside the phagocytic cell ***(Sagar et al., 2013)***. In the current study, ten *S. agalactiae* isolates, which showed β-hemolysis on sheep blood agar plates, were titrated for β-haemolysin. Six (60%) out of 10 isolates of *S. agalactiae* revealed titre of β-haemolysin ranged from 1:16 to 1:64 **(Photo. 2,3,4)**, while 4 (40%) of 10 isolates were negative (**Table 4**). The highest titre of beta toxins was 1/64 (3 isolates, 50%), followed by 1/32 (1 isolate, 16.67%), whereas 1/16 (2 isolates, 33.33%) was the lowest titre. This result was close to the results of ***Nizet et al. (1996)*** who found that the titre of haemolysin ranged from 1:4 to 1:64.

Genotypic characterization of cylE and hyl genes of S. agalactiae

PCR assay is a rapid, accurate, sensitive and specific method for identification of the virulence genes (*cylE* and *hyl* genes) of *S. agalactiae*. Thus, the present study detected the *cylE* gene encoding β-hemolysin in 6 (60%) of 10 *S. agalactiae* isolates (**photo. 5).** The present result was close to the result of ***Ding et al. (2015)*** who recorded 50% of *cylE* genes in isolates. On the other hand, this result was higher than those obtained by ***Spellerberg et al.*** **(2000)** and ***Bergseng et al. (2007),*** who found 34.3% and 23% of *cylE* genes in isolates, and lower than those obtained by ***Dmitriev et al. (2002)*** who recorded 100% of cylE genes. By PCR test, 6 isolates harbored *cylE* genes were phenotypically expressed β- haemolysin with variable titres, while other 4 isolates were negative for *cylE* gene without expression of beta haemolysin. The genotypes of *β*-haemolysin of *S. agalactiae* in this study seemed to be having a correlation with the expression of their phenotypes and also correlating well with the result of the titre of haemolysin as illustrated in **Table (4)**.

Hyaluronidase enzyme is an essential factor in enabling the spread of the pathogens from an initial site of infection ***(Girish and Kemparajuk, 2007***). It has been assumed to facilitate the spread of *S. agalactiae* through the tissues of the infected host ***(Pritchard et al., 1994***). Also, it has a strong influence on intracellular survival of *S. agalactiae* and proinflammatory cytokine expression ***(Wang et al., 2014)***. Therefore, the *hyl* gene encoding hyaluronidase was another gene identified in 3 (30%) of 10 *S. agalactiae* isolates by PCR amplification in the present study **(Photo. 6).** This result was close to the result of ***Aprini et al (2016)*** who recorded 38.8% of hyl genes in isolates. On the other hand, this result was lower than those obtained by ***Krishnaveni et al. (2014).*** Both CylE and Hyl genes could observe for 3 (30%) of 10 isolates (**Table 5**). These two genes are responsible for the intracellular survival of *S. agalactiae* inside macrophage ***(Sagar et al., 2013; and wang Z et al., 2014)***.



Photograph 1. β-haemolysis of *S. agalactiae* on Modified Edward media

Table 1. **Oligonucleotide primer sequences used in this study**

Target gene	Sequence	Amplified product	Reference
S. agalactiae cylE	TGACATTTACAAGTGACGAAG	248 bp	Bergseng et al., 2007
	TTGCCAGGAGGAGAATAGGA		
S. agalactiae Hyl	CATACCTTAACAAAGATATATAACAA	950bp	Krishnaveni et al., 2014
	AGATTTTTTAGAGAATGAGAAGTTTTTT		

Table 2. Cycling conditions during PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
S. agalactiae cylE	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 30 sec.	35	72˚C 7 min.
S. agalactiae Hyl	94˚C 5 min.	94˚C 30 sec.	52˚C 30 sec.	72˚C 50 sec.	35	72˚C 10 min.

Table 3. Incidence of *Streptococcal species* in mastitic milk

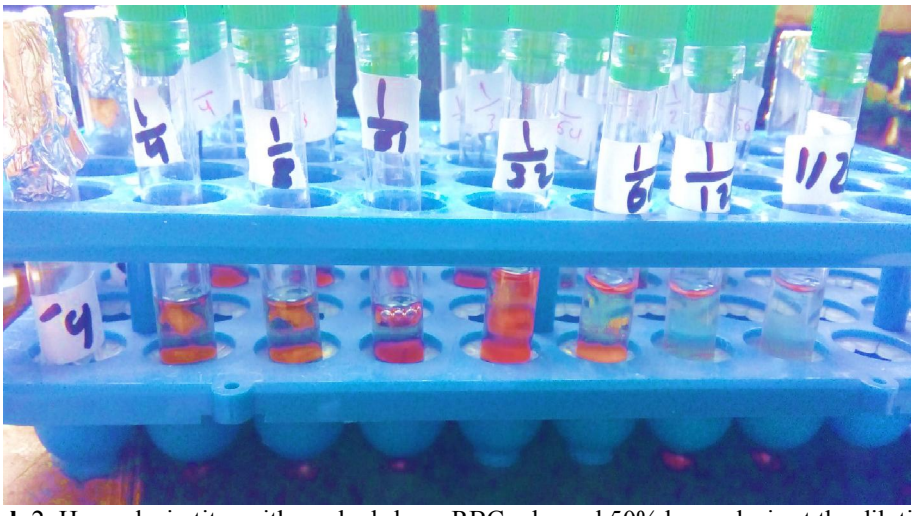
Isolates	Clinical mastitis	Subclinical mastitis	Total
S.agalactiae	19 (35.8%)	31 (54.4%)	50 (45.45%)
S.uberis	26 (49%)	20 (35%)	46 (41.82%)
S.dysgalactiae	8 (15%)	6 (10.5%)	14 (12.73%)
Total	53 (48.2%)	57 (51.8%)	110 (64.7%(

Table 4. the titer of hemolysin and the result of *CylE* gene by PCR

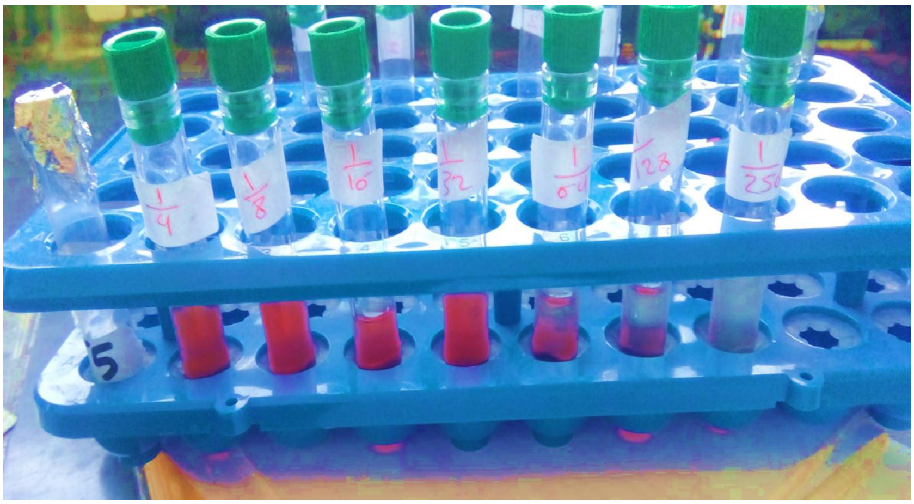
Code No. of tested isolates	Results	
	β-Haemolysin titre	***CylE***
1	1\64	+
2	-	-
3	-	-
4	1\16	+
5	-	-
6	1\64	+
7	1\32	+
8	1\64	+
9	-	-
10	1\16	+

Table 5. PCR amplification of *cylE* and *hyl* genes of *S. agalactiae*

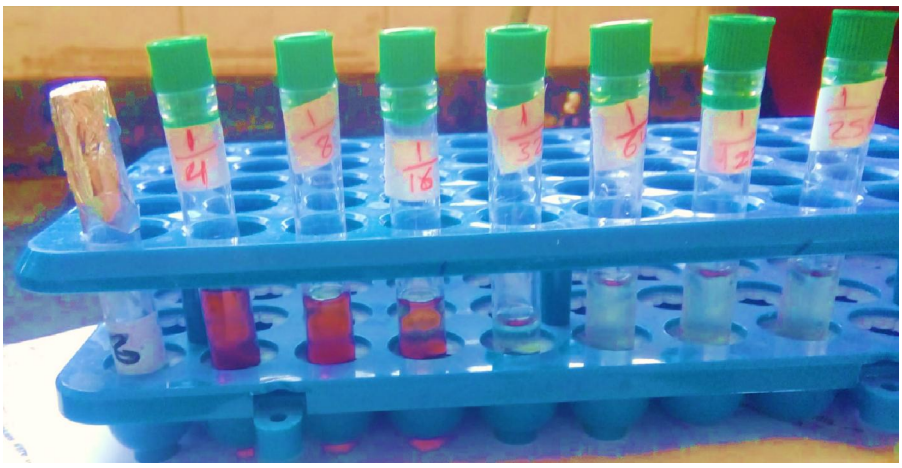
Code No. of tested isolates	Results	
	CylE	***Hyl***
1	+	-
2	-	-
3	-	-
4	+	+
5	-	-
6	+	+
7	+	-
8	+	-
9	-	-
10	+	+



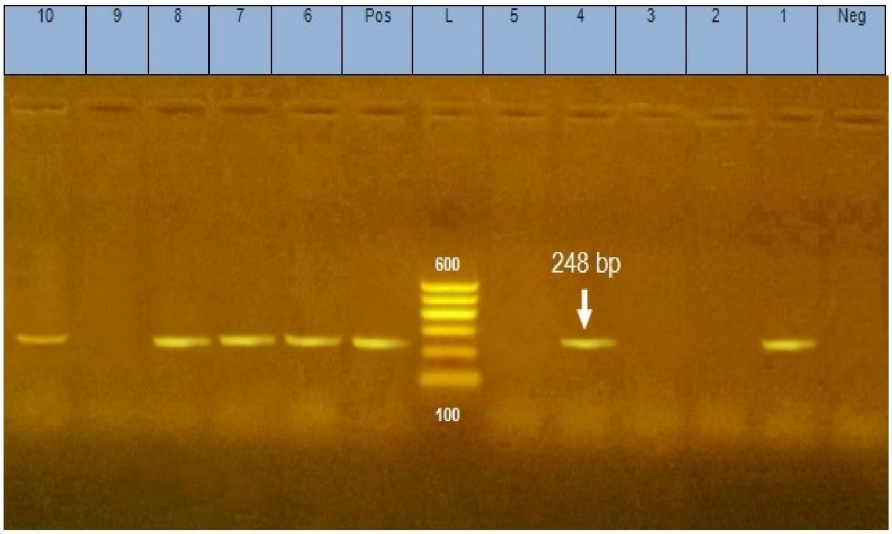
Photograph 2. Haemolysin titre with washed sheep RBCs showed 50% haemolysis at the dilution 1\32



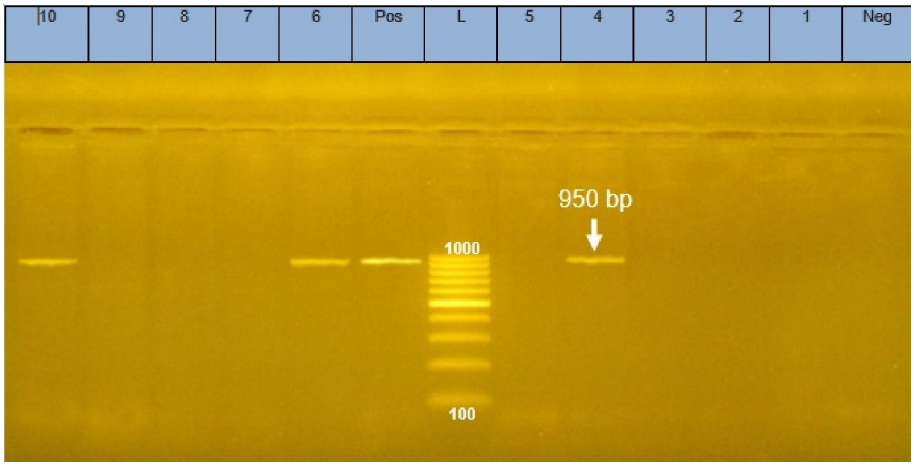
Photograph 3. Haemolysin titre with washed RBCs showed 50% haemolsis at the dilution 1\64



Photograph 4. Haemolysin titre with washed sheep RBCs showed 50% haemolysis at the dilution 1\16



Photograph 5. Agarose gel electrophoresis of PCR products showing amplification of *cylE* gene of *S. agalactiae*; Lane L: DNA molecular weight marker (100bp), lane Pos: positive control, lane Neg: negative control, lanes 1,4, 6,7,8,10: positive for cylE gene (248bp), lanes 2,3,5,9: negative for cylE gene.



Photograph 6. Agarose gel electrophoresis of PCR products showing amplification of hyl gene of *S. agalactiae*; Lane L: 100:1000 bp DNA ladder; lane pos: positive control, lane Neg: negative control, lanes 4,6,10: positive for hyl gene (950bp), lanes 1,2,3,5,7,9 negative for hyl gene.

Conclusions

The current study reported an overall prevalence of Streptococcal species, especially *S. agalactiae,* associated with mastitis that was a major health problem of dairy cows and will have a drawback on the production of dairy industry and hence warrants serious attention in Egypt. Particularly the prevalence of *cylE* gene encoding β-hemolysin and *hyl* gene encoding hyaluronidase in the present study help in the understanding of the distribution of *S. agalactiae* and contribute to the establishment of preventive approaches to reduce the spread of infection.

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