Identification of predominant SNPs as a novel method for genotyping bovine Staphylococcus aureus isolates

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Staphylococcus aureus is a formidable pathogen of both human and animal. Infection often gives rise to an economic loss resulting from the extended cost of treatment and hospitalization for humans, and loss of usable agriculture animal products from infected animals and treatment regimens. We describe here a protocol for the amplification and sequencing of predominant single nucleotide polymorphisms within the promoter region of hla (encoding α-toxin) that confers a hyper-producing α-toxin phenotype to S. aureus isolates associated with chronic and severe bovine mastitis infections. We validated our findings with a second round of analysis, confirming the SNPs as a valid genotypic marker for α-toxin hyper-producing bovine isolates. The identification of highly virulent isolates will allow for aggressive treatment of the infection and limit the disease and economic impact. With readily available reagents and facilities, this protocol can be completed in as little as 72 h once samples are isolated.

Introduction

Staphylococcus aureus is an important pathogen, causing infections ranging from mild skin and soft tissue infections, to life threatening necrotizing pneumonia, endocarditis and toxic shock syndrome in humans. It is also the major causative agent of bovine mastitis. The continual rise in multiple antibiotic resistance among S. aureus isolates further complicates treatment of these diseases and increases the economic burden associated with treatment in both the medical and veterinary fields. Additionally, S. aureus mastitis infections are generally subclinical, further exasperating the loss in milk yield and treatment costs. Identifying and classifying isolates of S. aureus is a common laboratory practice and is important epidemiologically to determine colony and prevalence of isolates and follow outbreaks. Many molecular techniques are utilized to group isolates of S. aureus: Multi-Locus Sequence Typing (MLST), antibiotic sensitivity testing, Pulsed Field Gel Electrophoresis (PFGE) and PCR based assays: SCCmec typing, spa typing, coa typing, agr typing, phage typing, Automated Repetitive PCR, Panton-Valentine leukotoxin (pvl) PCR. With the exception of direct PCR for virulence genes, these methods provide a general characterization of the isolate(s) based on previously collected empirical data. This protocol provides a direct method to identify isolates that have an α-toxin hyper-producing phenotype and are likely to cause severe bovine mastitis, allowing treatment to be initiated quickly and reducing the spread of the infection.

The bovine S. aureus ET3 clone is a common international isolate of clinical and sub-clinical bovine mastitis. The ST151 sub-type of ET3 is more virulent than other ET3 sub-types and has been shown to cause increased tissue damage and mortality in a mouse model of mastitis, as well as have elevated levels of RNAIII and cytolytic toxins, including α-toxin. Alpha-toxin is an important virulence factor in mastitis. Our lab and others have shown that S. aureus ET3-ST151, including RF122, produces an abundant amount of α-toxin, far more than other bovine and human isolates.
The ET3 lineage is primarily a bovine isolate, but has been associated with human infections. Additionally, the ST151 sub-type is 500 times more susceptible to vancomycin resistance gene transfer from enterococci due to mutations in the Sdr restriction modification pathway. This poses a serious potential public health issue if the ET3/ST151 lineage is able to become a successful human colonizer, especially in a hospital setting where vancomycin resistant enterococci and MRSA are commonly found.

Recently, our research group published a paper investigating the molecular mechanism of this α-toxin hyper-production. Through the use of comparative genomics we identified predominant single nucleotide polymorphisms (SNPs) within the ica promoter region that are associated with hyper-production of α-toxin in the virulent ET3/ST151 bovine mastitis S. aureus isolate, RF122. Additionally, we found many geographically diverse isolates from around the State of Minnesota also produced an abundant level of α-toxin. Of the bovine isolates we tested, 58.3% (7/12) produced α-toxin on a level similar to RF122, and of the hyper-producing toxin isolates, 85.7% (6/7) had an identical DNA sequence and SNPs to strain RF122 within the ica promoter region. The discovery of these SNPs provides a simple PCR/sequence based protocol for the direct identification of S. aureus isolates that may cause severe infections.

To further confirm the SNPs as a molecular typing protocol for S. aureus strains that hyper-produce α-toxin we received additional samples from the University of Minnesota Veterinary Diagnostic Laboratory (Table 1) and analyzed them for their α-toxin production on Sheep’s Blood Agar plates (BAP) and sequenced their ica promoter region. Thirteen of the 22 isolates (59%) produced α-toxin on a similar level to S. aureus RF122 (Fig. 1). DNA sequencing of the ica promoter region revealed 6 of 13 (46.1%) α-toxin hyper-producing isolates had sequences identical to RF122 (Fig. 2). All the isolates that contained the SNPs were from Minnesota or Wisconsin (Table 1). Importantly, 100% of the isolates with all three SNPs were α-toxin hyper-producing strains. The presence of the SNPs indicates the strain is an α-toxin hyper-producer. Furthermore, one isolate, Bsa 5, had 2 of 3 SNPs (-484/-483) and produced high levels of α-toxin. Our previous analysis of these SNPs indicated that the presence of these two SNPs was sufficient to promote elevated levels of α-toxin transcription. For comparison purposes, we included S. aureus Wood46 on the Sheep’s BAP (Fig. 1), which has previously been indicated to produce higher levels of α-toxin. Our analysis revealed Wood46 produces more α-toxin, but has the same ica promoter DNA sequence as WCUH29, suggesting other mechanisms of regulation are promoting α-toxin production this strain. Additionally, S. aureus ED133 was included in the DNA sequence alignment (Fig. 2). ED133 is known to produce high levels of α-toxin, but is clonally distinct from RF122. The ica promoter sequence obtained from the NCBI database indicates ED133 has a sequence similar to Bsa 5, having 2 of 3 SNPs (-484/-483), suggesting the high level of α-toxin expression is partly due to the SNPs.

Our analysis of the ica promoter region of S. aureus isolates revealed the presence of three SNPs at positions -484, -483 and -376. Secondary validation of this typing found the SNPs in 100% of isolates that hyper-produce α-toxin, although high production levels of α-toxin do not mean the strain contains the SNPs. The presence of the SNPs can be used as a molecular typing tool to group strains that produce high levels of α-toxin, as well as identify isolates that may cause severe infections.

Materials

Reagents. Genomic DNA purification kit (Promega, Qagen, Invitrogen) or Phenol/ Chloroform method were used. PCR reagents. a. High-fidelity polymerase, such as Pwo (Roche) or alike. b. dNTPs c. Primers

Table 1

| Sample ID | State of Origin | Presence of SNPs* |
|-----------|----------------|-------------------|
| 1         | Wisconsin      | -                 |
| 2         | South Dakota   | -                 |
| 3         | Maryland       | -                 |
| 4         | Minnesota      | -                 |
| 5         | Minnesota      | -484 and -483    |
| 6         | Iowa           | -                 |
| 7         | Illinois       | -                 |
| 8         | Wisconsin      | -                 |
| 9         | Wisconsin      | -                 |
| 10        | South Dakota  | -                 |
| 11        | Minnesota      | -484, -483 and -376 |
| 12        | Wisconsin      | -484, -483 and -376 |
| 13        | Minnesota      | -484, -483 and -376 |
| 14        | Wisconsin      | -                 |
| 15        | Minnesota      | -484, -483 and -376 |
| 16        | Minnesota      | -                 |
| 17        | Minnesota      | -484, -483 and -376 |
| 18        | Wisconsin      | -484, -483 and -376 |
| 19        | Wisconsin      | -                 |
| 20        | Minnesota      | -484, -483 and -376 |
| 21        | Delaware       | -                 |
| 22        | Minnesota      | -                 |

*The presence of a SNP is indicated by the nucleotide sequence number: -484, -483 or -376. (-) indicates the absence of all SNPs.
i. PhlaFor: 5’-TTTAATCCCATATCATTT-3’
ii. PhlaRev: 5’TTCATCATCCTTTATTTT-3’

Gel Extraction/PCR Cleanup Kit (Promega, Qiagen, Invitrogen)

Equipment and facilities. On/off-site sequencing facility was used.

Procedure

1. Obtain bovine S. aureus isolates from source. Use enrichment and collection from bulk tank milk supply or infected animals. The isolates for the initial publication and secondary validation experiment were obtained from the University of Minnesota’s Veterinary Diagnostics Laboratory, Mastitis Department.

Day 1 (Time: Overnight).
2. Pick one colony for each isolate and incubate in Trypticase soy broth (TSB) for 16–18 h at 37°C with vigorous shaking (220RPM). Prepare a large enough volume to prepare chromosomal DNA according to the genomic purification kit to be used.

Day 2 (Time: 1 Day).
3. Isolate genomic DNA using genomic DNA purification kit according to manufacturer’s instructions or perform Phenol/Chloroform genomic DNA isolation. Resuspend gDNA in TE Buffer at 65°C for 1 h.
4. Perform PCR using PhlaFor/PhlaRev primer set and Pwo DNA polymerase or alike. Prepare a mastermix according to the manufacturer’s protocol with enough reagents for a 50 μl PCR for each sample. Aliquot 49 μl of the mastermix into a PCR tube for each sample, add 1 μl of template DNA. Set a thermocycler with the following protocol:
   a. 95°C for 2 min, 1X
   b. 95°C for 30 sec; 52°C for 30 sec; 72°C for 60 sec; each 35X
   c. 72°C for 6 min, 1X
5. Load 3–5 μl of the PCR into a 0.8% or 1% mini-agarose gel and subject to electrophoresis for ~20 min at 130 V.
6. Using a handheld UV or Gel Dock station, ensure a single PCR product is apparent. If more than one band is visible, optimize the PCR to obtain a single band or load the rest of the PCR in a large well agarose gel and subject it to electrophoresis, then cut out and remove the ~750 bp band. Purify the PCR product with a Gel Extraction/PCR clean up kit according to the manufacturer’s instructions.
7. Submit samples to sequencing facility. Refer to sequencing facility’s sample submission guidelines for primer and template concentration. Use the PhlaFor primer for sequencing.

Day 3 (Time: 24–48 h).
8. Submit samples to sequencing facility if not yet submitted. Follow instruction #7.
9. Follow Day 4 protocol for samples submitted and sequenced on the same day.

Day 4 (Time: 1–2 h).
10. Obtain sequencing data for all samples. Use a ClustalW program to align the sequences. A web-based program can be found at www.ebi.ac.uk/Tools/msa/clustalw2 or a standalone program can be downloaded at www.clcbio.com/index.php?id=1052. Export data in a supported format. If more than three sequences are to be aligned, a file can be uploaded. Please follow the appropriate links on the webpage to find supported formats and instructions. The sequence containing the relevant SNPs (in bold) of the S. aureus RF122 PhlA is as follows: ATGTTGACTCAGTAAATT CCAGAAATTTGCAAACAT AATTTATTACCCCTTTTCCTGTAT TTCTATTATTTTACGTATGG TAATATTTGTTTATTTATATTATT.
**Figure 2.** Structural alignments of *Staphylococcus aureus* isolates. The symbol ‘−’ represents the upstream region from the start codon of *hla* and the ‘***’ represents nucleotides that are same among all isolates. Nucleotide differences among the isolates are indicated by the letters in the alignment.
Problem Handling

1. PCR Optimization. Other high-fidelity DNA polymerases can be used for the PCR step. It is suggested that an initial annealing temperature gradient PCR is set-up to optimize the annealing temperature to obtain a single band of PCR product, as each buffer and enzyme combination will produce different results. A Gradient Thermocycler works best, if available.

2. Low genomic DNA concentrations or unclear DNA can cause the PCR product to be weak. If the PCR product or unclean DNA can cause the PCR Thermocycler works best, if available.

3. Use 2 μl of genomic DNA for the template and 48 μl of mastermix or, increase the units of DNA polymerase by 0.5 to 1 unit and/or increase the template and 48 μl mastermix or, increase the units of DNA polymerase by 0.5 to 1 unit and/or increase the annealing temperature up to optimize the annealing temperature

4. Anticipated Results

This protocol provides the necessary information to sequence and analyze a defined region within the hla promoter region for SNPs that are associated with the hyper-production of 3-toxin in S. aureus. Once completed, an investigator will be able to compare the sequences of their unknown isolates and determine if the unknown isolates harbor the SNPs, linked to hyper-production of 3-toxin.

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References

1. Kapoor V, Smir W, Grose R, Whitman T, Mason J. Molecular population genetic analysis of Staphylococcus aureus recovered from cows. J Clin Microbiol 1995; 33:574-9. PMID:7714959

2. World Health Organisation (WHO). World Health Organization report on infectious disease, encompassing antimicrobial resistance, 2008 (Internet) [cited 2013 Jul 9]. Available from: http://www.who.int/infodis/en/.

3. Gaud D, Royl J, Buerant D, Waller A. Cures of healthcare-associated methicillin-resistant Staphylococcus aureus and its control. Clin Microbiol Infect 2010; 16:1721-34. PMID:20934424; http://dx.doi.org/10.1111/j.1469-0691.2010.03058.x

4. Sutra L, Poutrel B. Virulence factors involved in the pathogenesis of bovine intramammary infections due to Staphylococcus aureus. J Med Microbiol 1994; 46:79-85. PMID:8070966; http://dx.doi.org/10.1099/00221217-46-1-79.

5. Church DL, Chow BL, Lloyd T, Gregson DB. Potential risks and epidemic consequences of methicillin-resistant Staphylococcus aureus (MRSA) isolates from dogs in Portugal. Microbiog Dog Rev 2011; 15:533-5. PMID:21940410; http://dx.doi.org/10.1098/smdr.2010.0088

6. Li QT, Zhu YZ, Deng R, Sun C, Zhu Y, He JJ, et al. A novel insertion-based one-typing method to discriminate nosocomial methicillin-sensitive Staphylococcus aureus isolates. J Med Sci 2011; 31:865-9. PMID:21779961; http://dx.doi.org/10.1007/s11876-011-1315-5

7. Coello C, Teres C, Bahamonde H, Pinto L, Loureiro C, Grmec-Savi E, et al. Molecular detection and characterization of methicillin-resistant Staphylococcus aureus (MRSA) isolates from dogs in Portugal. Microbiol Dog Rev 2011; 15:533-5. PMID:21940410; http://dx.doi.org/10.1098/smdr.2010.0088

8. Li QT, Zhu YZ, Deng R, Sun C, Zhu Y, He JJ, et al. A novel insertion-based one-typing method to discriminate nosocomial methicillin-sensitive Staphylococcus aureus isolates. J Med Sci 2011; 31:865-9. PMID:21779961; http://dx.doi.org/10.1007/s11876-011-1315-5

9. Vekas A, Kostiuk S, Vitaliano, A., Voigt P, Szabolics S. Adapting genotyping for national laboratory-based surveillance of methicillin-resistant Staphylococcus aureus. Eur J Clin Microbiol Infect Dis 2011; 30:789-797. PMID:21721709; http://dx.doi.org/10.1007/s10096-010-1315-5

10. Fingold JR, Meaney WS, Hartigan JP, Smith GS, Kapoor V. Fluorescent-based molecular epidemiological analysis of Staphylococcus aureus recovered from cows. Epidemiol Infect 1997; 119:261-9. PMID:9363026; http://dx.doi.org/10.1017/S0950268897011757.

11. Grattad K, Venter A, Tormo-Mas MA, Tafuri K, O’Gorman E, et al. Pathogenicity analysis of the common bovine Staphylococcus aureus clone (ET3): Emergence of a virulent subtype with potential risk to public health. J Infect Dis 2008; 198:1721-8. PMID:18177250; http://dx.doi.org/10.1086/593689

12. Jonsson P, Lindberg M, Haraldsson I, Wadstrom T. Identification of single nucleotide polymorphisms associated with hypersusceptibility to resistance gene transfer among enterococci. Antimicrob Agents Chemother 1989; 49:765-9. PMID:4040889

13. Blower M, Paul AL, O’Rielly M, Foster R, Foster TJ. Roles of alpha-toxin and hla toxin in virulence of Staphylococcus aureus for the murine mammary gland. Infect Immun 1988; 56:2499-504. PMID:3359821

14. Li QT, Zhu YZ, Deng R, Sun C, Zhu Y, He JJ, et al. A novel insertion-based one-typing method to discriminate nosocomial methicillin-sensitive Staphylococcus aureus isolates. J Med Sci 2011; 31:865-9. PMID:21779961; http://dx.doi.org/10.1007/s11876-011-1315-5

15. Vainio A, Koskela S, Virolainen A, Vuopio J, Karuki M, Smyth DS, Villaruz AE, et al. Pathogenomic model: studies of alpha hemolysin, coagulase, and protein A as possible virulence determinants with host adaptation. Genome Biol Evol 2010; 2:524689. doi:10.1093/gbe/evq031

16. Guinane CM, Ben Zakour NL, Tormo-Mas MA, O’Gorman E, et al. Fine-structure molecular epidemiological analysis of the common bovine Staphylococcus aureus clone (ET3): Emergence of a virulent subtype with potential risk to public health. J Infect Dis 2008; 198:1721-8. PMID:18177250; http://dx.doi.org/10.1086/593689

17. Ohlsen K, Koller K, Hacker J. Analysis of expression of the alpha-toxin gene (hla) of Staphylococcus aureus by using a chromosomally encoded inactivated gene cassette. Infect Immun 1997; 65:5066-74. PMID:9280136

18. Gómez-Sanz E, et al. Molecular detection and characterization of methicillin-resistant Staphylococcus aureus. J Clin Microbiol 2011; 49:3595-55. PMID:21367215; http://dx.doi.org/10.1128/JCM.02254-10

19. Garin-Toledo S, Veiga C, Azamar S, Albericio F, Chaves D, et al. Virulence of Staphylococcus aureus in a murine mastitis model: studies of alpha hemolysin, coagulase, and protein A, as possible virulence determinants with protease function androgen cloning. Infect Immun 1985; 47:765-9. PMID:2640889

20. Bramley AJ, Paul AL, O’Rielly M, Foster R, Foster TJ. Roles of alpha-toxin and hla toxin in virulence of Staphylococcus aureus for the murine mammary gland. Infect Immun 1988; 56:2499-504.