Genotypic and phenotypic □-Lactam resistance and presence of PVL gene in Staphylococci from dry bovine udder

Vinodkumar Kulangara,Neetha Nair,Asok Sivasailam,Suchithra Sasidharan,Justin Davis and Radhika Syam

Abstract

Dairy cows affected with subclinical mastitis can be sources of virulent, antimicrobial-resistant Staphylococci to humans by the excretion of the bacteria through their milk. This study focussed on the phenotypic and genotypic antibiotic resistance patterns of Staphylococci isolated from dairy cows during the early dry period. Among 96 isolates of Gram positive cocci from 157 cows, 76 were identified as Coagulase Negative Staphylococci and the remaining twenty were *Staphylococcus aureus.* Typical amplicons of coagulase gene were obtained for all twenty samples of *S. aureus* with three major coagulase types being identified as giving 627 bp (40 %), 910 bp (35 %) and 710 bp (25 %) long PCR products. The *groEL* gene was amplified in PCR of all 76 isolates of Coagulase Negative Staphylococci, and incubation of PCR products with restriction enzyme PvuII yielded three distinct PCR-RFLP fragment patterns bearing resemblance to *S. chromogenes* and *S. hyicus.* Highest sensitivity of Coagulase Negative Staphylococci was noted for Azithromycin (92.5%) and the least to Tetracyclines (76.3%), whereas for *S. aureus,* it was Cefoperazone (95%) and Azithromycin (72.2%) respectively. Phenotypic resistance to Oxacillin (25 isolates), and Cefoxitin (11 isolates) was detected by dilution method with a commercial strip (Ezy MIC™). Genotypic resistance to b-Lactam antibiotics was found in 65 (34 with *mecA* gene and 31 with *blaZ* gene) isolates. Eighteen isolates possessed both the genes, with the PVL gene for virulence being detected in five of them. Nine isolates which had *mecA* gene were phenotypically susceptible to oxacillin while phenotypic resistance to oxacillin was observed in seven isolates that did not have either *mec*A or *blaZ* gene. This is the first report of persistent Staphylococcal infections possessing PVL gene and high level of genotypic resistance to b-Lactam antibiotics in small- holder dairy cattle from India.

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Protocol

Step 1.

Coagulase gene based typing for Staphylococcus aureusTwenty isolates of S. aureus were typed using the coagulase gene.Reagents

Step 2.

PCR reaction mix included Taq DNA polymerase, Taq buffer, dNTPs, MgCl2 (Sigma- Aldrich, U.S.A)2. Primers. Details of primers as discussed in Table-1.

Step 3.

DNA template

Step 4.
Millipore WaterTable 1: Coagulase gene specific primer sequences (Moon et al., 2007)Gene Sequence 5’-3’ Size of ampliconCoagulase coaF ATAGAGATGCTGGTACAGG 500 – 900 bpcoaR GCTTCCGATTGTTCGATGCProtocolPCR reaction mix (25 µl)Forward primer (10 p mol) 1.875 µlReverse primer (10 p mol) 1.875 µldNTP 0.5 µlMgCl2 3 µlTaq DNA polymerase 1 µl10X Taq buffer 2.5 µlNuclease free water 13.25 µlTemplate DNA 1.0µlPCR cycle conditions for coagulase gene specific based PCR

Step 5.
CNS amplification for groEL gene and PCR-RFLP using PvuII.MATERIALS AND METHODSThity-five isolates of coagulase negative S. aureus were subjected to PCR targeting the groEL region. DNA from all these isolates was extracted using the DNeasy blood and Tissue kit (Qiagen, Germany). Two microlitres of the DNA obtained were run on a 0.7% agarose gel to obtain clear single DNA bands. The groEL gene of 10 of these isolates was subjected to digestion using restriction enzyme PvuII and distinct RFLP patterns were obtained.Reagents

Step 6.
PCR reaction mix included Taq DNA polymerase, Taq buffer, dNTPs, MgCl2 (Sigma- Aldrich, U.S.A)2. Primers. Details of primers as discussed in Table-2

Step 7.
DNA template

Step 8.
Millipore WaterTable 2: groEL gene specific primer sequences (Goh et al., 1997)Gene Sequence 5’-3’ Size of amplicongroEL H279A GAIIIIGCIGGIGA(TC)GGIACIACIAC 550bpH280A (TC)(TG)I(TC)(TG)ITCICC(AG)AAICCIGGIGC(TC)TTProtocolPCR reaction mix (25 µl)Forward primer (10 p mol) 4 µlReverse primer (10 p mol) 4 µldNTP 0.5 µlMgCl2 2.5 µlTaq DNA polymerase 0.5 µl10X Taq buffer 2.5 µlDMSO 1.25 µlNuclease free water 7.75 µlTemplate DNA 2.0 µlPCR cycle conditions for groEL gene based PCRRESULTSAmplicons of the desired size (550bp) were obtained for all the 35 isolates subjected to PCR.The PCR products were incubated for 8 hours with restriction enzyme PvuII at 37ºC. Three distinct PCR-RFLP patterns were identified.

Step 9.
PCR amplification and RFLP of groEL gene using PvuII enzymePCR amplificationDegenerate primers H279A (5’- GAIIIIGCIGGIGA(TC)GGIACIACIAC-3’) and H280A (5’-(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCIGGIGC(TC)TT-3’) were designed for the chaperonin groEL, and used to amplify a 550-bp groEL gene fragment. The PCR reactions were performed in a Veriti® 96-well Thermal Cycler (Applied Biosystems) in a final volume of 25µl. The cycle conditions employed included an initial denaturation at 95 ºC for 3 min; 40 cycles involving denaturation at 94 ºC for 1 min, annealing at 37 ºC for 2 min and elongation at 72 ºC for 1 min; followed by 1 cycle of final elongation at 72 ºC for 10 min. The PCR products were analysed by an agarose gel electrophoresis on a 1.5% agarose gel.PCR-RFLPThe restriction fragment length polymorphism was studied using PvuII restriction endonuclease (Fermentas), as per the prescribed protocol. Briefly, digestions were performed with 10 µl of the PCR mix and 2 µl (20 units) of the restriction enzyme in a total reaction volume of 30 µl. The product was digested for 8 hours at 37 ºC. The size of the fragments was determined by comparison with the 50 -bp DNA ladder (Fermentas) on a 2.5% agarose gel.

Step 10.
mec-A gene based PCR for identification of methicillin resistance in coagulase positive S. aureusMATERIALS AND METHODSAll the 20 isolates of coagulase positive S. aureus were analysed for presence of mecA gene.Reagents

Step 11.

PCR reaction mix included Taq DNA polymerase, Taq buffer, dNTPs, MgCl2 (Sigma- Aldrich, U.S.A)2. Primers. Details of primers as discussed in Table-3

Step 12.
DNA template

Step 13.
Millipore WaterTable 3: mecA gene specific primer sequences (Strimmenger et al., 2003)Gene Sequence5'-3' Size of ampliconmecA Forward primer AAAATCGATGGTAAAGGTTGGC 532 bpReverse primer AGTTCTGCAGTACCGGATTTGCProtocolPCR reaction mix (25 µl)Forward primer (10 p mol) 1 µlReverse primer (10 p mol) 1 µldNTP 0.5 µlMgCl2 0.5 µlTaq DNA polymerase 0.5 µl10X Taq buffer 2.5 µlNuclease free water 17 µlTemplate DNA 2 µlPCR cycle conditions for mecA gene specific based PCRMec A Gene Amplification (alternate protocol)Primer sequences:Forward 5'- GTG AAG ATA TAC CAA GTG ATT -3'Reverse 5'- ATG CGC TAT AGA TTG AAA GGA T - 3'Amplification of DNAReaction Volume was set up in a total volume of 25µL.Reaction Mix:10X PCR Buffer 2.5µLdNTP 0.2mMTaq DNA polymerase 1.25UPrimersTemplate 10µLThe final volume was made up to 25µL with Autoclaved Triple distilled H2O.Mec A PCRMecA specific PCR primers, which amplify a 146 base pair product, were used at a final concentration of 3 pico moles for the reaction.Cycling conditions: initial denaturation 94oC for 4 minutes, followed by 38 cycles, each cycle consisting of a denaturation step of 1 minute at 94oC, an annealing step of 1 minute at 61oC and an extension step of 50 seconds at 72oC. Final extension was provided at 72oC for 8minutes.