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PREVALENCE, BIOCHEMICAL CHARACTERIZATION AND MOLECULAR DETECTION OF *STAPHYLOCOCCUS AUREUS* IN DIFFERENT CLINICAL CASES OF LIVESTOCK AND POULTRY IN COASTAL ANDHRA PRADESH

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Abstract- Introduction: - *Staphylococcus aureus* is a panzootic gram positive pathogenic bacterium of mammalian species which produces a host of diseases in humans and animals. Since the organisms are transmissible from animals to human beings, its occurrence in animals possess a potential threat to humans.
Aims and objectives: - A study was conducted in four coastal districts of Andhra Pradesh state
Materials and Methods:- In the present study, 60 clinical samples of various animal diseases were collected from four coastal districts of Andhra Pradesh and were analysed for the presence of *Staphylococcus aureus* using biochemical tests like catalase, oxidase, Voges – Proskauer, and coagulase test and cultural characteristics like mannitol fermentation and haemolysis. Their presences were confirmed by PCR using species-specific primers for *S. aureus*. From the results, species wise, district wise and disease wise incidence of *S. aureus* is calculated
Results: -The results indicate that 68% of bovine and 82% of poultry cases were positive for *S. aureus*. The district wise distribution varied from 56.53% to 100%. In disease wise, all the cases of bumble foot in poultry, 85.65% of gangrenous dermatitis in poultry and 68% of bovine mastitis cases were positive for *S. aureus*.
Conclusion: - *S. aureus* is a potential threat due to its presence among animal diseases, which may be of zoonotic importance to in contact human beings. Further studies are required for the identification of antibiotic resistance pattern and mechanisms of antibiotic resistance of the organism.

Keywords: -*Staphylococcus aureus*, Livestock, Poultry, Diseases, Biochemical tests, PCR.

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Introduction

Staphylococcus aureus is a gram positive pathogenic bacterium affecting all known mammalian species and cause wide range of diseases in both humans and animals. It colonises mucous membrane, skin and skin glands and cause infections both in human and animals such as inflammations of bones, rashes and the meninges as well as septicaemia[1]. In addition, *S. aureus* causes Staphylococcal Scaled Skin Syndrome (SSSS), food poisoning, pneumonia, postoperative wound infections, and nosocomial bacteremia in humans and it causes mastitis in bovine and the bumble foot disease in poultry [2,3]. It also causes tenosynovitis, yolk sac infection, spondylitis, osteomyelitis, staphylococcal septicaemia, endocarditis and granuloma. Gangrenous dermatitis (GD) in commercial broiler chickens and Mastitis in buffaloes and cows is responsible for substantial economic losses for the global poultry and the dairy industry [4,5].
Extensive and inadvertent use of antibiotics both in human and in veterinary medicine is the key reason for emergence of resistant strains of *S. aureus* [6]. The antibiotics generally used for the treatments of infections caused by *S. aureus* were penicillin and its derivatives, including methicillin [7]. However, certain strains of *S. aureus* developed resistance to methicillin known as Methicillin Resistant *Staphylococcus aureus* (MRSA). There are three different types of MRSA namely Hospital Acquired MRSA (HA-MRSA), Community Acquired MRSA (CA-MRSA) and Livestock Associated MRSA (LA-MRSA) [6].
Rising numbers of domestic animals suffering from infections with methicillin-resistant *Staphylococcus aureus* (MRSA) have been reported in the recent past. As the treatment, options for MRSA are limited and due to their high zoonotic potentials to humans, these infections have gained importance. There is a drastic rise in number of reports of MRSA isolation in domestic animals [8-13]. Cross-infection of certain strains of MRSA between humans and animals, were also reported[14,15]. Animals can thus act as potential source of MRSA infection to in-contact human beings [16].
Successful strategies to combat *S. aureus* are hence the need of the hour and have to be initiated and coordinated with integrated efforts of both the medical and the veterinary professionals [17].In the present scenario, it is an essential task to study the prevalence of *S. aureus* in livestock and poultry diseases, as they possess a potential threat of MRSA to human beings. Thus, the present study is designed to evaluate a total of 60 samples collected from different clinical cases of livestock and poultry and analysed for the presence of *Staphylococcus aureus*.

Materials and Methods

Sample Collection

A total of 60 samples were collected from different clinical cases in livestock from costal Andhra from poultry, buffaloes and cows. Four districts namely Guntur, Krishna, East and West Godavari were included in the study. Swab samples were collected from suspected clinical cases of gangrenous dermatitis and bumble foot disease. Milk samples were collected from buffaloes and cows with clinical mastitis. They were further processed for isolation of *S.aureus*.

Isolation, cultural and biochemical characterization of *Staphylococcus aureus*

The collected samples were inoculated in Tryptic Soy Broth (TSB) (M/S oxoid, UK) for enriched culture and further streaked on Mannitol Salt Agar (MSA) (M/S oxoid, UK). The inoculated plates were incubated at 37°C for 24 hrs. The

suspected isolates, which were gram-positive cocci, were picked up and maintained on MSA slants for further examinations [18]. These clinical *S. aureus* isolates were further characterized by cultural and biochemical tests.The suspected isolates were identified using Catalase Test, Spot Oxidase Test, Voges – Proskauer Test. Haemolytic activity was tested on 5 % Sheep Blood Agar [2].

These characterizations were further confirmed by molecular detection tools using species-specific primers as described below.

DNA extraction from *S. aureus* for PCR

The TSB was inoculated with provisionally confirmed *S. aureus* and incubated for 18 hrs at 37°C. Out of which, 2 ml of enriched culture was taken and centrifuged at 5,000 rpm for 10 min to pellet the bacterial cell mass. Further, the pellet was washed in phosphate buffer saline (PBS) at 5,000 rpm for 10 minutes followed by washing with TKM-1 solution. The obtained pellet was resuspended in TKM-2 solution and incubated at 37°C for 15 min where the cell lysis was achieved. This step was followed by adding 10% SDS and mixing gently. Subsequently 6M NaCl was added and mixed well and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and mixed with absolute alcohol of double the volume of supernatant and centrifuged at 10,000 rpm for 5 minutes. Finally, the pellet obtained was washed twice with 70% ethanol. The DNA pellet thus obtained was resuspended in 40 µl of sterile Milli-Q water and kept at -80°C for further use in PCR [19] with suitable modifications [20].

Molecular detection of *Staphylococcus aureus* by Polymerase Chain Reaction

S. aureus was detected by species specific primers by PCR in Master cycle rep gradient thermal cycler (Eppendorff, Germany). The forward and reverse primer sequences were 5'ACGGAGTTACAAAGGACGAC 3' and 5'AGCTCAGCCTTAACGAGTAC 3' respectively [21], synthesized at Bioserve Biotechnologies (India) Pvt. Limited, India.

PCR amplification

The reaction was performed in a 25 µl reaction mixture comprising of 12.5 µl master mix (New England Biolabs, UK), 1.0 µl of forward and reverse primer, 4.0 µl of DNA template and finally 6.5 µl of distilled water. The master mix contained 10 mM TrisHCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 50 units/ml Taq DNA Polymerase, 0.2 mM of each dNTP and 0.2 µM of each primer. The PCR conditions include initial denaturation and subsequent denaturation carried out at 94˚C for 2 min and 45 sec respectively, annealing at 64˚C for 60 sec, followed by extension and final extension at 72˚C for 2 min and 10 min respectively. A total of 35 PCR cycles were run under the above conditions.

The PCR amplicons were analyzed by electrophoresis on a 1.7% agarose gel stained with 0.5 µg of ethidium bromide / ml in Tris Borate EDTA (TBE) buffer. Electrophoresis were carried out at 90 V for 120 min in submarine gel electrophoresis unit (Atto Corporation, Japan) and the PCR products were visualized in InGenius Gel Documentation System, (Syngene, U.K) along with a ProxiO 100bp DNA ladder (BioLit, SRL,India).

Results

A total of 60 suspected clinical samples were collected from clinical cases of gangrenous dermatitis and bumble foot disease in poultry and mastitis in dairy animals occurred in four different districts of coastal Andhra Pradesh.

Morphology

A total of 60 suspected clinical samples were processed and from all the samples 60 suspected *S. aureus* isolates were identified. The microscopic morphology of all the 60 isolates showed Gram positive cocci.

Cultural characterization

All the 60 isolates were mannitol fermenting when grown on mannitol salt agar medium(MSA) indicating them as *S. aureus* [Fig-1].

Fig.-1 Mannitol Fermenting

Biochemical characterization

The 60 isolates were catalase positive, coagulase positive, Voges – Proskauer Test positive and spot oxidase test negative which confirms them as *S. aureus* [Fig- 2].

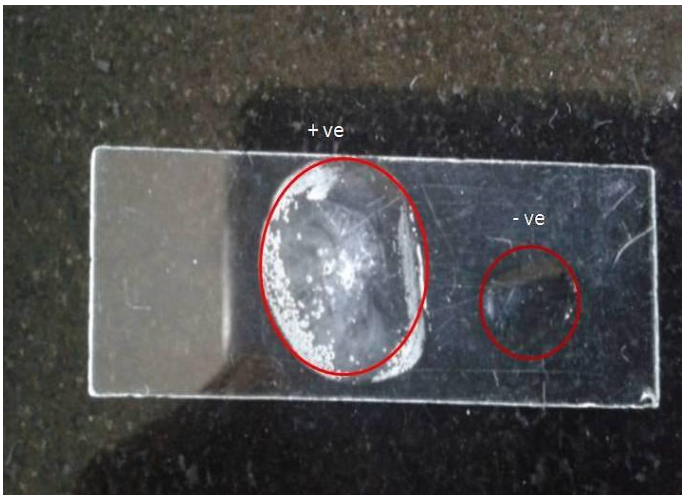

Fig.-2 Catalase Test

Haemolytic activity

Out of 60 provisionally confirmed *S. aureus*, 47 isolates shown haemolysis on 5% sheep blood agar medium which is a characteristic of *S. aureus* [Fig-3 and 4].

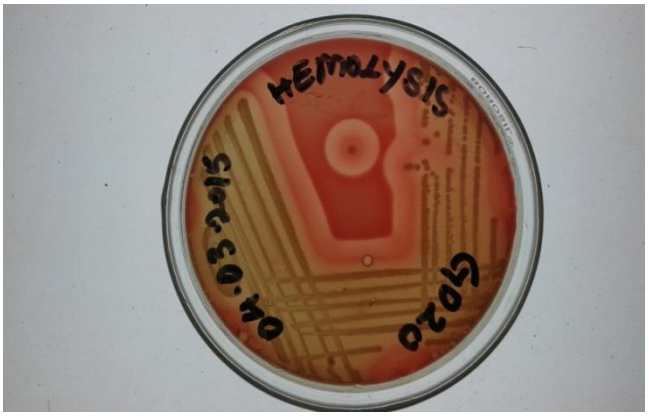

Fig.-3 Bacterial colony showing complete and incomplete haemolysis on 5% sheep blood agar medium

Fig.-4 Bacterial colony showing complete haemolysis on 5% sheep blood agar medium

Molecular detection of *S. aureus*

Provisionally confirmed 60 *S. aureus* isolates were further processed for species identification by PCR reaction. Out of 60 isolates, 42(70%) were positive for *S. aureus* [Fig-5].

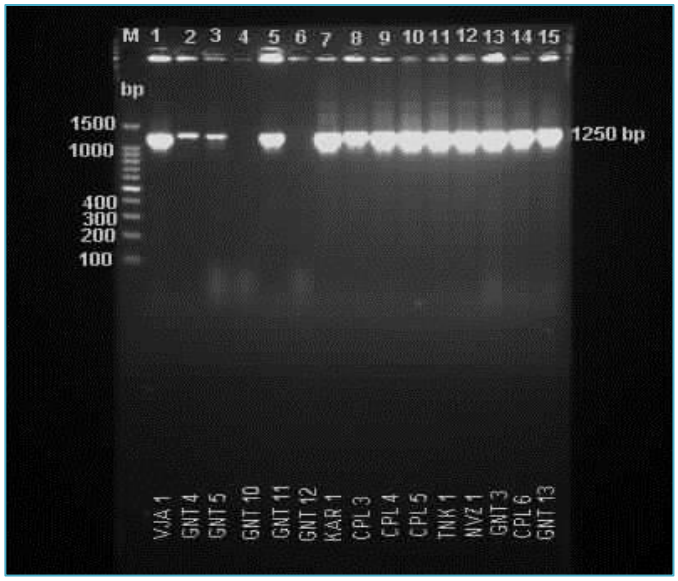

Fig.-5 PCR test with species-specific primers (Staur 4 and Staur 6) for detecting the *S. aureus*

Prevalence of *S. aureus*

S. aureus were present in 17 samples collected out of 25bovine cases (68%). In poultry 35 samples collected out of which the prevalence of *S.aureus* was in 29 (82.85%) cases.

District wise prevalence of *S. aureus*

Positive samples for *S. aureus* were obtained as shown in [Fig-6]. Highest incidence was observed in West Godavari (100%), followed by Krishna (92.85%), then Guntur (70%) and finally East Godavari (56.52%).

Species wise percentage incidence of *S. aureus* in clinical conditions

Species wise distribution is given in [Fig-7]. Out of the 25 bovine samples, 68% were positive for *S. aureus* whereas out of 35 poultry samples 82% were positive for *S. aureus.*

Disease wise percentage incidence of *S. aureus* in bovine and poultry

Disease wise distribution is given in graph [Fig- 8]. In mastitis cases, 68% were positive for *S. aureus*. In poultry with bumble foot diseases 100% were positive for *S. aureus* while in gangrenous dermatitis, 80.65% were positive.

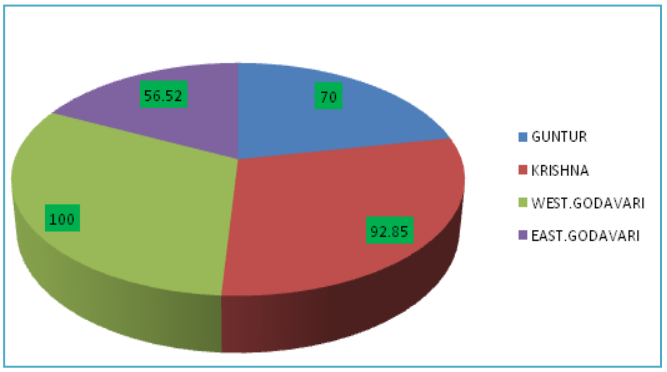

Fig.-6 District wise percentage distribution of *S. aureus* incidence in coastal Andhra Pradesh

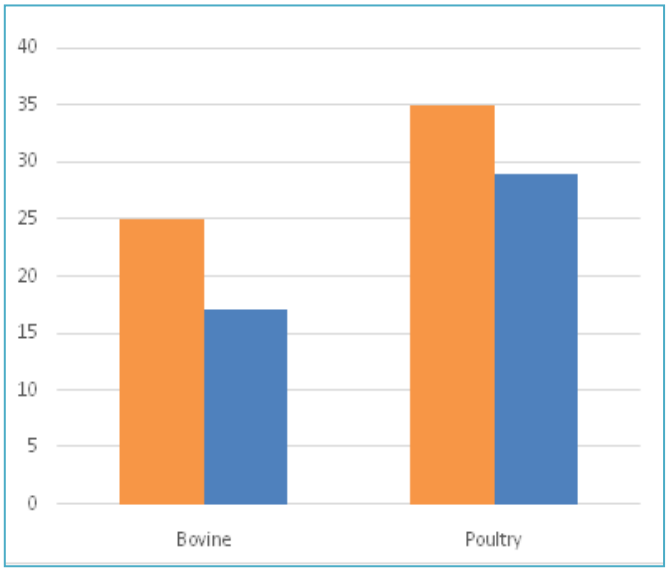

Fig.-7 Species wise percentage incidence of *S. aureus* in clinical conditions

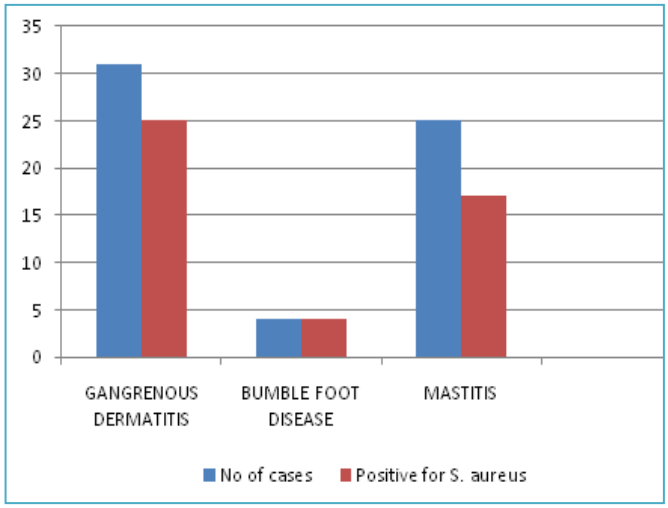

Fig.-8 Disease wise percentage incidences of *S. aureus* in bovine and poultry

Discussion

Staphylococcus aureus is the major cause of large-scale morbidity and mortality in both humans and animals [22]. Livestock-associated *S. aureus*, including multidrug-resistant *S. aureus* (MDRSA) and methicillin-resistant *S. aureus* (MRSA), can be exchanged between animals and humans [23, 24]. The nasal carriage has been observed among individuals who are in contact with livestock and poultry throughout Europe, the USA and Canada [25]. Though human-to-human transmission of livestock-associated strain may occur, they appear to be transmitted less effectively than human-adapted strains [26]. When compared to intermittent or non-colonisation *S. aureus*, persistent nasal colonisation with *S. aureus* is having an increased risk of infection in the clinical setting [27].The presence of LA-MRSA CC398 in the human food chain not only demonstrates the established risk through direct contact with animals it also shows a potential possible further pathway for the transmission of antimicrobial resistance from livestock and poultry to the broader human population [28].Inadvertent non-therapeutic use of antibiotics for prophylactic and probiotic purpose, aggravate the risk of development and propagation of antibiotic-resistant bacteria [29] and studies have proved that antibiotic-resistant bacteria can be transmitted to humans involved in livestock and dairy production management sites [25], and from these sites the bacteria are mobilised by means of multiple environmental pathways [30,31,32]. Previous studies of persistence have not investigated methicillin-susceptible *S. aureus* (MSSA) or MDRSA, though carriage of these bacteria may have important implications for clinical care and public health [27].

Keeping in view of the significance of *S.aureus*, the present work is designed to detect the prevalence of *S.aureus* in different clinical cases of gangrenous dermatitis and bumble foot disease in poultry and mastitis in dairy animals. A total of 60 samples were collected from clinical cases of bovine and poultry in four different districts of Coastal Andhra Pradesh i.e; Guntur, Krishna, East and West Godavari.

The prevalence of *S. aureus* in 25 mastitic milk samples collected from bovine, 17(68%) were positive for *S. aureus*. This signifies higher risk of *Staphylococcal mastitis* in the dairy animals in coastal Andhra Pradesh. In India, prevalence of *S. aureus* in cattle milk was found 34.01% [33], in Nepal it was 29.7% [34] and in Ethiopia it was 28.1% [35]. Another two studies in India revealed an incidence rate of 56% [36] and 50% [37].

In poultry, 35 samples were collected out of which the prevalence of *S. aureus* was 29 (82%). In the present study, the prevalence of *S. aureus* was found to be 82.65% in gangrenous dermatitis 100% in bumble foot cases. Infectious diseases of chicken flocks are a major economic burden on the poultry industry. The incidence and prevalence of Staphylococcal dermatitis was reported from long back [8 and 22], though currently there are few published reports of incidence from India.

In the present study, the occurrence of *S. aureus* varied widely among four coastal districts. In West Godavari district all the cases were positive while in East Godavari only 56.52% were positive. Regarding the disease wise occurrence in bovine 68% of cases contained *S. aureus* while in poultry all the cases of bumble foot and 80.65% were positive for *S. aureus.*

The present study indicates that the incidence of *S. aureus* is alarmingly high in diseases of cattle and poultry. Since *S. aureus* is a pandemic organism, the chances of zoonotic transmission to human beings remains to be a potential threat. Further studies are required for finding out the antibiotic resistance pattern of organism and possible mechanisms of antibiotic resistance to evaluate the danger potential of the bacteria. More extensive studies are required for the incidence of the organism in poultry, since very few studies are undertaken in this sector.

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