SUMMARY & CONCLUSIONS

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Staphylococcus aureus is an adaptable, opportunistic pathogen, its ability to persist and multiply in a variety of environments leads to wide spectrum of diseases in both humans and animals. In humans *Staphylococcus aureus* is the causative agent of many infections, ranging from superficial skin suppurations to life-threatening septicaemias associated with visceral or bone infections. Successful treatment is often hindered by the increasing prevalence of methicillin-resistant strains and by antibiotic inefficacy against the bacteria involved in chronic infections.

In lactating female animals, *Staphylococcus aureus* is a common cause of intramammary infections, frequently leading to chronic mastitis. Because this type of infection is very difficult to eradicate with antibiotic therapy, a premature culling of animals, involving substantial production losses, is the only efficient strategy to control this type of mastitis.

Various global studies are available characterizing the *Staphylococcus aureus* at molecular level, however, the studies are fragmentary from India on the current aspect and especially in *Staphylococcus aureus* isolates obtained from samples of animal origin.

Therefore, the present study was undertaken with the aim to evaluate the phenotypic and genotypic characters of *Staphylococcus aureus*, which might help to understand the characters of Indian *S. aureus* isolates obtained from human infections and from food samples of animal origin.

A total of Three thousand five hundred and fourteen clinical specimens and 1219 animal origin food samples were collected during June 2003 to March 2006. Of these 102 *Staphylococcus aureus* from

human clinical and 100 from animal origin samples were randomly selected for further study. Out of 102 *Staphylococcus aureus* studied from **clinical specimens**, 38 were from pus samples, 10 from urine samples, 1 from CSF, 2 from I/V catheter tips, 3 from body fluids, 3 from ear swabs, 3 from throat swabs, 14 from conjuctival swabs, 14 from cervical swabs, 7 from stool samples, 6 from vomitus samples and 1 from semen specimen. Out of 100 **animal-origin samples**, 29 *Staphylococcus aureus* were obtained raw milk, 4 from chamcha·n (a sweet prepared from milk), 3 from other sweets, 9 from khoa (milk concentrate used in sweets), 6 from paneer (cottage cheese), 27 from raw goat meat, 20 from raw buffalo meat, 1 from kabab (Meat cutlet) and 1 from salami sample were included in this study.

98.03% Clinical and 98% animal-origin isolates were found positive for slide coagulase test whereas they showed 100% positivity by tube coagulase test and by amplification of coagulase gene.

Out of 102 **clinical isolates** 80 (78.4%) were β-lactamase producers whereas of 100 **animal-origin isolates** 69 (69%) were found β-lactamase producers by iodometric method.

Among 102 **clinical isolates** maximum number of isolates were resistant to penicillin 100 (98.03%) followed by cotrimoxazole cotrimoxazole 71 (69.61%), tetracycline 70 (68.63%), amoxycillin 66 (64.7%), ciprofloxacin 62 (60.79%), erythromycin 56 (54.9%), amikacin 35 (35.3%), oxacillin 33 (32.35%), cefaclor 33 (32.35%), ceftriaxone 33 (32.35%), ceftazidime 33 (32.35%), cefepime 33 (32.35%), chloramphenicol 24 (23.53%) and gentamicin 28 (27.45%). While none of the isolates were found resistant to vancomycin and teicoplanin.

The isolates from **animal-origin samples** showed resistance to penicillin 93 (93%), erythromycin 51 (51%), tetracycline 49 (49%),

ciprofloxacin 39 (39%), cotrimoxazole 54 (54%), chloramphenicol 34 (34%), amikacin 27 (27%), oxacillin 27 (27%), cefaclor 27 (27%), ceftriaxone 27 (27%), ceftazidime 27 (27%), cefepime 27 (27%), gentamicin 20 (20%), and amoxycillin 19 (19%) in descending order. Only one isolate was found susceptible to all 16 antibiotics and none of the strains were found resistant to vancomycin and teicoplanin.

The drug resistance patterns of **clinical isolates** showed resistance to two drugs in 3 (2.94%) isolates, three drugs in 10 (9.8%) isolates, four drugs in 18 (17.65%) isolates, five or six drugs in 25 (24.51%) isolates, seven or eight drugs in 16 (15.67 %) isolates whereas resistance to more than ten or more drugs was found in 30 (29.47%) isolates. No isolate was resistant to only one drug.

In **animal-origin samples** the drug resistance patterns showed resistance to two drugs in 9 (9.0%) isolates, three drugs in 18 (18.0%) isolates, four drugs in 16 (16.0%) isolates, five or more than five drugs in 25 (25.0%) isolates whereas resistance to more than ten or more drugs was found in 23 (23.0%) isolates. One isolate was found sensitive to all 16 drugs.

Antimicrobial resistance has been noticed as one of the paramount microbial threats of the twenty-first century. *Staphylococcus aureus* has always been a stumbling block for antimicrobial chemotherapy and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of *Staphylococcus aureus*. Therefore, continuous surveillance on the resistance patterns and characterization of *S. aureus* in understanding new and emerging trends in human and animal from India is of utmost importance.

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen worldwide and is often difficult to detect due to the heterogeneous nature of expression of oxacillin resistance. A total of 102 clinical and 100 animal origin food isolates were tested for oxacillin resistant by disc diffusion method, by screen agar plate and detection of *mec* A gene by PCR. For the detection of *mec* A gene by PCR, the primers described by Prasad *et al.* (2000) were used, which gave a clear cut specific 604 bp amplified product.

Out of 102 **clinical isolates** 33 strains were oxacillin resistant by disc diffusion method, 32 strains by screen agar plate method and 34 strains were found positive for *mec* A gene by PCR.

In this study, 29% isolates from **animal-origin samples** were identified as MRSA by PCR, 27% by disc diffusion method and 26% by oxacillin agar plate method. The results of our study on PCR of MRSA seem to be promising for early and reliable identification of MRSA.

Bacteriophage typing was performed in 102 **clinical isolates**. Out of these isolates tested, only 55 (53.9%) isolates could be typed by the conventional set of phages at RTD. Distribution of isolates into phage groups revealed that maximum number of isolates 25 (24.51%) were typed in group III followed by 20 (19.61%) in group II, 7 (6.9%) in mixed group and (2.94%) isolates in group I. None of the isolates was typed at non allocated group.

47 non typeable isolates were further tested at 100 RTD, they showed 16.67% more typeability. Maximum number of these isolates belonged to group III (23.4%) followed by 8 5% in mixed group and 4.3% in group II.

The total typeability at 1 RTD and 100 RTD was observed in 70.58% of clinical isolates including 10 methicillin-producing strains.

MRSA phage groups were used to type methicillin resistant *Staphylococcus aureus* isolates of clinical specimens. A total of 14 (41.18%), out of 34 MRSA, were found typeable at RTD and 100 RTD using MRSA phages. Briefly, at RTD out of 15 pus isolates, 3 (20.0%) were found typeable and belonged to mixed group; 4 (100%) from cx. swab were found typeable (one in group III and 3 in mixed group); and 1 (100%) from drain tips (group I). Whereas all isolates from urine, conj. swab, ear swab, throat swab, stool, vomitus, Body fluid and CSF were found non typeable at RTD. The non typeable isolates at RTD could be typed at 100x RTD and on addition 16.6% isolates were typed. Briefly, at 100x RTD two pus isolates, out of 12, were typeable at mixed group; 3 urine isolates (one at group II and two at group III), one vomitus (at group III) were found typeable, whereas rest were non typeable.

Out of 100 **animal-origin isolates** none of the isolates were typeable at routine test dilution and at 100 RTD. MRSA phage groups were used to type methicillin-resistant *Staphylococcus aureus* isolates of food samples. Out of which only two isolates (one from milk and the other from buffalo meat) could be typed at 100 RTD which belonged to phage group II.

Two hundred and two coagulase positive *Staphylococcus aureus* isolates including 102 clinical and 100 animal-origin isolates were tested for the presence of thermostable nuclease (*nuc)* gene by polymerase chain reaction using Brakstad *et al.* (1992) method. All the 202 isolates showed a 270 bp amplified product after gel

electrophoresis, which is specific for the presence of *nuc* gene in *Staphylococcus aureus.*

The detection of the ***coa*** gene was done by Polymerase Chain Reaction using the primer set described by Goh *et al.* (1992). Twelve different electrophoretic patterns were observed in these isolates. Thirty strains (29.4%) showed a distinctive bands of 500 bp and 590 bp, 22 strains (21.6%) showed bands of 480 bp, 680 bp and 800 bp, 10 strains (9.8%) showed band of 500 bp and 580 bp, 8 strains (7.8%) showed bands of 500 bp, 720 bp and 800 bp, 7 strains (6.9%) showed bands of 500 bp, 580 bp, 700 bp and 800 bp, other 7 strains (6.9%) showed bands of 580 bp, 700 bp and 800 bp, where as other 5 strains (4.9%) showed bands of 480 bp, 500 bp and 580 bp, other 4 strains (3.9%) showed bands of 550 bp, 780 bp and 900 bp, 3 strains each (2.9)% showed 500 bp, 580 bp and 720 bp: and 550 bp, 800 bp and 900 bp respectively, 2 strains (1.9%) showed bands of 470 bp, 700 bp, 790 bp and 920 bp, whereas 1 strains (1.0%) showed bands of 550 bp 600 bp and 820 bp. Maximum numbers of strains showed a band pattern of 500 bp and 580 bp and were obtained from pus isolates.

In 100 animal-origin isolates 7 electrophoretic patterns were observed. Forty seven percent strains showed a band pattern of 900 bp, 17% strains showed bands of 650 bp and 700 bp, 13% strains showed bands of 590 bp, 680 bp and 700 bp, 8% strains showed bands of 600 bp, 7% strains showed bands of 650 bp, other 7% strains showed bands of 650 bp and 750 bp, whereas 1% strain showed bands of 500 bp and 820 bp.

The molecular typing of the clinical isolates and animal-origin isolates was carried out by Coa-RFLP, after the digestion with

Alu 1 enzyme as described by Goh *et al.*, 1992. A total 12 types of ***Coa* -RFLP** patterns were observed in **clinical isolates**. Majority of C***oa* -RFLP** patterns were observed in **clinical isolates**. Majority 30 (29.4%) of strains showed bands of 243 bp and 405 bp were classified in group XII followed by 22 strains (21.6%) in group III showing bands of 81 bp, 243 bp while 10 strains (9.8%) were characterized by distinctive bands pattern of 162 bp and 243 bp and were characterized in group VI. Eight strains (7.8%) having bands of 405 bp and 567 bp were categorized in group IX. Seven strains (6.9%) each showed bands of 81bp, 162 bp, 243 bp, 324 bp, 405 bp and 162 bp, 324 bp, 405 bp, respectively, and were classified in group I and group VIII. Five strains (4.9%) and 4 strains (3.9%) each showed bands of 81 bp, 162 bp: and 162 bp and 324 bp, respectively, were classified in group VII and group II, 3 strains (2.9%) each were classified in group IV and group X consisting of band patterns of 162 bp, 243 bp, 324 bp, 567 bp and 162 bp, 332 bp, 405 bp, respectively. Two strains (1.9%) showed band patterns of 162 bp, 243 bp, 405 bp belonged to group XI. However, 1 strain (1.0%) of group V showed band pattern of 243 bp, 324 bp. Heterogeneity was observed in specimens of a similar type. For example, *S. aureus* isolates from cervical swabs belonged to six different Coa-RFLP.

Among these 12 patterns, 7 group patterns, namely, group I, group II, group IV, group V, group VI, group VIII and group XI were noticed in MRSA isolates. Rest of the group patterns were in MSSA isolates. Briefly, out of 34 *Staphylococcus aureus* isolates from clinical specimens 7 (20.6%) belonged to *Coa*-RFLP pattern of groupI where majority of strains were resistant to six or more antibiotics; 10 strains (29.4%) belonged to *Coa*-RFLP group VI and 4 strains

(11.8%) of *Coa*-RFLP group II were also found resistant to six or more antibiotics. While 7 (20.6%) strains belonged to *Coa*- RFLP group VIII having resistance to five or more antibiotics. *Coa*-RFLP group IV and group XI having 3 strains (8.8%) and 2 (5.9%) strains respectively also showed resistance to five or more antibiotics, however, only one strain (2.9%) which belonged to *Coa*-RFLP group V showed resistance to five antibiotics.

In 100 **animal-origin isolates** 7 types of *coa* -RFLP were observed, maximum strains 47% were classified in group VI with bands of 405 bp followed by 17% strains characterized by distinctive band patterns of 243 bp and 324 bp in group V, 13% strains in group I with a band pattern of 162 bp, 250 bp and 405 bp, 8% strains showed bands of 81 bp and 324 bp and belonged to group VII. and 7% strains showed bands of 324 bp and were characterized as group II, 3% strains strain showed a band pattern of 162 bp and 405 bp classified in group IV. 1% strain of group III showed band patterns of 162 bp and 324 bp. Five each out of 29 raw milk isolates were classified in group VII and group II with band patterns of 81 bp, 324 bp and 324 bp. In the present study, *S. aureus* isolates from animal-origin samples and human clinical specimens were found to differ in their gene patterns whereas animal clinical specimens and animal-origin food samples were showed mixed Coa-RFLP gene patterns. This finding might provide a better understanding of the distribution of the *S. aureus* clones among human and animal-origin isolates and can be helpful in the control of *S. aureus* infections.

To appreciate the reproducibility of coa-RFLP the test were performed in duplicate. The reproducibility was good as similar results were obtained in both the tests.

Among these 7 patterns, 4 group patterns, namely, group II, group III, group I, and group VII, were noticed in 29 MRSA isolates from food samples. Rest of the group patterns were in MSSA isolates. Briefly, out of 29 *Staphylococcus aureus* isolates from food samples 8 (27.6%) belonged to *Coa* - RFLP pattern of group VII where majority of strains were resistant to six antibiotics. While 13 (44.8%) strains belonged to *Coa* - RFLP group I having resistance to five antibiotics. *Coa*-RFLP group III having resistance to seven or more antibiotics found in 7 strains (24.1%), however, only one strain (3.4%) showed resistance to three antibiotics were belonged to *Coa*-RFLP group III. Among these 7 patterns, 3 group patterns, namely, group IV, group V, and group VI were noticed in MSSA isolates. Antibiotic resistance patterns in relation to *Coa*-RFLP pattern could not be inferred in isolates obtained from food samples due to variability of resistance patterns; 40 different types of patterns. Majority 13 (44.8%) of the MRSA isolates showed *Coa*- RFLP pattern of group belonging to group I followed by group VII (27.6%) and group II (2.4%). However, no predominant resistance patterns were noticed.

We feel that *Coa*-RFLP technique could be incorporated as diagnostic tool to confirm the MRSA. It's also observed that the *Coa*-RFLP patterns of the MRSA strains were unique and distinct from the MSSA strains from both types of the isolates of *Staphylococcus aureus*. *Coa*-RFLP is performed with primers, homologous to a conserved region with in the *coa* gene, in order to

amplify the sequence encoding the C-terminals region of this molecule. Since the number of repetitive sequence varies within the *coa* gene, the resulting PCR products of individual strains can be of different lengths. Therefore we found different length and different patterns of *S. aureus* strains in *coa* RFLP. It is noted that there is extensive polymorphism with *coa* gene circulating in human and animal strains. We believe that the heterogeneity observed for the *coa* gene has a potential discriminatory power for further epidemiological studies of medical and veterinary importance.

For the detection of enterotoxin genes, namely enterotoxin A, enterotoxin B and enterotoxin C, PCR assay was used to amplify specific base pair products. A total of 202 isolates (102 human clinical isolates and 100 animal-origin isolates) were tested for the production of enterotoxin A, enterotoxin B and enterotoxin C. For the enterotoxin A and enterotoxin B modified method of Johnson *et al.* (1991) was used, which gave 120 bp and 478 bp gene specific products respectively while the modified method of Chen *et al.* (2001) used to amplify enterotoxin gene C which showed a gene specific 234 bp product.

Briefly, of the 102 **human clinical isolates** three were found enterotoxigenic, out of which one stool sample showed presence of enterotoxin A, 1 from vomitus sample showed enterotoxin B while one urine sample showed presence of enterotoxin C.

Out of 100 **animal-origin isolates** a total of 13 were found enterotoxogenic. Out of which 2 were positive for enterotoxin A (1 from raw milk sample and 1 from buffalo meat); 3 were positive for enterotoxin B (1 from raw milk sample, 1 from a sweet and 1 from cottage cheese) and 8 were found positive for enterotoxin C (1 from raw milk, 1 from paneer, 3 from goat meat and 3 from buffalo meat).

This is amongst the premier report regarding the prevalent enterotoxins in Indian *Staphylococcus aureus* and especially in clinical isolates. The phenotypic and genotypic results of the present study might help to understand the distribution of prevalent *S. aureus* clones in clinical and food isolates which can be the base to investigate and control the *Staphylococcus aureus* infections.

Conclusions:

On the basis of this study, following **conclusions** were drawn:

- 98.03% isolates from human clinical specimens and 98% isolates from animal-origin samples were positive for tube coagulase test, slide coagulase test and coagulase-PCR. Remaining 4 (1.98%) isolates were positive by tube coagulase test and coagulase-PCR, but missed detection by slide coagulase test.
- Multidrug resistance was noticed in both clinical and animal-origin isolates.
- None of the isolate was found resistant to vancomycin and teicoplanin in both human clinical and animal-origin isolates.
- Methicillin-resistance was found in 33.33% of clinical isolates and 29% of animal-origin isolates.
- Phage typing of the clinical isolates showed that only 70.58% clinical isolates were typeable using conventional phages at 1 RTD and at 100 RTD including 10 methicillin-producers. Amongst these, maximum number (35.3%) of isolates belonged to phage group III.
- Majority of the MRSA isolates were found non-typeable by conventional sets of phages. By using a set of MRSA phages they showed a typeability of 41.8% (14/34).
- In animal-origin isolates, only 2% isolates were found typeable using MRSA phages at 1 RTD and 100 RTD both of them belonged to group II.
- Disc diffusion, screen agar plate and polymerase chain reaction were used for detection of methicillin-resistance. MRSA were

detected in clinical isolates by disc diffusion method in 32.35%, by screen plate agar method in 31.41% and by PCR in 33.33%.

- One *mec* A positive isolate missed detection by disc diffusion while 2 isolates missed detection by screen agar plate method.
- 29% isolates from animal-origin samples were identified as MRSA by PCR, 27% by disc diffusion method and 26% by oxacillin agar plate method.
- PCR of MRSA seem to be promising for early and reliable identification of MRSA.
- All the human clinical and animal-origin isolates tested were positive for nuc gene.
- 12 Coa-RFLP patterns were observed in human clinical isolates.
- In 100 animal-origin isolates 7 types of *coa* –RFLP patterns were observed.
- In animal-origin samples and human clinical specimens different gene patterns were found.
- In animal clinical specimens and animal-origin food samples mixed Coa-RFLP gene patterns were observed.
- Coa-RFLP patterns were different for human clinical and animal-origin isolates.
- Coa-RFLP results suggest a divergence between *Staphylococcus aureus* isolates of human and bovine origin.
- Results of coagulase gene typing demonstrated that the MRSA and MSSA strains from clinical specimens could be grouped into 7 and

5 Coa-RFLP patterns, respectively. However, in animal-origin isolates 4 from MRSA and 3 from MSSA Coa- RFLP patterns were observed.

- The MRSA and MSSA strains did not share similar PCR-RFLP patterns. MRSA gave distinct *Coa*-RFLP patterns as opposed to those of MSSA.
- The ability of the PCR-RFLP typing method of the coagulase gene to differentiate between MRSA and MSSA in human clinical and animal-origin isolates was also noticed. It offers an attractive option to be considered for rapid epidemiological analysis of *S. aureus* strains.
- 102 human clinical isolates were tested for commonly countered enterotoxins; three of them were found enterotoxin producers, out of which 1 was positive for enterotoxin A, 1 for enterotoxin B and the other 1 for enterotoxin C.
- Out of 100 animal-origin isolates, a total of 13 were found enterotoxogenic. Of which 2% were positive for enterotoxin A; 3% were positive for enterotoxin B and 8% were found positive for enterotoxin C.
- Among the 16 enterotoxogenic *Staphylococcus aureus* isolates, 13 different antibiotic resistance patterns were observed in this study. All of the enterotoxogenic isolates from human clinical specimens were found resistant to methicillin. However all of the strains exhibited different antibiotic resistance patterns.
- In the remaining 13% enterotoxogenic animal-origin isolates 10 different antibiotic resistance patterns were observed. A total of 8% of them showed methicillin-resistance. Out of which, methicillin

resistance was noticed in 2% isolates, each, producing enterotoxin A and enterotoxin B, while in 4% isolates producing enterotoxin C.

In a nutshell diversity between human clinical and animal-origin isolates of *Staphylococcus aureus* was noticed and that the incidence of methicillin resistance was quite high in this collection of isolates. Concomitant high resistance to other classes of antibiotics was also noted. Phage typing was found to be of low discriminatory value whereas Coa-RFLP could discriminates a fairly large numbers of bacterial isolates and suggest that Coa-RFLP could be used as an epidemiological typing method for *Staphylococcus aureus*. The enterotoxins A, B and C were detected in our collection of human clinical, animal-origin food and animal clinical (raw milk) isolates. However, in animal-origin food isolates enterotoxin C was the predominant type.

A continuous surveillance on resistance patterns and characterization of *Staphylococcus aureus* in understanding new and emerging trends in India is of utmost importance for the formulation of infection control policies.