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

Wang fei 1&2
Yang hongjun 1*
He hong-bin 1
Wang changfa 1
Gao yundong 1
Zhong qifeng 1
Wang Xiaohong 3
Zeng Yanjun 3*

1Research Center of Dairy Cattle, Shandong Academy of Agricultural Science, Jinan 250100, China;
2College of Life Science, Shandong Normal University, Jinan, 250014)
3Biomedical Engineering Center, Beijing University of Technology, Beijing, 100022, China

KEY WORDS: Staphylococcus aureus, Mastitis, Hemolysin phenotype, genotype distribution

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 observed with the method of 5% blood plate. Hemolysin genes were assessed via polymerase chain reaction. Results: The typing method for Staphylococcal 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 endanger the safety 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. Low concentration of Alpha-toxin produce small pores, resulting in characteristic DNA fragmentation and cell-death through apoptosis. 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. 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. 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. 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. Hlb with phospholipase C-like activity, can specifically cracking sphingomyelin on cell membrane, caused the cell leaked 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. 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 cells. Hlb can damage the bovine mammary epithelial cells, promoting the adhesion of Staphylococcus aureus. Secondly, hlb can promote the multiplication of Staphylococcus aureus and increase the harm to the bovine by hla.

The aim of this study was to compare these hemolysin genotype 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 genotype. 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 pharmaicia bioted company; UV Gel Imaging System (GIS-1000A), purchased from Tanon company, etc.

**Reagents and medium**

Lysozyme (Shanghai sangon), dissolved by Tris.cl (PH 8.0), 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 at 37°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 proteinate 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>
<thead>
<tr>
<th>Table 1</th>
<th>PCR primer sequences and product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment</td>
<td>Primer sequence (5-3)</td>
</tr>
<tr>
<td>hla</td>
<td>GAAGTCTGGTGAAAACCCCTGA</td>
</tr>
<tr>
<td></td>
<td>TGAATCCTGTCGCTAATGCC</td>
</tr>
<tr>
<td>hlb</td>
<td>CAATAGTGCCAAAGCCGAAT</td>
</tr>
<tr>
<td></td>
<td>TCCAGCACCACAACGGAAT</td>
</tr>
</tbody>
</table>
min to inactivate the proteinase K. An equal volume of phenolchloroform was added and the mixture centrifuged at 12 000 g 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 12 000 g for 5 min. The DNA pellet was washed with ice-cold 70% ethanol, recentrifuged, and dried by tube inversion. The DNA was suspended in 100 μL 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 2 μL of target DNA (approximately 350 ng/μL), 1 μL of each of the primers (20 μmol/L), 2 μL of a mix of deoxynucleotide triphosphates (25 mmol/L each), 0.5 μL of Taq polymerase (5 μmol/mL), and 3 μL 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 25 μL with sterile water. Evaporation was prevented by the addition of 25 μL 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 1 min, 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 demonstrated 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 genotype 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 Marker 2000; 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 α or 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, explain 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 genotype. The information gathered could be used to develop control measures for mastitis caused by S. aureus.

REFERENCES