RESEARCH NOTE

MOLECULAR TYPING OF CLINICAL *STAPHYLOCOCCUS AUREUS* ISOLATES FROM NORTHERN INDIA USING COAGULASE GENE PCR-RFLP

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Abstract. Molecular typing of total 84 *Staphylococcus aureus* clinical isolates was performed using coagulase gene PCR. Out of 84 *S. aureus* strains total 33 different types of *S. aureus* strains were prevalent in this hospital and community. Types 2-7 and 9 were the most prevalent *S. aureus* strains accounting for more than 53% of total isolates. This technique is relatively inexpensive and is simple to perform and analyze.

INTRODUCTION

Staphylococcus aureus causes a variety of suppurative infections and toxinoses in humans. It causes superficial skin lesions as well as more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections, and deep-seated infections such as osteomyelitis and endocarditis (Lowy, 1998). *S. aureus* is a major cause of nosocomial infection of surgical wounds and infections associated with indwelling medical devices. However, methicillin resistant *S. aureus* (MRSA) strains continues to be problematic not only in hospital but in community as well (Chambers, 2001).

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Most epidemiological studies of staphylococci attempt to identify etiological agents and determine their source and distribution. Currently, there are numerous typing techniques for the discrimination of *S. aureus* isolates, which can be applied by clinicians and epidemiologists. The greatest challenge for a clinical laboratory is to carry out an analysis that can reliably group epidemiologically related strains and discriminate them from unrelated strains. These typing systems are used for the epidemiological investigation of outbreaks of nosocomial infection, and furthermore can aid in the clinical treatment of a patient, allowing for discrimination between successive and recurrent infections, and in a broader context they contribute to the understanding of the epidemiology of infections (Power, 1996; Arbeit, 1999).

Several genotypic techniques have been developed in the last decades. Initially, these techniques were only used by a few research laboratories, but they have been increasingly employed in clinical practice (Maslow *et al,*

1993). Production of coagulase, a product of coagulase (*coa*) gene, is the principal criterion in the clinical microbiology laboratory for the identification of *S. aureus*. The coagulase protein is an important virulence factor for *S. aureus.* The *coa* gene has polymorphic repeat regions that can be used for differentiating *S. aureus* isolates. The variable region of *coa* gene is comprised of 81 bp tandem short sequence repeat (SSRs) (van Belkum *et al,* 1998). Amplification of *coa* and RFLP analysis of the amplified products for the discrimination of *S. aureus* isolates have previously been reported (Goh *et al,* 1992; Shopsin *et al,* 2000; da Silva and da Silva, 2006).

In India studies have been carried out for typing of *S. aureus* using phage typing, PFGE, MLST and *spa* typing (Arakere *et al,* 2005; Javid *et al,* 2006). No work has hitherto been conducted on the molecular typing of *S. aureus* using coagulase gene PCR- RFLP in the northern part of India. The current study was done for the identification and epidemiological typing of *S. aureus* clinical isolates using coagulase gene PCR-RFLP in this part of India.

MATERIALS AND METHODS

Bacterial strains

A total of 84 *S. aureus* strains from different patients were isolated from various clinical specimens like pus, urine, blood, sputum, endo-trachial tube catheter tip during March 2003 to December 2005 from Sir Sundar Lal Hospital, Banaras Hindu University, Varanasi, a tertiary care university teaching hospital in northern part of India.

Identification

All clinical samples were plated on blood agar, whereas urine samples were plated onto CLED agar and incubated overnight at 37ºC. Bacterial genus was determined on the basis of Gram's staining, colony morphology and catalase testing. Colonies identified as *Staphylococcus* were further subjected to slide coagulase, slidex staph plus (Biomerurix India, New Delhi, India) and tube coagulase testing using rabbit plasma. Coagulase positive staphylococci were tested for thermonuclease production, acetoin production and aerobic fermentation of various sugars including sucrose, D- mannose, D- cellobiose, L- arabinose, raffinose, D- trehalose, maltose and D-mannitol (Bannerman, 2003). Strains identified as *S. aureus* were kept frozen at -20ºC in tryptic soy broth (Hi Media, New Delhi, India) containing 15% (v/v) glycerol for molecular characterization.

Coagulase gene PCR

Staphylococcal DNA was isolated using classical chloroform-phenol extraction method (Sambrook *et al*, 1989). Using NCBI data base (accession number NC_002758), primers *coa* F (5′-GGG ATA ACA AAG CAG ATG CGA TAG-3′) and *coa* R (5′-ACG TTG ATT CAG TAC CTT GTG G-3′) were synthesized for use in amplification of hypervariable region of *coa.* Extracted DNA (1 μl) was added to a 25 μl PCR reaction mixture containing 2.5 μl (20 pmol) of each primer, 2.5 μl of 10 x PCR buffer, 1.1 μl of $MgCl_2$, 1 μl of deoxynucleoside triphosphate mixture (dNTP mix), 0.33 μl of *Taq* polymerase (1.25 U) and 14.07 μl of MiliQ water. A Biometra DNA thermocycler was programmed as follows: initial denaturation, 4 minutes at 94ºC; 35 cycles with 1 minute annealing step at 54ºC and 2 minutes final extension step at 72ºC, holding step at 4ºC until sample was analyzed. PCR products were electrophoresed, stained with 10 μM ethidium bromide and visualized by using UV transillumination.

Restriction enzyme digestion of *coa* amplicon

Amplicons (10 μl) were digested overnight with 1 μl (2U) of *Alu*I (Fermatas, India), 2 μl of enzyme buffer and 7 μl of MiliQ water. Digested products were electrophoresed in agarose gel (2.5%) at 50 mV for 4 hours with ethidium

bromide solution and photographed under UV illumination.

Specificity testing

To test the specificity of the *coa* primer pair, we analyzed DNA of *S. epidermidis* ATCC 12228, *S. intermidius* 08/96PE-FUNED and *S. aureus* ATCC 25293.

Data analysis

The discriminatory power of coagulase gene PCR-RFLP was determined according to the numerical index described by Hunter and Gaston (1998). The D-value indicates the probability that 2 isolates randomly selected from the test population will be assigned to different typing groups. The following formula was used:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

with D = discriminatory index, s = total number of different types, *n*j = number of isolates representing each type, and *N* = total number of isolates in the sample population.

RESULTS

Detection by PCR of coagulase gene was carried out in 84 different *S. aureus* strains. PCR products were of 1456, 1150 and 710 bp (Fig 1). The PCR products generated different types of band patterns. Six *S. aureus* strains showed double bands, 2 had three bands and the remaining 76 strains showed a single PCR band.

Molecular typing of *S. aureus* (54 MRSA and 30 MSSA) was carried out by restriction digestion of *coa* PCR product with *Alu*I. Agarose gel analysis of *Alu*I RFLP patterns showed 33 different types of *S. aureus* strains (Fig 2 and Table 1). Types 1, 2, 3, 4, 7, 8, 10, 11, 14, 16, 18, 20 and 23 showing different 3-band RFLP patterns accounted for 54% of total population; types 5, 6, 13, 17 and 28 with different 4-band RFLP patterns 21%; types 12 and 15 with different 5-band RFLP

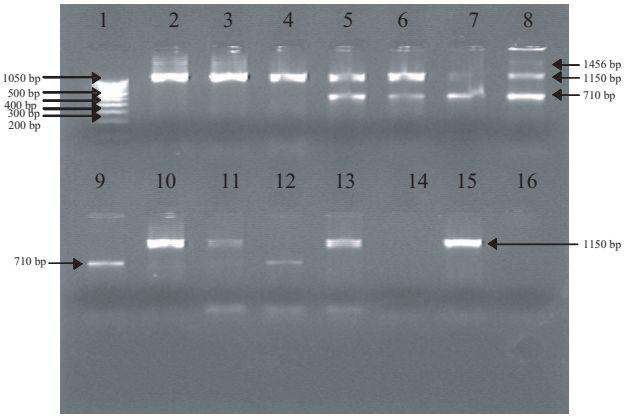

Fig 1–Detection of *coa* gene by PCR. After completion of 35 cycles of amplification, amplicons were loaded and electrophoresed in agarose gel with 10 μM ethidium bromide. Lane 2 *coa* PCR positive and lanes 14 and 16 negative control. Lane 1 has 1,000 kbp DNA ladder. Lanes 3, 4, 9, 10, 11, 12, 13 and 15 show single band; lanes 5, 6 and 7 double bands and lane 8 triple band PCR products.

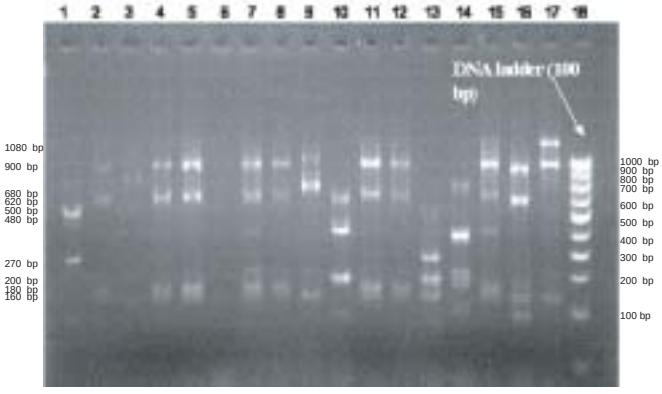

Fig 2–RFLP analysis of–*coa* gene in *S. aureus* by *Alu*I. *coa* PCR positive amplicons were digested with *Alu*I and products were electrophoresed in agarose gel with 10 μM ethidium bromide. Lanes 1-5 and 7-17, *Alu*I digested *coa* PCR products of different test strains of *S. aureus*; lane 6, negative control. Lane 18 has 100 kbp DNA ladder. Lanes 2, 4, 5, 7, 8, 9, 11, 12, 15 and 16 are RFLP patterns of strains with single band *coa* PCR product; lanes 1, 3 and 17 with double band *coa* PCR product and lanes 10 and 14 with triple band *coa* PCR product.

patterns 5%; types 9, 19, 21, 24, 25, 26 and 27 with different 2-band RFLP patterns 12%; and types 29, 30, 31, 32, 33 with different single band RFLP patterns 8%. PCR products of 7 strains were not digested by *Alu*I and therefore this method had 92% typability. The discriminatory index of this typing method was 0.95.

Sixty-one *S. aureus* strains were from different surgical departments and 21 *S. aureus* strains were from different medicine departments (Table 1). Of the samples from surgery departments, the majority of *S. aureus* strains were of types 2, 3, 5, 6, 8, 10, 11, 12, 14, 15 and 16. Types 2, 3, 4, 5, 6, 7, 9 and 14 showed multiple antibiotic resistance profile. Among medicine department's isolates no multiple antibiotic resistance profile was observed. Of the samples from surgery departments the majority was from orthopedic surgery departments.

DISCUSSION

Coagulase production is the principal criterion used for the identification of *S. aureus* isolates from human infections in clinical microbiology laboratory. In this study we have performed coagulase gene PCR of 84 *S. aureus* strains isolated from different clinical specimens. The *coa* PCR showed 100 % sensitivity and specificity. These results are in accordance with those of Aarestrup *et al* (1995) showing that among 187 strains of *S. aureus*, 10 strains of *S. intermedius*, 3 strains of *S. hyicus*, 1 strain of *S. delpheni* and 1 strain of *S. schleiferi* subspecies *coagulans* the presence of *coa* band is only in *S. aureus*. The variability in size and number of *coa* bands seen in this study may be due to presence of structurally different gene forms of coagulase in *S. aureus*, allowing one strain to produce one or more of these variants (Goh *et al,* 1992).

Goh *et al* (1992) have proposed typing of *S. aureus* on the basis of RFLP of PCR product of coagulase gene since this gene has a very high level of polymorphism in its core genome sequence. Of 84 different *S. aureus* strains 54 (64%) were MRSA and 30 (36%) were MSSA strains, isolated from different wards (40% MRSA and 9% MSSA) and different outpatients departments (29% MRSA and 26% MSSA). Of the 33 different types of *S. aureus* observed in our hospital setup 7 different types accounted for more than half of the *S. aureus* samples. Seventy-eight percent MRSA were found in types 2, 3, 4, 5, 6, 7, 8 and 9. This method could clearly classify *S. aureus* types consisting of either MRSA or MSSA or both. Types 4, 5, 8, 9, 15, 16, 19 and 23 consisted exclusively of different MRSA types whereas types 10, 12, 13, 17, 18, 20-28, 30 and 31 were consisting exclusively of different MSSA types and types 1, 2, 3, 6, 7, 10, 14, 29 and 32 of both MRSA and MSSA.

We have found 92% typability and 0.95 discriminatory index for *coa*-RFLP. Da Silva *et al* (2006) have reported discriminatory index of 0.92 for PCR amplification and 0.99 for *coa*-RFLP analysis. The coagulase gene RFLP performed here for typing of *S. aureus* isolates is much simpler to perform and analyze than other typing methods. In a similar study Goh *et al* (1992) classified 19 distinct groups out of 69 *S. aureus* isolates on the basis of coagulase PCR amplified gene products and unique *Alu*I RFLP profiles.

Type 3 *S. aureus* strains (8 out of 10), isolated from surgery departments, showed varying degrees of multiple resistance with non penicillin antimicrobials such as gentamicin, chloramphenicol, trimethoprim/sulphamethoxazole, erythromycin, ciprofloxacin and tobramycin (unpublished observations). Other strain types have also revealed multiple antibiotic resistances. Types 2, 3, 5 and 6 strains had almost similar type of antibiogram patterns. However, strains from medicine departments have demonstrated a certain degree of antibiogram similarity with some strains from

Table 1
RFLP patterns and origin of the 84 *S. aureus* strains studied.

Types	RFLP pattern	Strain no.	Strains/specimens/source	Frequency (%)
1	700/400/180	8	MSSA/Pus/ Surgery OPD	
		9	MRSA/Pus/ Surgery OPD	
		11	MRSA/ETT/ICU	4
2	720/540/230	6	MSSA/Pus/ Ped Surgery OPD	
		12	MSSA/Pus/Ortho OPD	
		41	MRSA/Pus/ F OPD	
		42	MRSA/Pus/ Burn Ward	
		43	MRSA/Pus/ Septic Ward	
		44	MRSA/Skin Exudates/ Skin and VD Ward	
		45	MRSA/Pus/ Ortho OPD	
		46	MRSA/Pus/Burn Ward	
		47	MRSA/Pus/MS Ward	11
3	900/680/160	1	MRSA/Pus/S-OPD	
		2	MSSA/Pus/Ortho OPD	
		14	MRSA/Pus/Ortho OPD	
		16	MRSA/Blood/Ped Surgery Ward	
		17	MRSA/Pus/Ortho OPD	
		19	MRSA/Pus/Ped Med Ward	
		20	MRSA/Pus/Ped Med OPD	
		21	MRSA/Pus/S OPD	
		23	MRSA/Pus/Ortho OPD	
		24	MRSA/Pus/Ortho OPD	12
4	710/400/205	26	MRSA/Pus/MS Ward	
		32	MRSA/Blood/Ped Ward	
		38	MRSA/Pus/S OPD	
		39	MRSA/Pus/F OPD	5
5	1100/890/720/420	30	MRSA/Pus/Ortho OPD	
		31	MRSA/Pus/MS Ward	
		33	MRSA/Pus/Plastic Surg Ward	
		34	MRSA/Pus/Ortho OPD	
		36	MRSA/Pus/Radiotherapy OPD	6
6	900/780/720/420	64	MRSA/Pus/Ortho OPD	
		67	MRSA/Pus/S OPD	
		68	MRSA/Pus/Ped Surg Ward	
		71	MRSA/Pus/Septic Ward	
		72	MRSA/Pus/Skin Ward	
		74	MSSA/Pus/Ortho OPD	
		75	MRSA/Pus/FS Ward	
		76	MRSA/Blood/Ped Surg Ward	9
7	840/780/400	82	MSSA/Pus/ S OPD	
		83	MRSA/Pus/Ortho OPD	
		84	MRSA/Pus/FS Ward	
		85	MRSA/Pus/Ortho OPD	5

Table 1 (continued).

Types	RFLP Pattern	Strain No.	Strains/specimens/source	Frequency (%)
8	980/780/620	77	MRSA/Pus/Plastic Surg Ward	
		78	MRSA/Pus/Ortho Ward	
		80	MRSA/Pus/S OPD	4
9	740/420	35	MRSA/Urine/Endo Ward	
		37	MRSA/Pus/Plastic Surg Ward	
		69	MRSA/Pus/F OPD	
		70	MRSA/Pus/Burn Ward	5
10	640/580/280	55	MSSA/Pus/S OPD	
		57	MSSA/Pus/Ped Surg Ward	2
11	900/780/620	40	MRSA/Pus/Ped Surg Ward	
		54	MSSA/Pus/Ortho OPD	2
12	900/810/720/480/280	58	MSSA/Pus/S OPD	
		59	MSSA/Pus/Ortho OPD	2
13	780/640/410/	52	MSSA/Urine/Endo Ward	
	220	60	MSSA/Pus/S OPD	2
14	900/780/680	86	MSSA/Pus/S OPD	
		87	MRSA/Pus/Ortho OPD	
		88	MSSA/Pus /Ortho OPD	4
15	910/780/510/	48	MRSA/Pus/ Plastic Surg Ward	
	410/190	50	MRSA/Pus/ Plastic Surg Ward	
16	750/550/490	49	MRSA/Pus/Ortho OPD	2
		51	MRSA/Pus/MS Ward	2
17	1000/750/290/180	4	MSSA/Pus/S OPD	1
18	900/290/180	5	MSSA/ Floor Swab Ortho Ward	1
19	480/270	13	MRSA/Pus/Ortho OPD	1
20	680/500/180	22	MSSA/Pus/S OPD	1
21	500/300/200/160	25	MSSA/Pus/Ortho OPD	1
22	1080/900/620/400	27	MSSA/Ear Pus/ENT Ward	1
23	900/620/160	28	MSSA/Pus/Neruo Surg Ward	1
24	1080/900/160	29	MSSA/Ped Surg Ward	1
25	780/600	56	MSSA/Pus/Ortho OPD	1
26	780/640	61	MSSA/Pus/Med OPD	1
27	900/800	62	MSSA/Pus/S OPD	1
28	900/700/620/380	63	MSSA/Pus/Ortho OPD	1
29	540	7	MSSA/Pus/S OPD	
		10	MRSA/Blood-Ped Ward	2
30	480	3	MSSA/Pus/S OPD	1
31	700	15	MSSA/Pus/Ortho OPD	1
32	900	53	MSSA/CSF/Ped Surg Ward	
		73	MRSA/Pus/Ortho OPD	2
33	780	65	MRSA/Urine/Endo Ward	1

MRSA= Methicillin resistant *S. aureus*, MSSA= Methicillin sensitive *S. aureus*, CSF= Cerebrospinal fluid, ETT= Endo tracheal tube, OPD= Out patient department, VD= Venereal disease.

surgery departments. The primers that we have designed for coagulase gene detection should be useful for identification as well for typing of *S. aureus* strains.

In summary, coagulase gene PCR-RFLP analysis was useful for typing *S. aureus*. We would recommend this typing method for *S. aureus* typing in a tertiary care hospital because of its good discriminatory power and typability and also for its ease of use and cost effectiveness.

ACKNOWLEDGEMENTS

Dr Hare Krishna Tiwari is grateful to University Grants Commission, Nepal for providing a partial fellowship for this work. We are grateful to Dr Gopalnath, Reader, Department of Microbiology, IMS, BHU, for his help in primer designing. We also thank Biomerurix India Ltd, New Delhi, India for providing slidex staph plus kit.

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