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Detection of *aroA* gene in staphylococci isolates from dog suffered with dermatitis

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Abstract
Dermatitis is a common problem in dog but to achieve the therapeutic goal is a challenge. There are various etiologic agents which are responsible for the occurrence of dog dermatitis but the opportunistic bacteria always aggravate the infection. Staphylococci mostly predominate in various dermatological disorders in canines. The study was conducted in order to find out which gene of staphylococci commonly present in canine dermatitis. To study the presence of the gene, PCR-RFLP was performed. A total of 10 staphylococci isolates from various clinical cases suffered from dermatitis were considered for molecular study and were confirmed the presence of *aroA* gene. Thus it can be inferred that presence of *aroA* gene confirms the presence *Staphylococci* spp. and mostly pathogenic for the occurrence of dermatological issues in canine.

Keywords: dermatitis, mannitol salt agar, pcr, *aroA*, staphylococci

Introduction
Globally dog has been the most closest companion animal and has been increasing remarkably in India in recent times. Dog not only provide security but simultaneously it also helps in rejuvenation of the mental health of lonely geriatric human being. The better health of dog is highly essential to restore this unconditional relationship between human and companion animal. Various diseases and disease syndromes are concerned for the owner and especially for the veterinarians in order to maintain good health. Amongst the various clinical diseases in canine dermatological diseases are most common problem in high humid areas like Assam. There are various etiological agents constantly trying to settle in dog skin leaving behind various clinical signs. Although the initial etiological agents subsides due to veterinary intervention but, the opportunistic bacteria and fungus sometime deteriorates the skin health leads to a complex situation and finally delays in recovery. In this regards proper confirmatory diagnosis is highly essential. Molecular means of diagnosis could be accurate to substantiate the study.

Materials and Method
Dog presented in Teaching Veterinary Clinical Complex, College of Veterinary Sceince, Assam Agricultural University, Khanapara, Guwahati – 781022, Assam, India and various private clinics in an around Guwahati city were subjected for the study. Cases were screened for various dermatological conditions and based on clinical findings dog dermatitis cases were considered for the collection of sample.

Isolation and identification of bacteria
Samples were collected with the help of sterile cotton swab for bacteriological study and had put it in to nutrient broth and incubated at 37 ^{0}C. Further, inoculates were processed for primary isolation in sheep blood agar and again kept at 37 ^{0}C for incubation. A pure colony has been picked up and was again processed for secondary isolation in nutrient agar. The grown colony was subjected for Gram staining to confirm the presence of gram positive bacteria. Colonies positive for Gram positive bacteria were further grown in Mannitol Salt Agar (MSA) to confirm the presence of staphylococcus. Under biochemical studies, isolates were subjected for catalase test to differentiate *Staphylococcus* from streptococcus. Further, coagulase test was performed to differentiate coagulase positive staphylococci from coagulase negative staphylococci.

In addition to above biochemical, Hugh leifson's test was performed to differentiate staphylococci from micrococci.

Extraction of DNA

Genomic DNA was extracted from each randomly selected isolates of Coagulase positive stapahylococci as per the methods mention in Gsure^{TM} Baterial Genomic DNA isolation kit from GCC Biotech kit. The concentration of purified DNA was determined by using Pico Drop Spectrophotometer.

Method 1

Genomic DNA was extracted from each randomly selected isolates of Coagulase positive staphylococci as per the method mentioned in Gsure^{TM} Baterial Genomic DNA isolation kit from GCC Biotech. The concentration of purified DNA was determined by using Pico Drop Spectrophotometer.

A single and pure colony from Mannitol Salt Agar was inoculated in to 5 ml Luria Bertani (LB) Broth and incubated it at 37 ^{0}C for 24 hours under aerobic condition. After overnight incubation, the growth was tested for its purity. The broth was transferred to a micro-centrifuge tube and was centrifuged at 12,000 rpm for 5 minutes. The pellet collected after centrifugation was suspended in 250µl GDB1 provided by the manufacturer to the cell pellet and the whole volume was transferred to 1.5 ml sterile micro-centrifuge tube in a floater. Tubes were incubated at 70 ^{0}C for 15 minutes and vortexed after every 2 minutes. Then 250 µl Buffer GDB2 was added and mixed by inverting the tube 4-6 times. The tubes were again incubated at 70 ^{0}C for 15 minutes. Later 350 µl Buffer was added GDB3 and the tube was inverted immediately. It was shaken vigorously to mix the solution. Then the micro-tubes were centrifuged for 10 minutes at 13,000 rpm in a table-top micro-centrifuge. Further, the supernatant was applied in the GMini Spin Column by pipetting. Again centrifuged at 13,000 rpm for 30-60 s and the supernatant were discarded. Then the GMini Spin Column was washed by adding 600 µl membrane wash buffer and centrifuged at 13,000 rpm for 30-60 sec. The supernatant was discarded and the washing steps were repeated and again re-centrifuged for 2 minutes to remove the additional supernatant. Then the GMini Spin Column was placed in a clean 1.5 ml micro-centrifuge tube. To elute DNA 50 µl nuclease-free water was added to the centre of each GMini Spin Column. The tubes were kept for 1 minute to stand and again centrifuged at 13,000 rpm for 1 minute on a table top micro-centrifuge. Finally, the GMini Spin Column was discarded and the eluted DNA present in pellet form in the micro-centrifuge tube was considered as the required DNA. As a precaution, whenever any sediment was found in any of the buffers provided, the container was warmed to 50 ^{0}C until the sediments get dissolved. The collected genomic DNA was stored in a micro-centrifuge tube at -20 ^{0}C for further use as template DNA for PCR.

Mehod 2: Extraction of DNA by Hot and Cold lysis

In this method 2-3 pure and isolated bacterial colonies were inoculated in to about 5 ml of enrichment broth (Luria Bertani broth) and then incubated overnight at 37 ^{0}C. Further, 1 ml of culture was transferred to a 1.5 ml sterile micro-centrifuge tube. The tubes were centrifuged at 10,000 rpm for 5 minutes and decanted the supernatant. The pellet was Re-suspended in 50 µl of 1X Tris EDTA (TE) buffer. The suspension was boiled in water bath at 100 ^{0}C for 10 minutes by placing the micro-centrifuge tube in a floating rack. After boiling, the micro-centrifuge tubes were placed immediately into ice for 10 minutes and later thawed the content and centrifuged at 10,000 rpm for 10 minutes. The clear supernatant containing the bacterial genomic DNA was collected in clean micro centrifuge tube without disturbing the pellet. The clean micro centrifuge tubes were stored at -20 ^{0}C.

Polymerase chain reaction (PCR) was conducted by preparing reaction mixture consisting of 4µl DNA template, 25µl 2X master mix, 1 ml each of specific forward and reverse primer (Integrated DNA technologies, Skokie, Illinois, 60076 USA) and making the final volume up to 50 µl with nuclease free water in a PCR tube. PCR tube containing the mixture was tapped gently, followed by a centrifuge at 8000 rpm for 3-5 seconds and the mixture was subjected to Thermocycler (Applied Biosystem, by life technologies, USA) for further processing.

Reference Culture

The reference culture of *Staphylococcus* used in the present study were obtained from Microbial Type Culture Collection (MTCC), Chandigarh. The strains were MTCC 96 and MTCC 902.

Primers

Oligonucleotide primers were obtained from integrated DNA technologies. The primers were *aroA*-F with sequence AAGGGCGAAATAGAAGTGCCG, size 21 and *aroA*-R with sequence ATTGGTTGAAGCATTGGTGT, size 20. The product length was 1,153-bp as per the Ref. Marcos *et al*. (1999) [1].

Visualization of Amplified Product

The PCR product once amplified was further visualized by electrophoresis in 1.5% agarose gel. Agarose gel was prepared by boiling 1.5% (W/V) agarose (Hi-Media Pvt. Ltd., Mumbai) in 1X Tris Acetate EDTA (TBE) buffer. The gel was cooled to 50 ^{0}C and then 5 µl of ethidium bromide (0.5 µl/ml) was added in the agarose solution. The gel was poured in gel casting tray and a fitting comb was applied for the formation of complete wells. The comb was adjusted in such a way that it hung 1 mm above the base of the tray. The sides of the tray were sealed with adhesive tape before pouring the gel to avoid leakage and later the tape was removed once solidification of gel was accomplished. The comb was removed once the gel got solidified and the gel tray was submerged in the horizontal tank (Bio Rad) filled with 1X TBE buffer.

The amplified products of desired size were visualized by submerging gel electrophoresis in 1.5% agarose gel by loading 20 µl amplified PCR product and 5 µl of gel loading dye (bromophenol blue) and electrophoresed at 80 volt for 1 hour. A standard Z 100bp / Z 1000bp DNA ladder (Thermo Scientific) was also loaded simultaneously in each gel. Amplified DNA fragments of specific size were visualized with UV light by Gel documentation system (Bio-Imaging system Mini Lumi, Israel) and the image was captured using Alpha imager EP software (Alpha InfoTech Corporation, Multi Image System, San Leandro, CA, USA).

Result and Discussion

In the result out of 10 isolates 6 (60.00%) isolates revealed positive for the presence of *aroA* gene as in the gel doc there was a thick band at 1,153-bp could be visualized. Sasaki, *et al.* (2010) [4] revealed that coagulase-positive staphylococci

(CoPS) other than *Staphylococcus aureus* have frequently been misidentified as being *S. aureus* strains, as they have several phenotypic traits in common. In this regards, the confirmed molecular diagnosis is highly essential to establish its presence. Staphylococci with special reference to *S. aureus* was found to be responsible for the cause of canine pyoderma. Most clinical cases from where the samples were collected were mostly having pustular dermatitis. Later, those pustules ruptured and slowly formed exfoliation. Simultaneously, new pustules were generated at other location. The significant lesions were scattered in ventral abdomen. Similar findings were also recorded by Reddy *et al.* (2011) [2]. In an experiment Hoekstra *et al.* (2002) [3] found that overall, *Staph. intermedius* was isolated more frequently (60.70% isolates) than *Staph. aureus* (39.30% isolates). *Staphylococcus aureus* is the causative agent of many opportunistic infections in humans and animals. In dog *S. aureus* causes superficial and, deep pyoderma and in skin and and soft tissue infections. The use of nucleic acid amplification by PCR has applications in many fields, especially for the rapid identification of bacteria. In this study we were able to identify *S. aureus* strains isolated from dog. The pair of primers used in this study did not recognize the other bacteria tested. However, in the present study, the prevalence of *Staph. aureus* was only studied, but the prevalence of *Staph. intermedius* in dog dermatitis clinical case could establish further involvement of virulence gene.

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