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Direct Detection of Methicillin Resistant Staphylococci: Comparison of Phenotypic Methods with Multiplex PCR and Direct Susceptibility Testing

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Authors' contributions

This work was carried out in collaboration among all authors. Author LB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author BAF supervised the conduct of the study. Author NUDW managed the analyses of the study. Author YN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Bloodstream infections are augean diseases characterized by a high morbidity and mortality, related with the lag in administration of the first adequate anti-infectious agent. Clinical and epidemiological data guide the clinicians towards empirical anti-infectious treatments whose effectualness remains questionable especially in the present day milieu of multidrug-resistant organisms. Early microbiological evidence of the causative agent is hierophantic of antimicrobial stewardship. We evaluated three rapid methods for the direct identification of *S. aureus* from 720 positive blood cultures in comparison to Vitek 2 automated microbial identification system. Early distinction *S. aureus* from CoNS was attempted with Direct Tube Coagulase. Multiplex PCR assay was used to separate MRSA from MSSA by the presence of mecA gene. Direct antibiotic susceptibility (DST) from positive blood culture bottles was performed in an endeavour to reduce time and compared to the routine Vitek 2 compact antimicrobial susceptibility. For Direct tube coagulase at 4 hrs of incubation sensitivity was 79.6% while specificity was 100% however, on

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overnight incubation sensitivity increased to 97.9% with 100% specificity. The positive predictive value and negative predictive values were 100% and 96.9% respectively when compared to Vitek 2 identification of staphylococcal species. By DST Categorical agreement of 95% was seen for coagulase negative staphylococci microorganism-antibiotic combinations. Categorical agreement of 89.7% was seen for *S. aureus* microorganism-antibiotic combinations. Direct multiplex PCR testing did not misidentify any *S. aureus* isolate compared to Vitek 2. In 12 methicillin resistant CoNS isolates mecA gene was not detected by PCR. In total 13(3.5%) strains of staphylococci identified by DST as methicillin resistant were not identified by PCR analysis. Each of the tests has positive qualities and all may have a place in a Gram positive cocci algorithm for testing blood cultures depending on the laboratory setting, workload volume and staffing. However, rapid detection methods are a pressing priority.

Keywords: Blood stream infections; Staphylococcus; MRSA; DST; multiplex PCR.

1. INTRODUCTION

Staphylococcus aureus is a serious threat to human health, as it causes wide range of infections. Serious infections include bacteremia, pneumonia, endocarditis, bone and joint infections, and toxic shock syndrome. *S. aureus* can also be responsible for outbreaks of food poisoning.

There are several other species of staphylococci with clinical relevance, collectively referred as Coagulase Negative Staphylococci (CoNS). The most CoNS associated with human disease include *S. epidermidis*, *S. saprophyticus*, *S. lugdunensis* and *S. haemolyticus* [1]. CoNS are abundantly inhabiting normal human skin and mucous membrane. They infrequently cause primary invasive disease and are most commonly encountered as contaminants. Differentiation of contamination from true infection is essential to enable appropriate patient management although clinically insignificant are considered significant by the clinician if the following conditions were present: (i) patient has multiple episodes of bacteremia (ii) intravascular catheters, prosthetic heart valves, or other risk factors are present *in situ*; or (iii) patient has pyrexia, leukocytosis [1].

CoNS often serve as inventories of antimicrobial-resistance determinants [2]. Therefore, the assortment of *S. aureus* from CoNS is of importance. The rise of antibiotic-resistant strains in the 1960s and 1970s; particularly methicillin-resistant *S. aureus* (MRSA) has created additional therapeutic challenges. USA reports 35% of their hospital strains of *S. aureus* as methicillin resistant which were declining till 2012 but has remained almost steady in recent studies [3]. In Europe 44% of the hospital strains of *S. aureus* were methicillin resistant which has increased in the previous years [4].

Methicillin resistant *S. aureus* infections account for 40-60% of all nosocomial *S. aureus* infections in many centers across the world. MRSA is now endemic in India, some cities reporting 70% of the strains to be resistant to methicillin. The incidence of MRSA varies from 25% in western part of India to 50% in South India [5]. In north India, the prevalence of MRSA was 46 % where MRSA isolates were found to be more resistant to other antibiotics than Methicillin sensitive *Staphylococcus aureus* (MSSA). There was a prevalence of 39.48% MRSA reported from Sher-i-Kashmir Institute of Medical Sciences in 2014 [6].

Detection of the *mec A*gene by Polymerase chain reaction (PCR) has been described as a rapid method for the identification of MRSA [7]. PCR assays improve clinical outcomes by decreasing the time to identification of CoNS, MSSA and MRSA and by allowing for early and more effective antimicrobial therapy. The limitation of use of PCR in resource poor countries can be substituted to some extent by DTC (Direct Tube Coagulase Test) and much time can be saved by performing direct susceptibility testing on the flag positive culture broth, therefore allowing rapid discrimination (2-5 hrs) between *S. aureus* and potential contaminant CoNS. Rapid microbiological investigations—identification of the causative agent and antimicrobial susceptibility testing (AST)–are therefore very important: 1) to adjust the anti-infectious therapy and to avoid inefficient treatment, 2) to reduce the spectrum of the anti-infectious therapy so as to limit the selection of resistant strains and 3) to limit the toxicity and negative impact on beneficial bacteria of some broad-spectrum antibiotics or combined therapy [8].

The present study was conceived with an aim of early distinction of *S. aureus* from CoNS with

Direct Tube Coagulase test. PCR assay was used to separate MRSA from MSSA by the prescence of mecAgene. We also compared antibiotic susceptibility directly from positive blood culture bottles with Vitek 2 to reduce time. All these efforts may prove to be an effective tool for antimicrobial stewardship.

2. MATERIALS AND METHODS

This prospective study was conducted in the Department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences (SKIMS); a 720 bed tertiary care hospital. The study was carried out over a period of 6 months from January to June 2015.

Positive blood culture bottles which showed Gram positive cocci in clusters were included for further analysis. Blood cultures of repeated samples from the same patient, positive cultures showing more than one organism and Gram negative bacteria on Gram stain were excluded.

2.1 Sample Processing

Positive blood culture samples from BacT/Alert microbial detection system were selected; aliquots from these bottles were subjected to Gram staining. Gram positive cocci in clusters when found was further processed. Ten (10) ml of fluid was aspirated aseptically from blood culture bottles and further subjected to DNA extraction and multiplex PCR amplification for *mecA* and *nucA* gene. Direct susceptibility testing and direct tube coagulase test was done from the positive blood culture broth. The same broth was sub-cultured and growth was identified and susceptibility to antibiotics checked by Vitek 2 compact.

2.2 DNA Extraction [9]

One ml of sterile, distilled water was added to 200 µl aliquot of each sample and mixed by inversion. The sample was incubated at room temperature for 5 mins then centrifuged at 14000 rpm for 1 minute. The supernatant was discarded and the pellet resuspended in 100 µl of Triton X-100 lysis buffer (100 Mm NaCl, 10 Mm of Tris HCL, 1 mM EDTA and 1% Triton x-100). Five (5) microliters of lysostaphin (1 mg/ ml, Sigma Aldrich) was added, mixed and incubated at 37ºC for 10 min. This suspension was boiled for 10 min. After cooling to room temperature for 5 min, the sample was centrifuged at 14000 rpm for 1 min and 1 µl of the supernatant was used for PCR.

2.3 Multiplex PCR Amplification

PCR was performed for the detection of *mecA* and *nucA* genes by the methodology as described by Mason, et al. [10] Multiplex PCR was performed in a 25 µl volume with 1× PCR Buffer, 3 mM $MgCl_2$, a 200 µM concentration of each deoxynucleoside triphosphate, 1 µl of *Taq*polymerase, 0.5 µl *nuc*and 0.5 µl *mec*A primer with 2.5 µl of template DNA. The following primers were used.

mec-A1 (5'-AAA ATC GAT GGT AAA GGTTGC C-3')

mec-A2 (5'- AGT TCT GCA GTA CCG GATTTG C- 3')

nuc-A1 (5'- GCG ATT GAT GGT GAT ACGGTT-3')

nuc-A2 (5'- AGC CAA GCC TTG ACG AACTAA AGC- 3')

Thermocycling conditions in a Gene Amp 9600 thermocycler were as follows: 94ºC for 5 mins, followed by 25 cycles of 94ºC for 30 sec, 59ºC for 30 sec and 72ºC for 30 sec, with final extension at 72ºC for 10 min. Thermocycling was completed in 2.15 h. ATCC 43300 was used as control strain. Electrophoresis at 100 V for 30 min was performed to separate the PCR amplicons on a 1% 1X TBE (Tris – borate - EDTA) – agarose gel. Gels were photographed under UV illumination.

2.4 Direct Susceptibility Testing

The aspirated sample from blood culture bottle was directly used for direct susceptibility testing. Five (5) ml of aspirated sample from blood culture bottles was centrifuged at 160× g for 5 min to pellet blood cells. The supernatant was then centrifuged at 650 × g for 10 min to pellet bacteria. The turbidity of the bacterial suspension was adjusted to match the Mac Farland 0.5 standard. The antibiotic susceptibility testing was performed using Kirby–Bauer disc diffusion method according to CLSI guidelines [11].

The following antibiotic discs procured from Himedia Mumbai India were used; Penicillin 10 units; Cefoxitin 30 µg; Vancomycin 30 µg; Teicoplanin 30 µg; Ciprofloxacin 5 µg; Cotrimoxazole 1.25/23.75 µg; Tetracycline 30 µg; Clindamycin 2 µg; Erythromycin 15 µg; Gentamycin 10 µg; Levofloxacin 5 µg; Linezolide

30 µg. The zone sizes were measured and interpreted using CLSI breakpoints. Though CLSI does not mention disc diffusion criteria of vancomycin and teicoplanin, disc contents were chosen as applied to *Enterococcus spp.* and interpreted accordingly. MIC by Vitek 2 compact system was taken as a comparative standard. *Staphylococcus aureus* ATCC 25923 was used as control strain.

2.5 Direct Tube Coagulase Test (DTC) [12]

Tube coagulase test was performed directly from blood culture bottle in 100 mm X 12 mm Pyrex glass tubes containing 1 ml of 10% pooled human plasma containing the anticoagulant EDTA. These were inoculated with 4 drops (0.1 ml) from a 1:10 dilution of the broth, prepared by suspending 10 drops (0.25 ml) of blood culture broth in 2.5 ml of 0.9% saline. The plasma tube was examined after every hour until 4 hrs of aerobic incubation at 35ºC. Then it was incubated overnight at room temperature and re-examined. The test was recorded as positive if a clot was observed at either time. *Staphylococcus aureus* ATCC 29213 was used as control strain.

2.6 Identification and Susceptibility by Vitek-2 Compact (V2C) [13]

Identification and susceptibility by Vitek-2 was done as per the manufacturer's protocol using V2C Card (AST-P628).The aspirated fluid from the positive blood culture bottle was Gram stained followed by subculture on blood agar and incubated at 37ºC overnight. The growths were examined and used for the preparation of V2C suspensions for test microbes using sterile loop, a homogenous organism suspension was made by transferring several isolated colonies from the plates to 4 ml of sterile saline and adjusted to the McFarland standard using a calibrated V2C Densi-Chek Meter 0.5-0.63 for GP. The suspensions were placed in the cassette and the V2C Card (AST-P628) was inoculated. *S. aureus* ATCC 29213 was used as control strain and tested weekly.

3. RESULTS

A total of 720 blood culture bottles were flagged positive by the BacT /Alert microbial detection system during the study period. Out of these, 350 blood cultures were excluded from the study as they did not meet the inclusion criteria.

The 370(51.3%) blood cultures which yielded Gram positive cocci in clusters were included in the study. Out of the 370 samples 321 (86.8%) were CoNS and 49 were (13.2%) *Staphylococcus aureus* isolates. Methicillin resistance was seen in 31 (63.3%) *Staphylococcus aureus* strains whereas 18 (36.7%) were methicillin sensitive. Among CoNS, 260 (81%) were methicillin resistant and 61 (19%) were sensitive to methicillin. Overall, 291 (78.6%) *Staphylococci* were resistant to methicillin and 79 (21.4%) sensitive to it.

Twelve different species of CoNS were identified from blood. The most common CoNS recovered was *S. hominis* 134 (41.7%), followed by *S. epidermidis* 107 (33.3%), *S. haemolyticus* 45 (14.0%), *S. capitis* 11 (3.4%), *S. warneri* 9 (2.8%), *S. lugdunensis* and *S. sciuri* 4 (1.2%) each, *S. cohenii* and *S. caprae* 2 (0.6%) each and *S. equorum*, *S. saprophyticus* and *S. auricularis* 1 (0.3%) each.

The patients recruited for the current study included 220 males (59.5%) and 150 (40.5%) females. Most of the patients from whom *Staphylococci* were isolated, were in the age group of 50-59 years, 96 (25.9%) followed by the age group of 0-9, 78 (21.1%); ≥ 60, 70 (18.9%); 40-49, 60 (16.2%); 30-39, 28 (7.6%); 20-29, 26 (7.0%) and 10-19 years, 12 (3.2%) respectively.

Samples received from neonatology yielded the maximum number of Staphylococcal isolates, 77 (20.8%), followed by Observation ward 41 (11.1%), surgical intensive care unit 33 (8.9%), gastroenterology 30 (8.1%), hematology 26 (7.0%), outpatient department 22 (5.9%), endocrinology 21 (5.7%), accident emergency 20 (5.4%), general medicine, nephrology and plastic surgery 14 (3.8%) each, post operative ward 12 (3.2%), neurology 10 (2.7%). In addition to this, 7 (1.9%) samples were received from kidney transplant unit and medical intensive care unit each, 6 (1.6%) from cardiovascular and thoracic surgery, 5 (1.4%) from cardiology, 4 (1.1%) from neurosurgery and pediatric surgery each, 2 (0.5%) from urology and 1 (0.3%) sample from general surgery.

Of the 49 *S. aureus* isolates as identified by Vitek, DTC showed 39 (79.5%) isolates with obvious gelling at 4 hrs while 10(20.4%) showed partial gelling. The 10 samples were further incubated at room temperature overnight, 9 of the samples showed gelling, while 1 sample remained partially jellified even after overnight

incubation. No samples from the CoNS group tested false positive by direct tube coagulase test. Vitek 2 results and DTC denoted very good agreement. (*kappa, k =0.871*).

Susceptibility results obtained by Direct Sensitivity were compared to antibiotic susceptibility by Vitek-2 and results are shown in Table 1 and in Table 2.

Multiplex PCR to detect the *nuc* and *mec-A* gene was done on all the blood culture bottles directly Figs. 1,2. Direct multiplex PCR testing did not misidentify any *S. aureus* isolate compared to Vitek-2. It confirmed the methicillin resistant *S. aureus* isolates to be positive for *nuc* and *mec-A* gene with the exception of one *S. aureus* isolate Table 3. However, only 248/260 methicillin resistant CoNS harbored the *mec-A* gene. In 12 methicillin resistant CoNS isolates *mec-A* gene was not detected by PCR.Overall 13 isolates of *Staphylococcus* were not picked by PCR as *mecA* harbourers; *S. aureus* (1), *S. hominis*(5), *S. hemolyticus* (6), *S. capitis*(1).

Total time required to perform the multiplex PCR assay directly from positive blood culture bottles was 3 hrs. Turnaround time of direct tube coagulase test was 4hrs. Direct susceptibility testing from flag positive bottles took 18 hrs on an average when compared to conventional AST which took around 32 hrs.

4. DISCUSSION

Bloodstream infections (BSIs) are severe diseases characterized by a high morbidity and mortality, related with the lag in administration of the first adequate anti-infectious agent [14].

Table 1. Antibiotic susceptibility profile of CoNS isolates by DST and Vitek 2

Antibiotic	DST (n=321)						VITEK (n=321)						Kappa
	S		I		R		S		I		R		
	n	%	n	%	n	%	n	%	n	%	n	%	
Penicillin	12	3.7	0	-	309	96.3	10	3.1	2	0.6	309	96.3	0.914
Cefoxitin	61	19	0	-	260	81	61	19	0	-	260	81	1.000
Vancomycin	-	-	-	-	-	-	321	100	0	-	0	-	-
Teicoplanin	321	100	0	-	0	-	321	100	0	-	0	-	-
Ciprofloxacin	90	28	-	-	231	72	90	28	3	0.9	228	71.0	0.977
Levofloxacin	128	39.9			193	60.1	121	37.6	7	2.1	193	60.1	0.955
Erythromycin	73	22.7	0	-	248	77.3	73	22.7	0	-	248	77.3	1.000
Clindamycin	120	37.4	0	-	201	62.6	120	37.4	0	-	201	62.6	1.000
Co-trimoxazole	55	17.1	-	-	266	82.9	53	16.5	2	0.6	266	82.9	0.978
Tetracycline	94	29.3	0	-	227	70.7	94	29.3	0	-	227	70.7	1.000
Linezolid	321	100	0	-	0	-	321	100	0	-	0	-	-

DST=Direct Susceptibility Test. n= number, S=Sensitive, I= Intermediate sensitive, R=Resistant

Table 2. Antibiotic susceptibility profile of *Staphylococcus aureus* isolates by DST and Vitek-2

Antibiotic	DST (n=49)						VITEK (n=49)						Kappa
	S		I		R		S		I		R		
	n	%	n	%	n	%	N	%	n	%	n	%	
Penicillin	4	8.2	0	-	45	91.8	4	8.2	0	-	45	91.8	1.000
Cefoxitin	18	36.7	0	-	31	63.3	18	36.7	0	-	31	63.3	1.000
Vancomycin	-	-	-	-	-	-	49	100	0	-	0	-	-
Teicoplanin	49	100	0	-	0	-	49	100	0	-	0	-	-
Ciprofloxacin	12	24.5	0	-	37	75.5	12	24.5	1	2	36	73.4	0.947
Levofloxacin	17	34.7	0	-	32	65.3	15	30.6	2	4	32	65.3	0.913
Erythromycin	9	18.4	0	-	40	81.6	9	18.4	0	-	40	81.6	1.000
Clindamycin	14	28.6	0	-	35	71.4	14	28.6	0	-	35	71.4	1.000
Cotrimoxazole	13	26.5	0	-	36	73.5	12	24.48	0	-	37	75.5	0.946
Tetracycline	21	42.9	0	-	28	57.1	20	40.8	1	2	28	57.1	0.959
Linezolid	49	100	0	-	0	-	49	100	0	-	0	-	-

DST=Direct Susceptibility Test. n= number, S=Sensitive, I= Intermediate sensitive, R=Resistant

Table 3. Identification of positive blood cultures comparing direct PCR testing from culture bottles with Vitek-2 results

Organism	Total No. of strains	Direct PCR results (mecA/nuc) no of strains picked	Vitek 2 results (mecA) no of strains picked
MRSA	31	(+/+) 30	(+) 31
MSSA	18	(˙/+) 18	(−) 18
MRCoNS	260	(+/-) 248	(+) 260
MSCoNS	61	(-/-) 61	(-) 61
Total	370	357	370

+, presence of PCR product; -, absence of PCR product

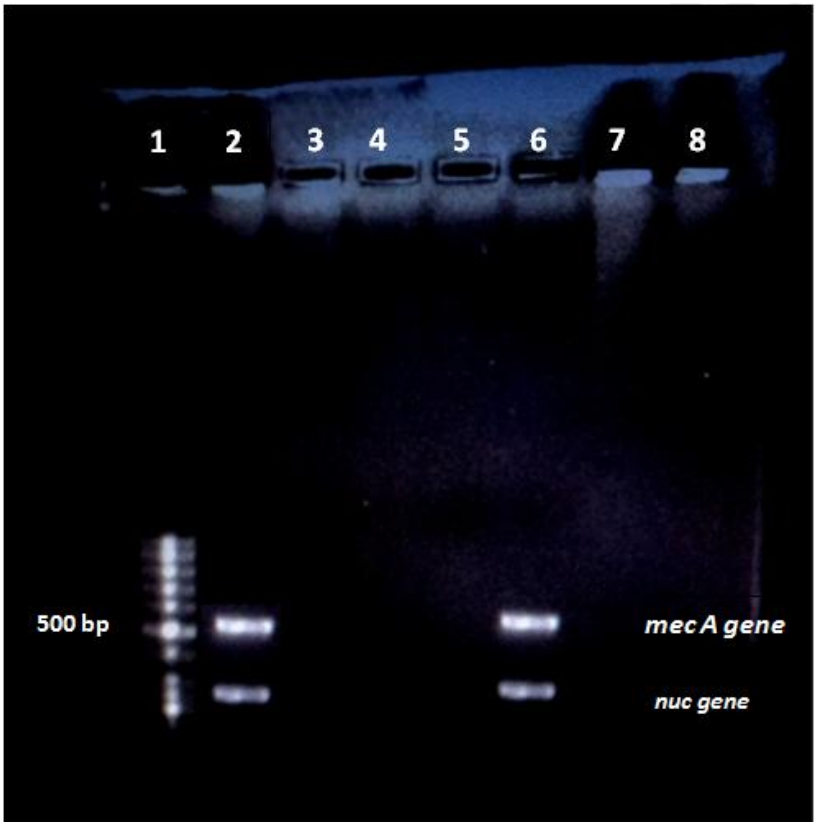

Fig. 1. Agarose gel showing PCR amplified products of mec A and nuc genes

Lane 1: 100 bp DNA ladder, Lane 2: MRSA ATCC 4330 showing amplification products (mec A at 533 bp, nuc at 270 bp), Lane 3,4,5: MSCoNS showing no amplified products; Lane 6: MRSA showing both amplification products (mec A and nuc); Lane 7: Negative control showing no amplification products

Empirical anti-infectious treatments are selected on the basis of the clinical and epidemiological data and are administered immediately after the sampling of blood; however their effectualness remains questionable, especially in the present day milieu of multidrug-resistant organisms [15]. When gram-positive cocci in clusters are seen in Gram stains of signal-positive blood culture bottles, two important questions arise. The first is whether the organism is *S. aureus* or a CoNS. The second question is whether the organism is susceptible to methicillin or not. The first distinction requires 18 to 24 hrs by conventional techniques. Determination of susceptibility of an organism in pure culture requires an additional 6 to 10 h. Our study was aimed at detection and characterization of the cause of bacteremia with an aim to answer the above mentioned queries as soon as possible.

Out of 720 samples 370(51.3%) blood cultures yielded Gram positive cocci in clusters on Gram

staining. Agger WA, et al. demonstrated the efficacy of Gram stain where a preponderance of Gram positive cocci in clusters was 100.0% sensitive (186/186) and 93.1% specific (95/102) for staphylococci [16]. Out of 370 Gram positive cocci, 321 (86.8%) were CoNS and 49(13.2%) proved to be *Staphylococcus aureus*. Increasing trends in sepsis etiologies towards gram positives has also been seen in long term studies [17].

In our study Methicillin resistance was seen in 31 (63.3%) *Staphylococcus aureus* strains whereas 18 (36.7%) were methicillin sensitive. Hasani A et al. reported similar results in their study where *mec-A* gene was detected in 81 (54%) isolates (considered as MRSA), and the remaining 69 (46%) isolates were identified methicillin sensitive (MSSA) [18]. On the contrary Prabhu K, et al. while working on bacteriological profile of blood isolates showed the incidence of MRSA to be 29.26% in *S. aureus* [19]. *S. hominis* was the most common CoNS, 134 (41.7%) recovered in our study. Results obtained by Weinstein MP, et al. found *S. epidermidis*, *S. hominis* (both subspecies) and *S. haemolyticus* accounted for nearly 98% of CoNS isolates to be clinically significant in blood [20]. The higher prevalence of gram positive bacteria in blood stream infections can be attributed to their hardy nature. *S. aureus* can survive in the environment for a relatively long time and fairly widely distributed in the hospital environment and therefore have the potential for being transmitted from the environment to the patients through practices that breach infection control measures.

Majority of the samples were received from inpatient departments. Samples received from neonatology yielded the maximum number, 77 (20.8%) of Staphylococcal isolates. Al-Mazroea, et al. made similar observations, 30% of positive blood cultures in their study were isolated from pediatrics department [21]. Contrary to our results Charoo BA, et al. observed the frequency of Coagulase-negative staphylococcal (CoNS) in only 10.5% among neonates with thrombocytopenia [22]. Naïve immune systems may be an explanation for higher rates of isolation from the neonatology section of our hospital.

Fig. 2. Agarose gel showing PCR amplified products of mec A and nuc genes

Lane 1:100 bp DNA ladder; Lane 2: MRSA ATCC 43300 showing amplification products (mec A at 533 bp, nuc at 270bp); Lane 3,4,7 : MSSA from samples showing only nuc products; Lane 5: MSCoNS showing no amplified product; Lane 6: MRCoNS showing only mec A product. Lane 8: Negative control showing no amplified product

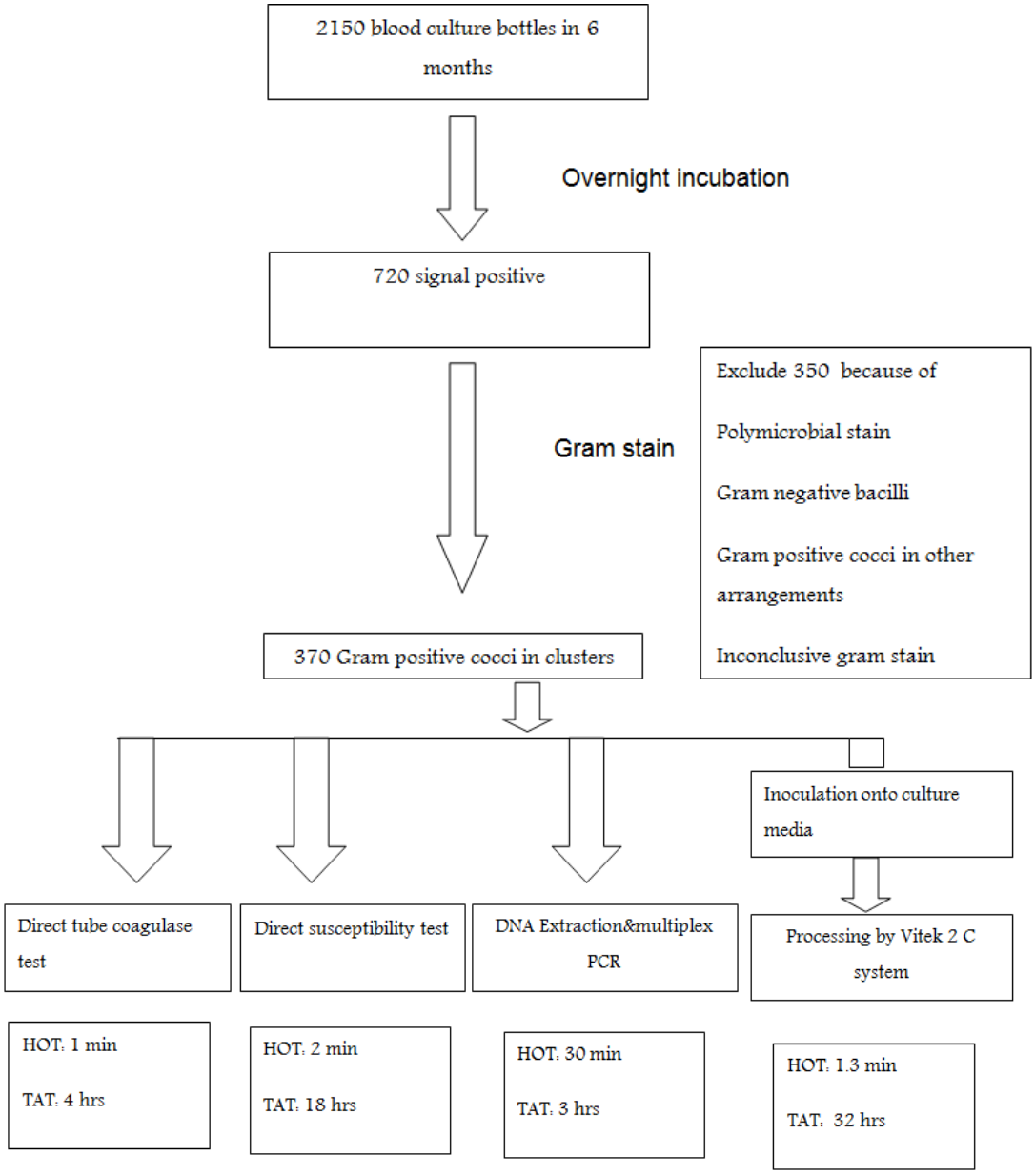

Chart 1. Study algorithm: Showing flow of sample from flag positive through different methods. HOT-Hands on time, TAT-turnaround time

Our series received 33 (8.9%) samples from surgical intensive care unit which were similar to the isolation rates of staphylococci by Burton DC et al. who reported 7.4% of central line–associated BSIs caused by MRSA and 4.7% due to MSSA. Al-Mazroea, et al. [23] also reported 31.9% prevalence of CoNS from positive blood cultures which was highest in the intensive care units. Ekpe K, et al. [21] studied ICU-acquired bloodstream infection where 9.7% were due to MRSA [24]. Profound degrees of immune compromise, repeated invasive procedures and intravenous accesses may predispose this group of patients to staphylococcal bacteremias in our hospital as also supported by above mentioned studies.

The patients in current study included 220 males (59.5%) and 150 (40.5%) females. Most of the patients from whom *Staphylococci* were isolated, were in the age group of 50-59 years, 96 (25.9%). Observations similar to ours were also

made by Hugonnet S, et al. who observed male-to-female ratio 622/446 and the median age to be 62.9 years [25]. Laupland KB, et al. sought to define the incidence and microbiology of severe bloodstream infection and assess risk factors for acquisition and death. Male gender including age > or =65 yrs were found as significant risk factors [26]. Babay HA, et al. reported similar proportion of males in their study of pediatric patients [27]. Gender differences with higher propensity in males may be explained by several factors like high body mass index, less compliance with hand hygiene behavior, the effect of female hormones to ward off virulence factors of microorganisms, these factors may explain higher colonization and infection rates in male gender [28].

Rapid tube coagulase test was performed directly on blood culture samples with the aim to answer the question whether the isolate was positive for coagulase producing staphylococci or Cons. Observations similar to ours were noted by Varettas K, et al. who found that only 62% *S. aureus isolates* were correctly identified by Tube coagulase test after 4 h of incubation and 91% were correctly identified after overnight incubation [29]. In discordance to our results Sturm PDJ, et al. observed the sensitivity of the DTCT at 4 h was 96%, with 100% specificity [30]. A reduced sensitivity of DTCT at 4 hrs could be due to several factors which include repeated freezing and thawing of the plasma, subjective differences in the interpretation of weakly positive results or to variation in batches of plasma from different suppliers. Varettas K, et al. have also reported the carryover of anticoagulant to influence clot formation. Since the sensitivity of direct tube coagulase increases at 24 hrs, reading the test at 4 hrs and again at 24 hrs may be appropriate, therefore negative results not to be reported until 24 hrs when simultaneously a susceptibility profile by direct susceptibility will also be available to the physician. On comparison of DTC with vitek 2 identification by the kappa test very good agreement was found with *K* value of 0.871.

As shown in Table 2 and Table 3 the direct disc diffusion testing had a similar bacteriological susceptibility profile as reported by AJ et al. where *S. aureus* and CONS were frequently found to be penicillin resistant (>89%). Resistance percentage to other antimicrobials like erythromycin, gentamicin, tetracycline and ciprofloxacin were above 40%. None of the strains showed resistance against vancomycin or teicoplanin and these drugs therefore can be effectively used if methicillin resistance is suspected during treatment. Tariq, et al. chalked out a similar profile of staphylococci isolated from blood cultures where all Staphylococci were resistant to penicillin, majority were resistant to gentamicin, 3rd generation Cephalosporins, Fluoroquinolones and cotrimoxazole [31]. As none of the isolates showed resistance to vancomycin, linezolid or teicoplanin the observation was comparable to the study of Rajeevan S, et al. [32].

The results obtained from DST interpretation were compared to susceptibility provided by Vitek-2 system. Concordance or discordance between the results of the two were calculated according to the following FDA definitions [1]. Essential agreement or “Minor errors” [2], Categorical agreement or “No Error” [3], Major errors [4], Very major errors [33].

No major errors or very major errors were seen for CoNS, for *S. aureus* one very major error was seen in the interpretation of cotrimoxazole for *S. aureus* (2.7%) as also noted by Waites KB, et al. they observed that drugs with the most errors on gram-positive panels were clindamycin, oxacillin and trimethoprim-sulfamethoxazole [34]. The results from DST were compared with AST from Vitek-2 by kappa test k values ranging from 0.914 to 1.00 were seen for the panel of antibiotics tested which imply perfect or very good agreement.

Our results are in accordance with the guidelines on the error rates as provided by FDA. Correlations of DST results with Vitek-2 system susceptibility are comparable to other studies. Bhattacharya S et al. advocated the use of DST of Gram-negative isolates from BacT/Alert bottles by using disk diffusion method with 96.2% essential agreement and 83.7% categorical agreement [35].

In present study Multiplex PCR was performed to detect the *nuc* and *mec-A* gene for all the 49 *S. aureus* isolates and 321 CoNS directly from the blood culture bottles. Automated BacT/Alert microbial detection system in our laboratory flag a blood culture bottle positive with inoculums sizes of >= 100 CFU/bottle within a range of 12.2 to 15.6 hrs [12]. The smallest amount of template DNA which can be detected by the multiplex PCR, i.e., 100 pg, corresponds to 10^4 staphylococcal cells (calculation according to www.molbiol.ru/ger/scripts). The quantities of

microbes present in the blood during BSIs ranges from 1 to 10 CFU/mL to 1 × 10^3 and 1 × 10^4 CFU/ml. The detection limit of the multiplex PCR assay for the simultaneous detection of the *mec-A* and *nuc*was calculated to be 10^5 CFU/ml, based on the seeding experiments by Louie L, et al. [9].

In Multiplex PCR 13(3.5%) strains of staphylococci (*S. aureus* & CoNS) identified by DST as methicillin resistant were not identified by PCR analysis. Very good agreement was seen on comparison of PCR and Vitek-2 results(*k*= 0.901). Results similar to that of our study were observed by Ak S, et al. where despite of being methicillin resistant phenotypically only 94% of the samples were genotypically *mec-A* positive [36].

Also accurate determination of methicillin resistance in *S. aureus* by conventional laboratory tests is subject to variations, including inoculum size, diameter, pH and salt concentration of medium, incubation time, etc [33]. Failure to amplify the *mec-A* gene can be attributed to many factors: PCR-negative isolates could be penicillinase hyper producers that hydrolyze the penicillinase-resistant penicillins, the borderline phenotypes have been attributed to other mechanisms: Production of an inducible, plasmid-mediated methicillinase or different alterations in the penicillin-binding protein genes due to spontaneous amino acid substitutions in the transpeptidase domain [37].

There may be differing levels of *mec*A gene expression of methicillin resistance, occurring every 10^4 or 10^6 cells, or absence of penicillinase plasmid, which plays an important role in the stability and phenotypic expression of the *mec* A gene [38].

Some authors have attributed negative results of PCR to PCR inhibitors which are a heterogeneous class of substances that act at different steps of the diagnostic procedure. They are present in a large variety of sample types and may lead to decreased PCR sensitivity or even false-negative PCR results. Al-Soud WA, et al. postulated similar findings while applying nucleic acid amplification techniques to blood samples ,they observed that the amplification capacity can be dramatically reduced or blocked by the presence of PCR-inhibitory substances [39]. Inhibitors in blood which have been identified are either natural components of blood, mainly heme and leukocyte DNA, or added anticoagulants such as EDTA [40]. Recently, immunoglobulin G present in humanplasma was identified as a major inhibitor of diagnostic PCR in blood [41].

5. CONCLUSION

In conclusion, we have shown that direct antimicrobial susceptibility testing of bacteria isolated from blood is often feasible, useful, and most importantly, highly reliable provided that the criteria outlined are followed. Results can be available as early as 24 to 36 h after blood for culture is drawn from a patient. DST of positive blood cultures can help clinicians to tailor antibiotic treatment about 24 hours earlier than final AST. In an effort to reduce the time lag for reporting of BSI's by carrying out rapid methods accurate and direct detection of methicillin resistance in *S. aureus* isolates was achieved 1 day earlier for blood culture samples. Vitek-2 though required the least technical time per test (1.3 min) time taken to provide results was the longest i.e. 32 hrs. Results from PCR analysis were obtained at the earliest i.e. 3 hrs. Direct tube coagulase could be read after 4 hours, however prolonging the incubation to overnight increase the sensitivity of the test. Results from direct susceptibility testing were obtained in 18 hrs approximately 14 hrs prior to Vitek antimicrobial susceptibility. Each of the tests have positive qualities and all may have a place in a Gram positive cocci algorithm for testing blood cultures depending on the laboratory setting, workload volume and staffing.

There are certain limitations to this study. The methods evaluated here were applied to a single system in operation; therefore the performance of the methods cannot be validated for other systems. Also blood culture contamination has no gold standard for assessment therefore the significance of CoNS as true pathogens could not be ascertained. Therefore, these tests can provide preliminary results while awaiting the results of the standardized antibiotic susceptibility testing and aid in implementation of antimicrobial stewardship.

CONSENT

As per international standard or university standard written patient consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

Ethical approval was obtained from the Institute's ethical clearance committee with order no. SIMS131/IEC-SKIMS/2016-143.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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