Study on the Hemolysin Phenotype and the Genetype Distribution of Staphyloccocus aureus Caused Bovine Mastitis in Shandong Dairy Farms

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ABSTRACT

Object: To develop a typing method for Staphylococcal hemolysin genes, and to research on the distribution of hemolysin phenotype and hemolysin genes in Staphylococcus aureus isolates obtained from milk of cow suffered mastitis and to analysis there relevance. **Methods:** The hemolysin phenotype was oberserved with the method of 5% blood plate. Hemolysin genes were assessed via polymerase chain reaction. **Results:** The typing method for Staphylococccal hemolysin phenotype and genes with PCR was developed. The isolates with α- hemolysis are 56 in 129 isolates, account for 43.4%; 43 show beta-hemolysis, account for 34.11%; Another 29 isolates showed no hemolysis phenotype, and it account for 22.48%. The isolates with hla gene account for 34.88%, hlb gene account for 42.60%.Conclusion: In the hemolytic phenotype, α- hemolysis are more than beta-hemolysis, however the hlb gene detection rate is higher than the hla gene. The distribution of hemolysin phenotype and hemolysin genes is not a corresponding relationship of Staphylococcus aureus isolates from raw milk of cow. The study can provide a basis for prevention and treatment of mastitis in dairy cows caused by Staphylococcus aureus.

Staphylococcus aureus can cause a range

of illnesses from minor skin infections, such as pimples, impetigo (may also be caused by Streptococcus pyogenes), cellulitis folliculitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia and sepsis, it can also cause bovine's mastitis. S. aureus have many virulence factors, such as hemolysin toxins, Panton-Valentine leukocidin (PVL), coagulase, DNAse, and many enterotoxins. S. aureus not only threat to dairy cattle health, but also endangere the safty of milk categories of food.

Hemolysin is recognized as potential virulence factor of S. aureus. Hemolysin toxins that act on cell membranes include alpha-toxin, beta-toxin, delta-toxin,etc. They can damage platelets, caused lysosome destruction, caused ischemia and necrosis of the body. Alpha-Toxin (a-HL) produced by Staphylococcus aureus is a bacterial toxin and its cytotoxicity is mediated through transmembrane pore formation[1-2]. Low concentration of Alpha -toxin produce small pores, resulting in characteristic DNA fragmentation and cell-death through apoptosis[3-4].Firstly, Alpha-toxin secreted into the bacteria extracellular as a form of soluble monomer,then it gathered in a variety of cell membrane as a form of seven polymer, small molecules within the cytoplasm such as ATP can leakage through the pore into the extracellular. For this, cell osmotic pressure can be changed irreversibly, the integrity of cell membrane be undermined. At the same time, the inflow of Ca2+ triggered a series of secondary reactions, including the production of arachidonic acid factors, the ATP, Mg2+ and GTP were secreted from intracellular, endonuclease was activated,cytokines such as IL-2 were released, membrane structure was changed, Hla-mediated epithelial cell death, IFN-β1-integrin-mediated pathogen-host cell interactions inhibited the adhesion and internalization of S.aureus. Hla can dissolve many types of cells in human and animal, including monocytes, lymphocytes, red blood cells, platelets and endothelial cells,et al. In 2009,a study demonstrate that membrane bound monomer of Staphylococcal a-Hemolysin can induces caspase activation and apoptotic cell death despite initiation of membrane repair pathway and show the range of cellular changes and responses that take place immediately after the binding of the monomeric form of staphylococcal a-hemolysin[5]. Nicky C. Caiazza have found that alpha-toxin, plays an integral role in biofilm formation, and he propose that alpha-hemolysin is required for cell-to-cell interactions during biofilm formation[6]. As known, staphylococcus aureus biofilms formed on many host tissues and implanted medical devices can often causing chronic infections.Hla antisera can inhibit the toxic effects of staphylococcus aureus on cells, and also can reduce the adhesion to bovine mammary epithelial cell layer of S. aureus. After Hla vaccination that can induce neutrophils to increase, it will effectively kill the bacteria in milk, suggesting that Hla has a good immunogenicity for Staphylococcus aureus infection[7-8]. Hlb of chromosomes in the 4-kb ClaI DNA fragment and encoding more than 330 amino acid peptide, molecular weight of about 37 ~ 39 kD esterase magnesium dependent nerve[9]. Hlb with phospholipase C-like activity, can specifically cracking sphingomyelin on cell membrane, caused the cell leakaged by cell lysis, and metal ions can promote the activity of hlb. Hlb does not lyse most types of host cells, but leaves them vulnerable to a number of other lytic agents, such as hla and Panton-Valentine leukocidin[10-12]. In fact, the cytotoxic effect of hlb is highly cell type and species-specific, suggesting that its primary virulence activity is to modulate host processes that affect pathogenesis rather than to directly kill host celles[13]. Hlb can damage the bovine mammary epithelial cells, promoting the adhesion of Staphylococcus aureus. Secondly, hlb can promot the multiplication of Staphylococcus aureus and increase the harm to the bovine by hla [14].

The aim of this study was to compare these hemolysin genetype and phenotypic characteristics among a variety of S. aureus

isolates. Study the distribution of hla and hlb gene in Shandong dairy farms, to research there relevance between hemolysin phenotype and hemolysin genetype. The date gathered could be used to develop control measures for mastitis caused by S. aureus.

MATERIALS AND METHODS

Materials

Bacterial Strains

129 S. aureus isolates were collected from the milk of cows with mastitis.The milk samples had been obtained between 2006 and 2009 from 50 different dairy herds in ShanDong province, identified by ATB system and kept frozen at -20°C in tryptic soy broth (TSB) containing 15% glycerol until molecular tests were carried out.

Instruments and equipment

Polymerase chain reaction apparatus(PTC-200),purchased from MJ RESEARCH Company; Clean Benches(MSC-12), purchased from jouan company; Refrigerated Centrifuge (GL-20G-II) ,purchased from Anke company; Electrophoresis(DYY-8C),purchased from Beijing Liuyi company; Horizontal electrophoresis tank (EPS301),purchased from amersham pharmacia bioted company; UV Gel Imaging System (GIS-1000A) ,purchased from Tanon company,etc.

Reagents and medium

Lysozyme(Shanghai sangon),dissolved by Tris.cl(PH8.0), preparation of the storage solution concentration 10mg/ml,store at 4°C. Nutrient agar,purchased by Hangzhoutianhe company.Protein K,sheep blood,reagent for PCR,et al.

Primer design and synthesis

The primers used for amplification and sequencing of the S. aureus genes were designed on the basis of the publicly available genome sequences and synthesized by Shanghai Sangon.

Methods

Hemolysin phenotyping

Identification of Staphylococcus aureus, hemolytic observation

S. aureus biochemical incubated 18-24h at37 °C, identified by ATB system.

Agar with 5% sheep blood was prepared to detect the presence of cytolysin activity. In Sheep blood agar plates,three phenomenon were appeared, α, β hemolysin phenotype and nonhemolytic phenotype in our study ,nonhemolytic phenotype means neither α nor β phenotype. Each strain inoculated at the same time on sheep blood agar media which were subsequently incubated at 37°C for 16 h. The sheep blood agar was then incubated at 4°C for a further 4 h (because the β haemolysin is a 'hot-cold' haemolysis). Then, observed their hemolytic phenotype(α,β, nonhemolytic phenotype).

Gene typing

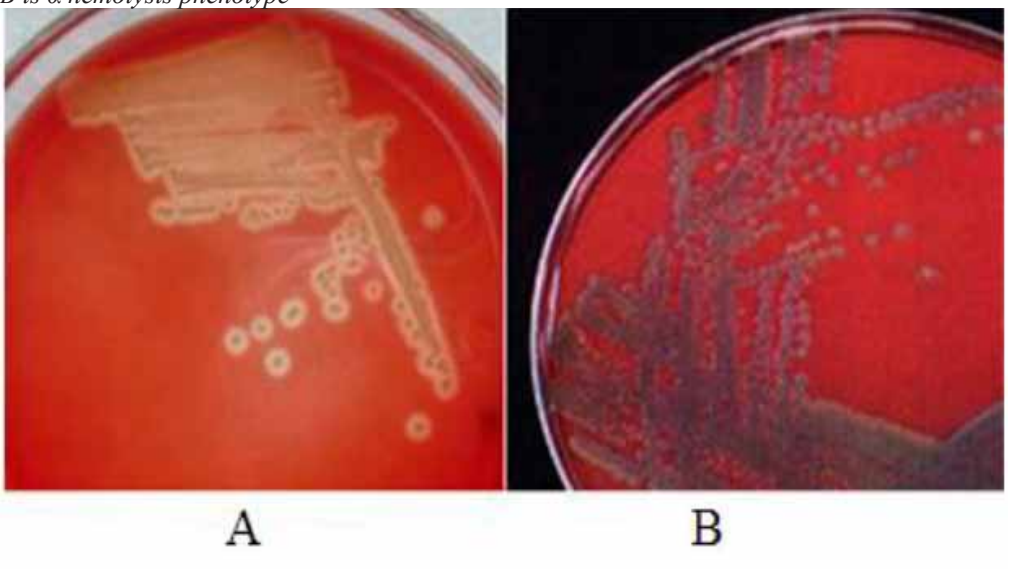
Extraction and purification of DNA

Bacterial cell lysates were prepared from 1.5mL of overnight TSB cultures. After centrifugation at 12000g for 10 min, the bacterial pellets were washed with 500μL of Tris-hydrochloride-ethylene diamine tetraacetic acid (EDTA) (TE) buffer (10 mM Tris-HCl,pH 7.5; 1 mM EDTA), and centrifuged again. The pellets were resuspended in 200uL of TE buffer, pH 7.5, with 15 U of lysostaphin (2 mg; Sigma) per milliliter, and incubated at 37°C for 1 h. Next, 15μL of proteinase K, 20 mg/mL, was added and the suspension incubated at 56°C for 1 h. The suspension was then heated at 95°C for 15

Table 1 *PCR primer sequences and product size*

Fragment	Primer sequence (5-3)	product
hla	GAAGTCTGGTGAAAACCCTGA	704bp
	TGAATCCTGTCGCTAATGCC	
hlb	CAATAGTGCCAAAGCCGAAT	496bp
	TCCAGCACCACAACGAGAAT	

Figure 1 *blood agar plate test of α and β hemolysis phenomenon A is β hemolysis phenotype; B is α hemolysis phenotype*



min to inactivate the proteinase K. An equal volume of phenolchloroform was added and the mixture centrifuged at 12 000g for 10 min. The supernatant was extracted with an equal volume of phenol-chloroform and then chloroform. The DNA in the supernatant was mixed with 2 volumes of 95% ethanol and stored overnight at-20°C. The mixture was then centrifuged at 12 000g for 5 min. The DNA pellet was washed with ice-cold 70% ethanol, recentrifuged,and dried by tube inversion. The DNA was suspended in 100μL of sterile TE, pH 7.5, quantified in a spectrophotometer(at 260 nm), and kept frozen at -20°C.

Double Polymerase chain reaction (PCR) amplification

For PCR, each reaction mixture contained 1 to 2μL of target DNA (approximately 350 ng/μL), 1μL of each of the primers (20μmol / L),2μL of a mix of deoxynucleotide triphosphates (25mmol / L each), 0.5μL of Taq polymerase (5μmol/mL), and 3μL of PCR 10X buffer (500 mM of KCl;100 mM of Tris-HCl, pH 8.4; 1% Triton X-100; and 15 mM of MgCl2).The volume of this mix was adjusted to 25μL with sterile water.Evaporation was prevented by the addition of 25μL of sterile mineral oil. Amplification was carried out in a thermal cycler (Takara Jap) as follows: initial denaturation at 94°C for 3 min, 30 cycles of amplification (denaturation at 94°C for 1min, annealing at 60°C for 45 s,and extension at 72°C for 1 min), and extension at 72°C for 10 min.

Agarose gel electrophoresis

The PCR products were separated in 1% agarose gel, respectively, with 10 mg/mL of an aqueous solution of ethidium bromide, and photographed under ultraviolet illumination.

Sequencing of the PCR product sample sequenced and compared the results on the NCBI to verify the accuracy of PCR results.

RESULTS AND ANALYSIS

Identification results

129 strains of Staphylococcus aureus identified by Gram Staining Microscopic Examination and ATB system .

Sequencing

PCR products sequenced by Shanghai Sangon.Then the sequences were submitted to NCBI, and BLAST sequence analysis showed that α-HL and β-HL has been 100% and 97% accorded with the sequences published in GenBank.

Hemolysis phenotype

The isolates with α- hemolysis was 56 in 129, account for 43.4%;43 showed β-hemolysis, account for 34.11%; Another 29 isolates showed unhemolysis phenotype, and it account for 22.48%.The data demonstated that α-hemolysis phenotype is higher than β- hemolysis phenotype.

The distribution of hemolysis gene

1% agarose electrophoresis showed the bands of hla and hlb gene products were in the right range corresponding with expectation(704 bp and 496 bp).

The detection of the hemolysin genetype of 129 s .aureus isolates by PCR method.47 with hla gene, the detection rate of 34.88%; 55 with hlb gene, the detection rate was 42.6%; 27 strains have both hla and hlb genes, the detection rate of 20.93%; 52 have neither hla gene nor hlb gene, the detection rate of 40.30%. Table.2 showed the distribution of hemolysin phenotype and hemolysin genes.

DISCUSSION

Study of Cifrian E [15] showed pathogenicity of hlb of Staphylococcus aureus. He pointed out that the key virulence factors of S.aureus's implication in red blood cell dissolution is beta-toxin. Alpha-toxin can promote the dissolution on cow red blood cell by beta -toxin while alpha-toxin exists in supernatants of S. aureus can not increase the dissolution in cow red blood cell. Purified beta -toxin had the cytotoxic effect on bovine mammary epithelial cell, but its Cytotoxicity was weaker than alpha-toxin. His study showed that beta-toxin can damage bovine mammary epithelial cell and increase the damages on the body by alpha-toxin. Alpha-toxin can also increase the adhesive forces around the bovine mammary epithelial cell caused by Staphylococcus aureus. For this, the proliferation of Staphylococcus aureus was increased,then the infection was increased in the body.We also found that the bovin suffered mastitis detected hla and hlb genes simultaneously appeared more severe than the bovine only detected only hla or hlb gene. The date we found confirmed the argument of Cifrian E from the side that hla and hlb have a synergistic effect in bovin mastitis.

The isolates with α-hemolysis was 56 in 129, account for 43.4%;43 showed β-hemolysis , account for 34.11%; Another 29 isolates showed unhemolysis phenotype, and it account for 22.48%. The isolates with hla gene account for 34.88%, hlb gene account for 42.60%; isolates with both no hla and hlb was 52, the detection rate of 40.30%.The data suggested that in the phenotype of α-hemolytic strains, the detection rate of hlb gene are higher than hla. However in β-hemolytic phenotype strains, it seems that the hlb gene detection are as the same as the hla genes.In the overall detection rate, Hlb gene is higher than hla gene. The results show some unhemolytic phenotype strains, but the hla and hlb genes are

Figure 2. *the specific test of the primers α-HL M Marker 2000; 1 E.coli ATCC 35218; 2 Sta.aureus ATCC 25923; 3 Str. agalactiae CVCC 1886*

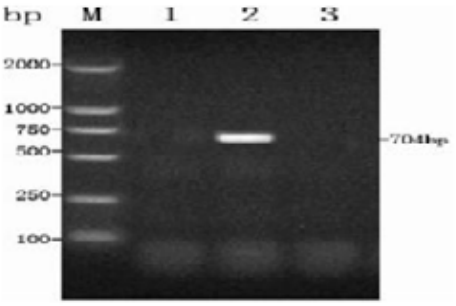
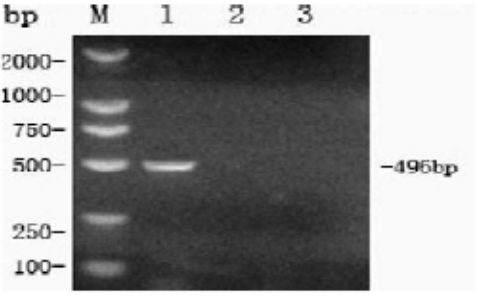


Figure 3. *the specific test of the primers β-HL M Marker2000; 1 Sta.aureus ATCC 25923; 2 E.coli ATCC 35218;3 Str. agalactiae CVCC 1886*



detected in PCR typing, the hla and hlb gene maybe silenced, the hla and hlb are not be expressed, so it does not appeared hemolytic phenomenon. Hemolysin phenotype strains as αor β, but both hla and hlb gene were not detected, there may be other reasons,maybe γ or δ-hemolysin were expressed, and they had an alone or synergistic effect on the blood agar plate, resulted in the emergence of the above , it also may be a limited number of the PCR test. In future experiments we can increased the number of the hla and hlb gene, combined with the detection of γ or δ hemolysin gene,explanat the phenomenon of the above.

A typing procedure based on polymorphism of the hemolysin gene was used to discriminate S. aureus isolated from Shandong dairy cows with mastitis.In conclusion, our results demonstrate that although mastitis caused by S. aureus strains that there is no strict correspondence between phenotype and genotype. Further studies are needed to determine the relevance between phenotype and genetype. The information gathered could be used to develop control measures for mastitis caused by S. aureus.

REFERENCES

[1] S. Bhakdi, J. Tranum-Jensen, Alpha-toxin of Staphylococcus aureus,, *Microbiol. Rev.* 55 (4) (1991) 733–751.

[2] R. Fussle et al., On the mechanism of membrane damage by Staphylococcus aureus alpha-toxin, *J. Cell Biol*. 91 (1) (1981)83–94.

[3] A. Hildebrand, M. Pohl, S. Bhakdi, Staphylococcus aureus alpha-toxin, Dual mechanism of binding to target cells., *J.Biol. Chem*. 266 (26) (1991) 17195–17200.

[4] D. Jonas et al., Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation,*Infect. Immun.* 62 (4) (1994) 1304–1312.

[5] Saumya S. Srivastava, Satyabrata Pany, Amita Sneh, Neesar Ahmed, Aejazur Rahman, Krishnasastry V.Musti. Membrane Bound Monomer of Staphylococcal a-Hemolysin Induces Caspase Activation and Apoptotic Cell Death despite Initiation of Membrane Repair Pathway. PLoS ONE 4(7): e6293.

[6] Nicky C. Caiazza,G. A. O'Toole. Alpha-Toxin Is Required for Biofilm Formation by Staphylococcus aureus [J] *JOURNAL OF BACTERIOLOGY*, 2003,5:3214-3217.

[7] Cifrian E,Guidry AJ,Obrien CN,et al.Effect of alpha-toxin and capsular exopolysaccharide on the adherence of Staphylococcus aureus to cultured teat,ductal and secretory mammary epithelial cells *[J].Res Vet Sci,*1995,58(1):20-25.

[8] Herbelin C,Poutrel B,Gilbert FB,et al.Immune recruitment and bactericidal activity of neutrophils in milk of cows vaccinated with Staphylococcu alpha-toxin[J].*J Dairy Sci,*1997,80(9):2025-2034.

[9] Marshall M J,Bohach G A,Boehm DF.Characterization of staphylococcus aureus beta-toxin induced leukotoxicity[J].*J Nat toxins*,2000,9(2):125- 128.

[10]. Doery HM, Magnusson BJ, Cheyne IM, Sulasekharam J: A phospholipase in staphylococcal toxin which hydrolyses sphingomyelin. Nature 1963, 198:1091–1092

[11]. Marques MB, Weller PF, Parsonnet J, Ransil BJ, Nicholson-Weller A:Phosphatidylinositol-specific phospholipase C, a possible virulence factor of Staphylococcus aureus. *J Clin Microbiol* 1989, 27:2451–2454

[12]. Walev I, Weller U, Strauch S, Foster T, Bhakdi S: Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of Staphylococcus aureus. *Infect Immun* 1996, 64:2974–2979

[13] Atsuko Hayashida, Allison H. Bartlett,Timothy J. Foster, et a1. Staphylococcus aureus Beta-Toxin Induces Lung Injury through Syndecan-1[J]., *The* A*merican Journal of Pathology*2009,2:509-518

[14] Cifrian E, Guidry A J, Bramley A J, et al. Effect of staphylococcal beta toxin on the cytotoxicity, proliferation and adherence of Staphylococcus aureus to bovine mammary epithelial cells[J]. *Vet Microbiol*, 1996, 48(3/4) :187-198

[15] Cifrian E, Guidry AJ, O'Brien CN, et a1. Effect of antibodies to staphylococcal alpha and beta toxins and Staphylococcus aurcus on the cytotoxicity for and adherence of the organism to bovine mammary epithelial cells[J] *Am J Vet Res,* 1996, 57(9):1308—11.

[16] Ajmone-Marsan, P., Negrini, R., Crepaldi, P., Milanesi, E., Gorni, C., Valentini,A., Cicogna, M., 2001. Assessing genetic diversity in Italian goatpopulations using AFLP markers. *Anim. Genet.* 32, 281–288.

[17] Canon, J., Garcia, D., Garcia-Atance, M.A., Obexer-Ruff, G., Lenstra, J.A.,Ajmone-Marsan, P., Dunner, S., 2006. Geographical partitioning of goat diversity in Europe and the Middle East. *Anim. Gene*t. 37, 327–334.

[18]Gjerset, B., Storset, A.K., Rimstad, E., 2006. Genetic diversity of smallruminant lentiviruses: characterization of Norwegian isolates of Caprine arthritis encephalitis virus. *J. Gen. Virol*. 87, 573–580.

[19] Glaria, I., Reina, R., Crespo, H., de Andre′ s, X., Ramı′rez, H., Biescas, E., Pe′ rez, M.M., Badiola, J., Luja′ n, L., Amorena, B., de Andre′ s, D., 2009. Phylogenetic analysis of SRLV sequences from an arthritic sheep outbreak demonstrates the introduction of CAEV-like viruses among Spanish sheep. *Vet. Microbiol*. 138 (July (1–2)), 156–162 Epub 2009 Mar 13.