Prevalence of Methicillin Resistance Staphylococci (MRS) from Neonatal Septicemia and Comparative Evaluation of Newer Rapid Latex Agglutination Method with Conventional Methods for diagnosis of MRS

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Abstract

Background & Aims: Neonatal septicemia is a major cause of neonatal mortality in developing countries. Rapid treatment with antimicrobials is very crucial for essential outcome. Methicillin Resistance Staphylococcus (MRS) are important cause of nosocomial infection, which causes septicemia especially in neonates. They are frequently resistance to other antibiotics also. So, rapid, sensitive and specific procedures are required urgently to initiate timely treatment. This study was undertaken to perform comparative evaluation of conventional methods with rapid agglutination method in diagnosis of MRS and to estimate the prevalence of MRS among neonatal septicemia cases. **Materials and Method:** This study was done in one of the major tertiary care hospitals in Ahmedabad, Gujarat. All the blood samples collected from the patients with sign and symptoms of septicemia, from January 2012 to July 2012, were cultured and identified by standard microbiological method. MRS detection was done by conventional methods and latex agglutination method. **Results**: *Staphylococcus* coagulase negative constituted 61.96% and *S.aureus* 4.33% of total bacterial isolates. Methicillin resistance in *S.aureus*and in CONS, was 44.44% and 25.91% respectively. Prevalence of MRS among neonates was 32.72%. The sensitivity and specificity of oxacillin disk was 68% and 100% respectively for detection of MRS. Latex agglutination test method shows sensitivity and specificity of 100% for detection of MRS. **Conclusion**: Along with cefoxitin disc diffusion method, latex agglutination method is to be used for early diagnosis & treatment and forprevention of over use of vancomycinfor methicillin resistant *staphylococc*i.

Keywords: Septicemia, MRS, Blood culture, *S.aureus*, Cefoxitin, CoNS

Introduction

Neonatal sepsis is one of the commonest causes of neonatal mortality in developing world accounting for 20-30% of 1.6 million neonatal deaths per year.[1] In India, the National Neonatal Perinatal Database (NNPD) reported an incidence of 8.5 per 1000 live births for blood culture proven sepsis for the year of 2002-2003. Previously, Gram negative organism like *Klebsiella* has been reported as a main isolate in neonates with a high incidence (47.5%-64%) of bacteremia. However, in last three decades, the isolation of Gran positive organisms has been increased significantly.[2]

After the introduction of benzylpenicillin in to clinical use in early 1940, isolates of *Staphylococcus aureus* that were resistance to penicillin due to production of β - lactamase were found and by 1948, over

50% of isolates in many hospitals were found resistant. Methicillin resistant strains of *Staphylococci* were identified immediately after a year upon the introduction of methicillin into clinical practice in UK in 1961. Resistance to methicillin was mediated by expression of novel penicillin - binding protein with low binding affinity not only for methicillin, but also for all licensed β-lactams.[3]The rate of resistance to methicillin has been increasing now a days and the empirical treatment of choice is often vancomycin. In MRS (Methicillin Resistance *Staphylococci*), the production of a modified penicillin binding protein - PBP 2a is specified by the expression of mecA gene. MecA gene detection by PCR is very sensitive but is not feasible in all laboratory set up. So, it is desired to use new method which is easy to perform and gives rapid and reliable result to detect MRS. A latex agglutination (LA) test detects PBP2a by using latex particles sensitized with monoclonal antibodies specific for PBP 2a of *Staphylococcus aureus*. The aim of this study is to evaluate a latex agglutination (LA) test for rapid detection of Methicillin resistance in *staphylococci* and to estimate the prevalence of MRS in neonatal septicemia cases.

Materials and Method

The study was carried out from January 2012 to July 2012 in Microbiology department of one of the largest tertiary care hospitals of Ahmedabad, Gujarat. A total of 2646 samples of blood culture from the patients admitted in the hospital, showing sign and symptoms of septicemia were processed for study. Blood for the culture was collected in BacT Alert blood culture bottles, following aseptic precautions. The bottles were loaded immediately into BacT Alert machine. Positive signalled bottles were unloaded and subcultures were done on MacConkey agar, blood agar & chocolate agar and incubated at 37°C for 24 hours. Gram stain was also done from signalled positive bottles to give presumptive report. Growth on solid media was identified and confirmed by their colony characteristics, culture smear and biochemical tests. Catalase test was performed to distinguish *Staphylococci* from *Streptococci* and coagulase test & mannitol fermentation test were performed to differentiate coagulase positive *Staphylococci (S. aureus)* from coagulase negative *Staphylococci* (CoNS).[4,5,6] Antibiogram was performed on Muller Hinton agar plate by standard Kirby-Bauer disk diffusion method and results were interpreted according to CLSI guidelines.[4-8] MRS were identified by following methods.

- Cefoxitin disc diffusion method:[7, 8] cefoxitin 30 microgram disc was used by diffusion method and results were interpreted according to CLSI guidelines (10^{th} edition, 2009 and 23^{rd} supplement, 2013).
- Agar dilution method:[9,10] MRS is detected by agar dilution method. Oxacillin sodium powder was used and serial double concentration ranging from 0.12 mg/L to 128 mg/L was used to determine minimum inhibitory concentration (MIC). The stock solution was prepared by using following formula.

  W = C x V x 1000/P (P= potency given by manufacture, which was 900 µg/mg, V= volume, C= final concentration of solution-multiples of 1000, in mg/L, W=weight of antibiotics to be dissolved in volume V in mL).

  By this formula first 10240 mg/L stock solution was prepared, which was further diluted to get stock solution of other concentration as shown in table 1.

  The density of the inoculum was standardized to give 10^4 colony forming unit per spot. For that four to five colonies of a pure culture were taken to avoid selecting an atypical variant. The inoculum was prepared by emulsifying overnight colonies from an agar medium. A 0.5 McFarland standard was used for visual comparison to adjust the suspension to a density equivalent to approximately 10^8 CFU/ml. The suspensions of the organism were diluted in 0.85% saline or broth to give 10^7 CFU/ml and within 30 minutes of standardizing the inoculum, plates were inoculated. About 1 µl (10^4 CFU/ml/spot) of diluted bacterial suspension was transferred to series of well-marked Mueller Hinton agar plates supplemented with 2% NaCl, including the control plate (without antimicrobial agent). The inoculum spots were allowed to dry at room temperature before inverting the plate for incubation at 30°C for 24-48 hr. The MIC was calculated by observing the lowest concentration of antibiotic agar plate that completely inhibits visible growth as judged by naked eye.

Table: 1 Preparation of dilutions of antimicrobial agent (oxacillin) for use in agar dilution susceptibility test

Antimicrobial concentration (mg/L) in stock solution	Volume stock solution (ml)	Volume distilled water (ml)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 19 ml of agar
10240	1	0	10240	512
10240	1	1	5120	256
10240	1	3	2560	128
2560	1	1	1280	64
2560	1	3	640	32
2560	1	7	320	16
320	1	1	160	8
320	1	3	80	4
320	1	7	40	2
40	1	1	20	1
40	1	3	10	0.5
40	1	7	5	0.25
5	1	1	2.5	0.125
5	1	3	1.25	0.06
5	1	7	0.625	0.03
0.625	1	1	0.3125	0.015
0.625	1	3	0.1562	0.008
0.625	1	7	0.0781	0.004

3. Latex agglutination test:[11]

   It is based on reaction of latex particles sensitized with monoclonal antibodies against PBP 2a of *S.aureus*. Test was performed according to manufacturers' instruction. Result was read for presence or absence of agglutination.

4. E- Strip method:[7, 8]

   An E-strip was placed onto Muller Hinton agar plate supplemented with 2% NaCl. Plates were inoculated by swabbing the surface with direct colony suspension. After incubation at 37°C for 24 hr, MIC was read at the point of intersection between the zone edge and E strip. For *S.aureus* the strain is considered as sensitive if it is $\leq$ 2 µg/ml and resistance if it is $\geq$ 4 µg/ml. For CoNS the strain is considered as sensitive if it is $\leq$ 0.25 µg/ml and resistance if it is $\geq$ 0.5 µg/ml.

Results

Study comprised total of 2646 blood culture samples from patients suspected of having septicemia, out of which growth was obtained in 783 samples. Among which 160 isolates were *Bacillus subtilis*(most common cause of contaminants) and they are omitted from the study. From remaining 623 positive isolates, 459 (73.68%) Gram Positive Cocci (GPC), 143 (22.95%) Gram Negative Bacilli (GNB) and 21 (3.37%) Candida species were isolated. Distribution of the bacteriological profile of septicemia cases is shown in table 2.

Among 459 Gram Positive Cocci isolates, 84.10% (386/459) were Coagulase Negative *Staphylococci*. Other isolates were *enterococci*, *S. aureus* and *Streptococcus* species which comprised of 6.97% (32/459), 5.88% (27/459) and 3.05% (14/459) respectively. All *staphylococcus* isolates were subjected to CLSI approved cefoxitin disk diffusion as well as other antimicrobial sensitivity test (Disk diffusion method) to find out MRS and resistance pattern among different antimicrobials respectively. The observation of AST was carried out by using CLSI disc diffusion criteria to define resistance. [7,8]Out of total 413

staphylococcus isolates, 112 (100 CoNS and 12 *S. aureus*) were found to be resistant to cefoxitin. Table 3 shows antibiotics used for AST and resistance pattern of all MRS.

Table 2: Bacteriological profile of septicemia cases

Organisms isolated from neonatal septicemia	Number of positive isolates	% of positive isolates
Staphylococcus coagulase negative	386	61.96%
Klebsiella species	60	9.63%
Acinetobacter species	42	6.74%
Enterococcus species	32	5.14%
Staphylococcus aureus	27	4.33%
Candida species	21	3.37%
Pseudomonas aeruginosa	19	3.05%
Streptococcus species	14	2.25%
E. coli	13	2.09%
Proteus vulgaris	02	0.32%
Providential species	02	0.32%
Citrobacter species	02	0.32%
Salmonella paratyphi A	02	0.32%
Salmonella typhi	01	0.16%
Total	623	100%

Table 3: Resistant pattern of all MRS isolates.

Sr. No.	Gram positive species			Percentage Resistant	
	Antibiotic	Code	Concentration	Coagulase Negative MRS	MRSA
1	Penicillin G	PR	10 unit	100%	100%
2	Cotrimoxazole	DA	25 g	48.86%	66.67%
3	Azithromycin	AS	15	82.95%	83.33%
4	Clindamycin	CS	2	35.22%	33.33%
5	Ciprofloxacin	RC	5	70.45%	25.00%
6	Levofloxacin	LVX	5	29.54%	0%
7	Gentamycin	GM	10	38.63%	33.33%
8	Tetracycline	PE	30	29.54%	25%
9	Chloramphenicol	LM	30	19.32%	0%
10	Linezolid	LZ	30	0%	0%
11	Teicoplanin	AT	30	0%	0%
12	Oxacillin	CLO	1	69%	75%
13	Vancomycin	VAN	30	0%	0%
14	Tigecycline	TG	15	0%	0%

Age and gender distribution of septicemic cases is shown in table 4, showed male preponderance. Neonatal sepsis may be categorized as early onset (day of life 0-3) or late onset (day of life 4 or later). Among 342 neonatal septicemic cases 303 (88.60%) neonates were suffered from early onset septicemia whereas 39 (11.40%) were suffered from late onset septicemia.

Out of total 112 MRS isolates, 100 isolates (12 *S. aureus* and 88 CoNS) including of neonates (72 isolates), were processed for various other phenotypic methods like oxacillin agar dilution method & E tests for oxacillin MICs and MRSA screen (PBP 2a – latex agglutination) test to detect methicillin resistance and compared with CLSI approved cefoxitin disc diffusion method. 10 Methicillin sensitive staphylococci (MSS) isolates were also tested with above methods, and all were found MSS by all other methods. Detection of methicillin resistance by various methods is shown in table 5.

Table 4: Age and gender distribution of septicemic cases

Sr no	Age	Number (Percentage)	Male	Female
1	≤ 28 days	342 (54.90%)	210	132
2	≤ 13 years	76 (12.20%)	158	123
3	> 13 years	205 (32.90%)		
4	Total	623 (100%)	368	255

Table 5: Methicillin resistance in CoNS and S. aureus by various methods & comparison of them with cefoxitin disc diffusion method (taking it as gold standard).

Test Name	CoNMRS (n=88)	Sensitivity	Specificity	MRSA (n=12)	Sensitivity	Specificity
Oxacillin disc diffusion method	60	68.18%	100%	9	75%	100%
E- test for oxacillin MICs	88	100%	100%	12	100%	100%
Agar dilution method for oxacillin	87	98.86%	100%	12	100%	100%
MRSA screening test (latex agglutination test)	88	100%	100%	12	100%	100%

Comparison of different methods used to detect MRS is shown in table 6.

Table 6: Comparison of the different parameters between the tests to detect MRS:

Method	Speed	Cost	Overall Sensitivity in our study	Overall Specificity in our study
Cefoxitin disc diffusion method	24 hours	Low	100%	100%
Oxacillin disc diffusion method	24 hours	Low	69%	100%
Oxacillin agar dilution method	24 hours	Medium	99%	100%
Oxacillin E strip method	24 hours	Medium	100%	100%
Latex agglutination test method	15-20 mins	higher but lesser than PCR	100%	100%

Discussion

Septicaemia is a clinical syndrome characterised by systemic signs and symptoms due to generalised bacterial infections with a positive blood culture and neonatal septicemia is which, detected in the first four weeks of life. Bacterial infection are the commonest cause of morbidity and mortality during the neonatal period. Fulminate and fatal course of infection may result from complications such as shock, disseminated intravascular coagulation (DIC) and multisystem organ failure, mandating early and rapid diagnosis of this life-threatening condition for a timely treatment and a favourable outcome.[12] In this study, a total of 2646 blood culture samples from patients suspected of having septicemia, were processed in automated blood culture system and 623 samples were found positive. Gram positive cocci were isolated maximally followed by Gram negative and candida species. Highest positivity of gram-positive organisms is due to a greater number of neonates samples (342/623), and CoNS is one of the most common organisms causing neonatal septicemia. S*taphylococci*were isolated in 413 cases from which, 112 were found to be resistant to cefoxitin, so overall prevalence of MRSin all septicemic patients was 27.12%. Methicillin resistance among isolated *Staphylococcus aureus* was 44.44% (12/27) and among isolated coagulase negative *Staphylococci* was 25.91% (100/386). Incidence of MRSA in India ranges

from 30 to 70 %[13] and our study is well corelated with it. Among 112 MRS isolates from septicemic cases, 89.29% were MRCoNS and 10.71% were MRSA. Antibiogram of MRS is shown in table 3, which suggests that MRCoNS tend to resistant to more antibiotics in comparison to MRSA. Antibiotics like clindamycin, ciprofloxacin, levofloxacin, gentamycin, tetracycline and chloramphenicol are more resistant in MRCONS than MRSA. As shown in table 4, among all 623 culture positive septicemia cases, 342 were neonates, 76 were children of 13 years or less than it & 205 were more than 13 years old, showing highest prevalence of septicemia (54.90%) among neonates. Male to female ratio was 1.59 and 1.44 in neonates and total septicemic cases respectively showing male predominance. *Staphylococci* were isolated from a total of 220 (64.33%) neonates, from which 72 cases were found with MRS. So, prevalence of MRS among neonates was 32.72%. A study carried out by HilalOzkan et al found similar outcome. Intheir study also, staphylococci were isolated more (85/151-56.29%) than any other organisms in neonatal septicemia cases.[14]

Accurate and early diagnosis of methicillin resistance is vital in the management of patients with infection caused by *staphylococci*. Current gold standard for MRS detection is identification of the mec A gene by molecular method. However, use of molecular method to identify MRS as a routine clinical practice may not be feasible in a budget constrained setting.[15] So, it is desirable to identify accurate, rapid, easy to performed and cost-effective phenotypic method for detection of MRS. We compared various MRS detection methods like Oxacillin disc diffusion method, oxacillin agar dilution method, oxacillin E test and latex agglutination method with current gold standard cefoxitin disc diffusion. Comparative results of various tests to detect methicillin resistance in reference to sensitivity and specificity and to different parameters were shown in table 5and table 6 respectively.

As shown in table 5 and 6, oxacillin agar dilution method is overall 99%sensitive (98.86% for CoNMRS and 100% for MRSA) and 100% specific, but it is time consuming, tedious and have higher cost than disc diffusion method, whereas Oxacillin disc diffusion method is least sensitive (69% overall)method among all methods tested. Comparing cefoxitin disc diffusion test with oxacillin disc diffusion test; 3 MRSA and 28 MRCONS give discrepant result for methicillin resistance by oxacillin disk diffusion methodmakingits least sensitive(69%). Higher sensitivity to cefoxitin can be explained as being an inducer of mec A gene, there is an increased expression of mec A-encoded protein PBP2a by cefoxitin disc.[15] Low sensitivity of oxacillin disc diffusion test could be due to heteroresistant strains to methicillin, which are not able to be picked up by oxacillin disc and resistance among them is influenced on culture conditions such as temperature, medium, pH, salt concentration and inoculums size.[16]

In other hand, PBP-2a Latex agglutination test (MRSA screen test) and E tests to detect oxacillin MICs are very well compared with cefoxitin disc diffusion test as all three gives similar results with 100% sensitivity and 100% specificity. The PBP2a latex agglutination screen assay is latex particles sensitized with anti-monoclonal antibody against PBP2a and react specifically with MRSA to cause agglutination. It is reported to have 97.6% sensitivity and it distinguishes between very low levels MRSA from MSSA. Bowers et al reported that latex agglutination test is a reliable and rapid detection test to do it from broth pure culture as well as from selective media and it is a reliable alternative test to mecA PCR for definitive diagnosis of MRSA[17]. It also fulfils criteria like accurately detection of MRCoNS, and turnaround time is also shorter than other susceptibility testing methods. i.e., gives result within 15 to 20 minutes, easy to perform and cost is lower than molecular method. The study carried out by Datta Priya et al found that detection of MRSA by LA had 100% sensitivity and 99.2% specificity. This method could detect even low level of PBP2a.[15]

Many studies suggested to use oxacillin induction,[18] to keep extended agglutination time[19] or to use large inoculum[20] to increase the sensitivity of the LA test without sacrificing the specificity particularly for MRSA isolates. In the present study, none of *S.aureus* isolates required increased inoculum volume or induction by oxacillin or extended agglutination time for detection of PBP2a. However, two out of 88 isolates of MRCoNS show weak agglutination with latex agglutination test. We repeated the agglutination test with overnight oxacillin induction and found that out of two isolates, isolate no 1 gave strong

agglutination but isolate no 2 gave same result, probable reason behind it could be borderline oxacillin resistance *staphylococcus* (BORS) that may have very low production of PBP2a to give weak agglutination and that could have given non consistence result in other phenotypic methods tested with. The strain termed borderline in susceptibility in methicillin or oxacillin may have altered PBPs, may be penicillinase hyperproducer or carry mecA gene but highly heterogenous in expression of resistance with only small proportion of cell express high level of resistance[21]. According to ChariyaChomvarin et al, most BORS isolates do not possess the mec A gene. Also, there may be possibility that BORS isolates may have low PBP2a expression or lack of mec A repressor expression either due to mecI mutation or mec I deletion[22]. We tested MIC of oxacillin by agar dilution method and E strip method. Sensitivity and specificity of both were good and comparable. In 23 MRS isolates, we found difference in MIC by both methods, though both gave over all result as resistant. We could not able to repeat the tests and find out the reason behind it because of time constrains and limited stock availability of oxacillin E strips. However, more research is required to find out inoculum effect, storage temperature of drug or E strips right from the manufacture, batch to batch variation in preparation of medium and variation in testing conditions etc. in such kind of isolates.

Thus, lacunae in the sensitivity of other phenotypic tests may not ensure appropriate and timely treatment of MRS infected patients and leads to LA test as a superior test.

Conclusion

Prevalence of MRS among all septicemic patients was 27.12% & in septicemic neonates is 32.72%. The cefoxitin disc, as recommended by CLSI is a good method for MRS detection but it should be supplemented with some other method. It is always advisable to combine two methods, one with high sensitivity and other with high specificity. According to our result best combination is cefoxitin disc diffusion method and LA test. A PBP2a latex agglutination can be performed in 20 mins with minimal technical training and besides MRSA, it can also be used to detect MRCoNS. So, for critical cultures like blood & sterile fluid, it will allow physicians to initiate or change a patient's antimicrobial regimen more appropriately & prevent overuse of vancomycin.

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