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SOME STUDIES ON THE FACTORS OF THE PATHOGENICITY OF STAPHYLOCOCCUS AUREUS ISOLATED FROM BOVINE MASTITIS

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ABSTRACT

This work aimed to study some factors of the pathogenicity of *S. aureus* isolated from bovine mastitis. Holstein-Friesian cows raised in two farms in El-Bohera governorate were examined from winter 2008 through spring 2009. Clinically, 208 out of 1000 cows had mastitis. Milk samples were collected and subjected for bacteriological examination for isolation and identification of *S. aureus* as well as further identification of *S. aureus* enterotoxin A (SEA) via PCR using species specific primers. Sixty-five (31.25%) isolates of staphylococci could be isolated. Twenty-two (33.85%) of the isolates were positive for coagulase activity. Two isolates out of eight (25%) biochemically identified *S. aureus* were positive for SEA. As detected by PCR.

INTRODUCTION

Worldwide bovine mastitis is the most common infectious disease affecting milk producing cows causing economic losses higher than any other disease of dairy cattle (Gillespie and Oliver, 2005). Also, it possesses food safety and anti-microbial resistance threats (Kim et al., 2001), as it is the primary contamination source of milk and milk products especially in case of defective pasteurization (Joffe et al., 2006).

In bovine mastitis, coagulase positive staphylococci (CPS) (*S. aureus* and some strains of *S. hyicus*) are more common pathogenic than coagulase negative staphylococci (CNS) isolates (Ali-Vehmas and Sandholm, 1995). The tube coagulase test has the potential to detect not only *S. aureus* but also other coagulase positive staphylococci in milk.

The pathogenic staphylococci produce a “battery” of toxins and enzymes (Quinn et al., 1994) which make a contribution to the ability of this organism to cause disease on the mammalian host (Salasia et al., 2004). These exotoxins include haemolysins, various enzymes and a family of related pyrogenic toxins, namely staphylococcal enterotoxin (SE), toxic shock syndrome toxins (TSST) and exfoliative toxins (ET) (Dinges et al., 2000). Recently a novel gene cluster encoding staphylococcal exotoxins-like protein has been described (Williams et al., 2000).

The aim of this work was to determine virulence factors of staphylococcus auveus isolated from mastitic milk through detection of haemolysis factors, coagulase enzyme and enterotoxin . A.

MATERIAL AND METHODS

Animals

A total of 1000 of Holstein-Friesian cows raised in two farms in El-Bohera governorate were examined clinically for mastitis during winter 2008 and spring 2009. Milk samples were collected from those suffering from clinical mastitis as indicated by the changes in milk colour and its consistency.

Bacteriological examination

Media used were nutrient agar (Oxoid), Baired-Parker's agar medium (Baired-Parker, 1962), brain heart infusion broth (ICMSF, 1982), mannitol salt agar (Difco, 1953), blood agar medium (Quinn et al., 1994), and nutrient broth (Oxoid). Milk samples from clinically mastitic cows were inoculated on brain heart infusion broth overnight at 37 C. A loopful from the inoculated broth tubes was streaked onto mannitol salt agar and Baired Parker's agar medium, and incubated at 37 C for 24 hours. The suspected *S. aureus* colonies were picked up and streaked on asterile nutrient agar for purification and studying the cultural characteristics. Meanwhile, the morphological characteristics were done, and biochemical activities of the recovered isolates according to Quinn et al. (2002) and Boerlin et al. (2003).

Coagulase activity

Each isolate was tested for production of coagulase by tube agglutination test (APHA, 1984) using asterile rabbit plasma in Wassermann tubes. The positive result was visible fibrin clot formation which was examined every 2 hours up to 24 hours.

Haemolysis on blood agar (Bailey and Scott, 1974)

Each Staphylococcus isolate was inoculated on defibrinated sheep blood agar and incubated for 24 hours at 37 C, then examined for haemolysis.

Biochemical reactions

Each suspected Staphylococcus isolate was examined for the following biochemical tests:

- Catalase, test (Jean and Macfaddin, 1976).
- Oxidation-fermentation of glucose (Hugh and Leifson, 1953).
- Urease (Cruickshank et al., 1975).
- Gelatin liquefaction (Jean and Macfaddin, 1976).
- Mannitol fermentation (Bailey and Scott, 1974).

The pathogenic determinants (coagulase, haemolysin, urease production and salt mannitol fermentation test) were studied according to Quinn et al. (2002) and Boerlin et al. (2003).

Detection of Staphylococcus aureus enterotoxin A (SEA) by PCR

Eight isolates of the coagulase positive Staphylococci which gave haemolysis on sheep blood agar were tested for the SEA by PCR using the following oligonucleotide sequence:

Gene[a]	Primer	Oligonucleotide sequence (5'–3')	Location within gene	Size of amplified product (bp)
SEA	GSEAR-1	GGTTATCAATGTGCGGGTGG	349-368	102
	GSEAR-2	CGGCACTTTTTTCTCTTCGG	431 - 450	

[a]nucleotide sequence and location were derived from the published sequence for SEA (Betley et al., 1988).

SEA detection by PCR was carried out using the following thermal cycling profile: An initial denaturation at 94 C for 5 min. followed by 35 cycles of amplification (denaturation at 94 C for 2 min., annealing at 57 C for 2 min., and extension at 72 C for 1 min.), ending with a final extension at 72 C for 7 min.

RESULTS

Incidence of clinical mastitis and characteristics of clinically mastitic milk samples:

Clinical observation of 1000 Holstein-Friesian cows raised in two dairy farms revealed that, 208 (20.8%) cases of clinical mastitis as indicated by the deviating characteristics of milk colour and consistency. Milk colours recorded were yellow, serum like or bloody. Consistency of mastitic milk ranged from watery to thick and coagulated and purulent.

The bacteriological examination of the clinically mastitic milk samples was performed to determine the prevalence of *S. aureus* as a causative agent of clinical mastitis. The incidence of *S. aureus* was 33.85%. The recovered isolates were identified bacteriologically as Gram-positive spherical cocci 0.8 – 1 µm in diameter arranged in grape-like clusters with some single or paired cocci, non-sporing, non-motile, and non-capsulated). Morphologically, suspected colonies were cream-buff to golden yellow or even orange colouration on nutrient agar and mannitol salt agar, and black shiny and glistening on Baired-Parker medium. Culturally, suspected colonies as *S. aureus* were 1-3 mm in diameter and well isolated colonies reached 4 mm in diameter and were smooth with glistening surface. Biochemically, suspected colonies were identified by urease test, gelatin liquefaction test (proteolytic activity), salt mannitol fermentation, coagulase activity and haemolytic activity on sheep blood agar.

Pathogenic determinants*:*

Out of 65 isolates tested for coagulase activity (TCT), 22 with a percentage of 33.85% were positive for TCT. Twenty-two isolates out of the tested 65 ones with a percentage of 33.85% have shown haemolytic activity on sheep

blood agar. Meanwhile, examined isolates were positive for salt mannitol fermentation with a percentage of 84.62%, urease production with a percentage of 87.69% and gelatin liquefaction test (proteolytic activity) with a percentage of 84.62% (Table 1).

***Results of amplification of S. aureus enterotoxin A (SEA) using PCR**:*

Eight isolates of the biochemically identified *S. aureus* were randomly studied for detection of SEA using PCR technique and a species specific primer to this gene. The specificity of the primers was conformed by positive amplification of fragment with the extracted DNA of the bacterial isolate as shown in photo (1). Out of the eight tested isolates, two isolates (25%) were positive for the SEA (photo 1).

DISCUSSION

S. aureus is regarded as an important pathogenic bacterium causing bovine mastitis since it is often isolated from acute and chronic mastitis (Hata et al., 2006). Furthermore, *S. aureus* is a contagious pathogenic bacterium that resists elimination by antibiotics therapy and persists for long periods, without overt symptoms (Erskine et al., 1994). For these reasons, the control of bovine mastitis if it is caused by *S. aureus* is very difficult (Hata et al., 2006), and need a rapid method for detection and identification for it.

Bacteriological examination of milk samples from clinically mastitic udders revealed an incidence of 33.85% for *S. aureus*. This result is in agreement with that of Misra et al. (1973) who found an incidence of 33.1%, as well as the value of 34% reported by Verma et al. (1978).

The incidence of *S. aureus* clinical mastitis is a matter of controversy; some authors reported every high incidence, e.g. 65% (Nakagawa, 1958), 62.75% (Sinha and Prased, 1969), 65% (Morselt et al., 1995), 62.8% (Gianneechini et al., 2002) and 74% (Olderiekerink et al., 2006). Other researchers obtained a high incidence of 58.8 and 47%, respectively by Rottschedt (1994) and Abdella (1996). On the other hand, low incidence of *S. aureus* mastitis was recorded by El-Mossalami et al. (1974), 3.73%; Nada and Fahmy (1975), 7.15%; Miltenburg et al. (1996), 14.4%; Sargeant et al. (1998), 6.7% and AK (2000), 13.3%. The results are in contrast with Roberson et al. (1996) who revealed that the percent of *S. aureus* was very high (82.7%). Also, the results disagree with Capurro et al. (1999) as they recorded an incidence of 97%.

The relatively high incidence of *S. aureus* in the present study may be attributed to the fact that *S. aureus* is known to spread easily among cows, especially as the time of the study was between December and May since in winter season the incidence increases due to indoor pattern. So that one case may infect more cases (Nickerson et al., 1995). As well, subclinically infected cows may act as a permanent source of infection through a continuous bacterial shedding, increasing the incidence of intermittent infection (Lam et al., 1996).

Production of toxins and extracellular enzymes, e.g. coagulase has a role in the pathogenicity as they are directly related to the establishment and progress of *S. aureus* infection

(Colqunavarro et al., 2000).

The results of PCR for detection of gene of SEA showed that out of the eight isolates tested by PCR, two (25%) were positive for SEA. This result is nearly similar to that of Mehrotra et al. (2000) and Lim et al. (2004) who reported incidence of 19.6 and 19.27%, respectively for SEA by PCR. The PCR is characterized than the other methods of *S. aureus* detection in its ability to detect SEA and NUC genes of *S. aureus* which cannot be detected by the other methods (Ikeda et al., 2005).

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Table (1): Biochemical activities of suspected *S. aureus* isolated from clinical mastitic milk samples

Test	Number	%
Biochemical	22	33.85
Tube coagulase test (TCT)	22	33.85
Urease	57	87.69
Gelatin liquefaction (proteolytic activity)	55	84.62
Aerobic fermentation of mannitol	55	84.62

Total number of tested isolates = 65.
Percentage was calculated according to the number of clinical mastitic milk samples (208).

Table (2): Pathogenic determinants of isolated *S. aureus*

Test	Number	%
Coagulase	22	33.85
Haemolysis on sheep agar	22	33.85

Number of tested isolates = 65.

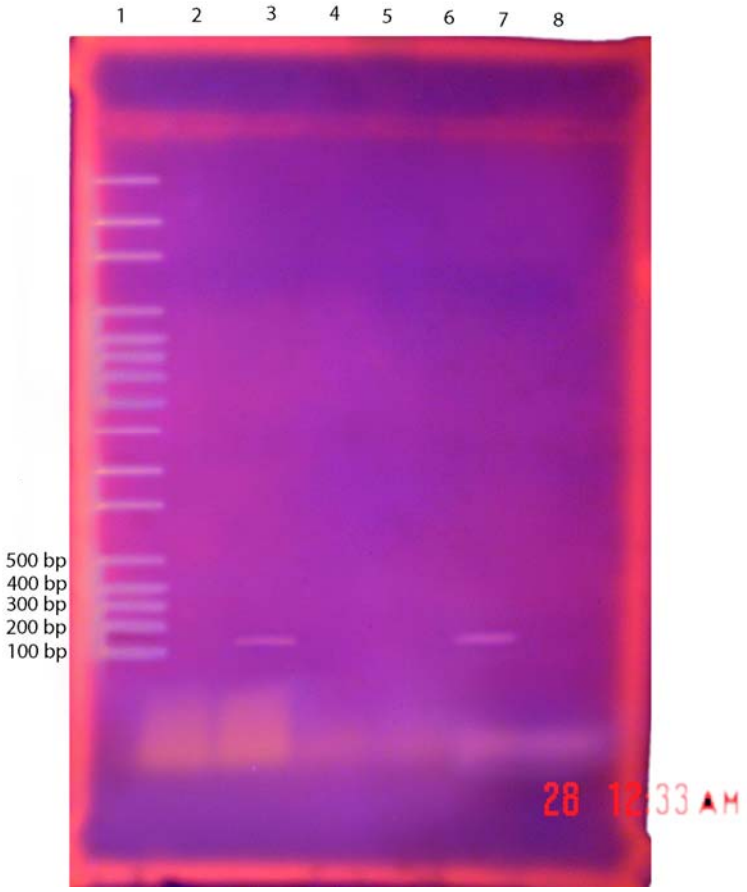

Photo (1): Results of electrophoresis of PCR product for detection of *staph aureus* enterotoxin. A, lane 1, DNA molecular size marker (100bp ladder). Lane 3 and 7 are positive. Lane 2,4,5,6 and 8 are negative staph aureus for enterotoxin A.

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