Effect of Variable Incubation Temperatures and Salt Concentration on Susceptibility Testing of Methicillin Resistant Staphylococcus Aureus (MRSA)

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The effect of variable incubation temperatures and variable salt concentration in the sensitivity agar on susceptibility testing of MRSA was evaluated. For this purpose 58 MRSA strains were tested. For each MRSA strain nine Mueller-Hinton agar plates, three plates each with 2%, 5% and 7.5% sodium chloride (NaCl) supplement were inoculated. One plate from each of the salt concentration was incubated at 30°C, 37°C and 40°C. It was found that with increase in temperature of incubation the size of inhibition zones increased. In the presence of 2% NaCl supplement in the sensitivity agar, 5 MRSA appeared falsely intermediate sensitive on incubation at 40°C. While on incubation at 37°C although all MRSA remained methicillin resistant, statistical analysis revealed the effect on inhibition zone size to be approaching significance level (0.1>P>0.05). The effect of incubation temperature on inhibition zone size was statistically insignificant (P>0.05) if 5% or 7.5% NaCl was incorporated in the sensitivity agar. Therefore we recommend the use of 5% NaCl supplemented sensitivity agar and incubation temperature of 37°C for the accurate detection of methicillin resistance in Staphylococcus aureus.

Key words: MRSA, Staphylococcus aureus, salt, temperature

Staphylococcus aureus is a common pathogen encountered in nosocomial infections[1,2]. It is involved in a wide range of diseases varying from infections to toxaemia[3]. Acquisition of antibiotic resistance by Staphylococcus aureus is a major health problem. In the 1940s and 1950s penicillins[4] and cephalosporins[5] were available for the effective treatment of these infections[6]. However continued use of these antibiotics lead to emergence of resistance to these beta-lactam drugs[7,8]. Bacteria developed resistance to them via production of plasmid mediated enzyme, pencillinase.[9] Pencillinase inactivates the beta-lactam drugs by splitting the beta-lactam ring[10].

To solve the problem of inactivation by pencillinase, pencillinase resistant pencillins (PRPs) such as methicillin were introduced[11]. However with continued use of these PRPs there has been an alarming increase in the proportion of infections caused by MRSA worldwide[12,13]. Infections caused by MRSA are generally also resistant to many other antibiotics[9]. Therefore it poses serious therapeutic difficulties.

In the standard disk diffusion antibiotic susceptibility methods, organisms are tested against different antibiotics at incubation temperature of 37°C. Use of this incubation temperature fails to detect all the MRSA strains[14]. Therefore to determine methicillin resistance among Staphylococcus aureus isolates incubation temperature of 35°C and/or incorporation of sodium chloride in the sensitivity agar is recommended[15,16]. In Pakistan the ambient temperature in most parts of the country remains above 30°C for more than six months of the year. Thus it is sometimes difficult to keep the temperature inside incubators at ≤35°C. The present study was carried out to evaluate different incubation temperatures and sodium chloride cocentrations in the sensitivity medium for accurate determination of methicillin resistance among Staphylococcus aureus.

Materials and Methods

Fifty-eight MRSA strains were tested under variable temperature and salt conditions. These strains were isolated from a variety of clinical specimens from patients admitted in Services Hospital, Jinnah Hospital and Shaikh Zayed Hospital, Lahore. Before determination of methicillin sensitivity, the organisms were confirmed as Staphylococcus aureus on the basis of colony morphology on blood agar, positive catalase, DNase and tube coagulase tests[17]. All the strains were kept stored at room temperature as stab cultures in nutrient agar[18]. The isolates were labelled as methicillin resistant based on results of methicillin susceptibility tests by Kirby-Bauer method on media containing 2% sodium chloride and incubation at 30°C[15].

Sensitivity pattern of these MRSA isolates against methicillin at variable temperature and salt conditions was determined using Stokes method[19] In the present study ATCC 25923 was used as control methicillin sensitive Staphylococcus aureus strain.

For each test organism, nine Mueller-Hinton agar plates were inoculated. Out of these, three plates had 2% sodium chloride supplement, three plates had 5% sodium chloride supplement, while three plates had 7.5% sodium chloride supplement. Separate sterilized cotton swabs dipped in suspension of test/control organisms were used for inoculation of each plate.

In the present study, the control organism was swabbed on the middle third of the dried Mueller-Hinton agar plate. While the upper and lower third portions of the plates were inoculated with two different test organisms, leaving a distance of not more than 5mm between the control and test organisms. The inocula were allowed to dry for a few minutes. Then 5µg methicillin disks were placed between test and control organisms with the help of a sterile forceps. The disks were gently pressed. One plate from each of the different salt concentration was incubated at 30°C, 37°C and 40°C. Thus for each organism three plates were incubated at 30°C, three at 37°C and the last three plates at 40°C. Next day, the plates were examined and radius of zone of inhibition was measured with the help of a ruler from the edge of the disk to the edge of zone of inhibition of the growth.

Test organisms were considered as sensitive, intermediate or resistant to methicillin according to the radius of zone of inhibition in comparison with that of the control organism. In sensitive strains (S) zone radius was larger than, equal to or not more than 3 mm smaller than that of control. In intermediate(I) the zone radius was more than 3 mm smaller than the control but not less than 3mm. In resistant strains (R) there was no zone of inhibition or zone radius was measuring 2 mm or less.

Results

The size of inhibition zones of MRSA increased with rise in temperature of incubation. This effect was observed irrespective of concentration of salt in the medium. The zones of inhibition were significantly larger ($P<0.05$) at 40°C as compared to 30°C and 37°C (Table 1). The results showed that incubation at 40°C lead to 5 MRSA strains to falsely appear as intermediate sensitive. While at 30°C and 37°C, all the MRSA remained resistant (Table 2).

Table 1 Zones of inhibition of MRSA isolates at various temperatures irrespective of salt concentration

Temperature of incubation	Zones of inhibition (mm)			
	0	1	2	3
30°C	147	24	3	0
37°C	146	25	3	0
40°C	126	30	13	5

$P<0.05$ (Significant) between 30°C vs 40°C and 37°C vs 40°C at 0 and 2 mm zones of inhibition.

$P>0.05$ (Non-significant) - 30°C vs 37°C at 0 and 2 mm zones of inhibition between different temperatures at 1 and 3 mm zones of inhibition.

"Zones of inhibition" indicates the size of inhibition around the resistant test organisms

The effect of variable temperatures on size of inhibition zones in the presence of variable sodium chloride concentrations in the medium was also studied. In the presence of 2%, sodium chloride in the medium, zones of inhibition were larger when plates were incubated at 40°C as compared to plates incubated at 30°C and 37°C. Statistical analysis revealed that at 40°C significantly ($P<0.05$) less number of isolates had 0 mm zone of inhibition. Moreover, 4 isolates could be labelled as intermediate sensitive on incubation at 40°C (Table 3). However, temperature of incubation had no significant ($P>0.05$) effect on the zones of inhibition when the MRSA isolates were inoculated on plates containing 5% and 7.5% sodium chloride (Tables 4-5).

Table 2 Resistance pattern of MRSA isolates at 30°C, 37°C and 40°C irrespective of salt concentration

Temperature of incubation	Resistant	Intermediate
30°C	174	0
37°C	174	0
40°C	169	5

$P>0.05$ but <0.1 (approaching significance level) between 30°C vs 40°C and 37°C vs 40°C

$P>0.05$ (non-significant) between 30°C vs 37°C

Table 3 Zones of inhibition of MRSA isolates at various temperatures using 2% sodium chloride supplemented media

Temperature of incubation	Zones of inhibition (mm)			
	0*	1**	2**	3**
30°C	51	6	1	-
37°C	49	7	2	-
40°C	39	9	6	4

*$P<0.05$ (Significant) between 30°C vs 40°C at 0mm zone of inhibition

*$P>0.05$ but <0.1 (Approaching significance level) between 37°C vs 40øC 0 mm zone of inhibition

**$P>0.05$ (Non-significant) between different temperatures at 1, 2 and 3 mm zones of inhibition

"Zones of inhibition" indicates the size of inhibition around the resistant test organisms

Table 4 Zones of inhibition of MRSA isolates at various temperatures using 5% sodium chloride supplemented media

Temperature of omcibatopm	*Zones of inhibition (mm)			
	0	1	2	3
30°C	46	12	-	-
37°C	45	13	-	-
40°C	40	13	5	-

*$P>0.05$ (Non-significant) between the different groups at 0, 1, 2 and 3 mm zones of inhibition

"Zones of inhibition" indicates the size of inhibition around the resistant test organisms

Table 5 Zones of inhibition of MRSA isolates at various temperatures using 7.5% sodium chloride supplemented media

Temperature of incubation	*Zones of inhibition (mm)			
	0	1	2	3
30°C	50	6	2	-
37°C	52	5	1	-
40°C	47	8	2	1

*$P>0.05$ (Non-significant) between the different groups at 0, 1, 2 and 3 mm zones of inhibition

Discussion

Antibiotic sensitivity testing in routine laboratories is generally performed on media without salt supplement followed by incubation at 37°C, irrespective of bacterial species to be tested. Under these conditions methicillin resistance in staphylococci may be missed.[20] Accurate detection of methicillin resistance can be done on media without salt by incubation at 30°C[21-23] or at 35°C[9,24]. Incubation temperature of 35°C has an added advantage that it can be used for sensitivity testing against other antibiotics as well[20,24]. However successful detection of methicillin resistance in Staphylococcus aureus requires strict control of temperature inside the incubators at 35°C. Increase in the temperature of incubator above 35°C may result in resistant strains to appear as sensitive[20,25].

In majority of southern areas of Pakistan the ambient temperature remains higher than 30°C for more than six months of the year. Particularly in the months of May to August, the mean maximum temperature remains more than 35°C[26]. In the presence of such high ambient temperature it is difficult to maintain the temperature inside the incubators close to 30°C for about seven months and 35°C for four months of the year. During these months this problem can be solved by installation of air-conditioners in the laboratories. To keep these air-conditioners functioning round the clock, continous power supply is required. In our country frequent power failures are a regular occurrence. Therefore, keeping in view all these facts it is difficult to maintain the optimum temperature (≤35°C) required for accurate detection of methicillin sensitivity of Staphylococcus aureus.

Methicillin resistant Staphylococcus aureus are multiple drug resistant organisms. The drug of choice for treatment of infections with MRSA has been vancomycin[27] But recently low level vancomycin resistance in MRSA strains has been reported from Japan[28], France[29] and America[27] In this scenario accurate identification of methicillin resistance in Staphylococcus aureus has acquired added importance.

In the present study, strains inoculated on 2% sodium chloride supplemented media showed larger zones of inhibition when sensitivity plates were incubated at higher temperature. The result of the present study are in agreement with results of studies carried out by other workers[21,30,3132]. In these studies Kirby-Bauer method was used to see the effect of temperature on inhibition zones of Staphylococcus aureus. They also concluded that the sensitivity of methicillin resistant strains of Staphylococcus aureus tends to increase with rise in temperature of incubation.

The effect of variable temperatures on inhibition zones of MRSA isolates inoculated on media containing 5% and 7.5% sodium chloride supplement was also studied. It was observed that on these salt supplemented media different incubation temperatures (30°C, 37°C and 40°C) had no significant effect (P>0.05) on the sizes of inhibition zones. In the light of these results we conclude that in our environment 5% sodium chloride supplemented media should be used for accurate detection of MRSA. On such media plates can be incubated at routine incubation temperature of 37°C.

References

1. Qureshi AH, Hannan A. The prevalence and susceptibility pattern of methicillin resistant Staphylococcus aureus. Pak J Pathol 1991; 2(1): 41-4.
2. Otkun M, Akata F, Teker B, et al. Nosocomial infections in the Trakya University Hospital: results from 1995. Turkish J Infect 1997; 11(1): 23-27.
3. Soares MJS, Tokumaru-Miyazaki NH, Noleta ALS, Figueiredo AMS. Enterotoxin production by Staphylococcus aureus clones and detection of Brazilian epidemic MRSA clone (III::B:A) among isolates from food handlers. J Med Microbiol 1997; 46: 214-21.
4. Foster JW, Karow EO. Microbiological aspects of penicillin VIII. Penicillin from different fungi. J Bacteriol 1945; 49: 19-29.
5. Burton HS, Abraham EP. Isolation of antibiotics from a species of cephalosproium. Cephalosporins P1, P2, P3, P4 and P5. Biochem J 1951; 50: 168-74.
6. Williams JD. Antibiotic guidelines. BMJ 1984; 288: 343-44.
7. Bergogne-Berezin E. Who or what is the source of antibiotic resistance? J Med Microbiol 1997; 46: 461-64.
8. Wada A, Watanabe H. Penicillin-binding protein 1 of Staphylococcus aureus is essential for growth. J Bacteriol 1998; 180(10): 2759-65.
9. WHO. External quality assessment in microbiology. WHO collaborating center for external quality assessment in clinical microbiology. Leuven 1997.
10. Lowy FD. Staphylococcus aureus infections. N Engl J Med 1998; 339(8): 520-32.
11. Resende CA, Figueiredo AMS. Discrimination of methicillin-resistant Staphylococcus aureus from borderline-resistant and susceptible isolates by different methods. J Med Microbiol 1997; 145-49.
12. Frenay HME, Theelen JPG, Schouls LM, et al. Discrimination of epidemic and nonepidemic methicillin-resistant Staphylococcus aureus strains on the basis of protein A gene polymorphism. J Clin Microbiol 1994; 32(3): 846-47.
13. Hafiz A. Methicillin resistant Staphylococcus aureus (MRSA). Infect Dis J Pak 1996: 12-13.
14. Mouton RP, Mulders SL, de Knijff J, Hermans J. Comparison of test systems for recognition of methicillin resistance in Staphylococcus aureus. Eur J Clin Microbiol Infect Dis 1989; 8(11): 968-73.
15. Richard P, Meyran M, Carpentier E, Thabaut A, Drugeon HB. Comparison of phenotypic methods and DNA hybridization for detection of methicillin resistant Staphylococcus aureus. J Clin Microbiol 1994; 32(3): 613-17.
16. Siddiqui GM, Hannan A. Comparison of standard Kirby-Bauer and modified Kirby-Bauer disk diffusion methods for the detection of methicillin resistance among staphylococcus. Pak J Pathol 1996; 7(1): 37-41.

17. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC Jr. Color Atlas and Textbook of Diagnostic Microbiology. 5th Ed. Philadelphia: Lippincott, 1997: 69-120, 539-76, 1311.
18. Barrow GI, Feltham RKA. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd Ed. Great Britain: University Press, 1993: 50-93, 242.
19. Cheesbrough M. Medical Laboratory Manual for Tropical Countries. Volume II: Microbiology. Great Britain: ELBS: 1984: 58-69, 196-205.
20. Jolly J, Goldberg M. Methicillin resistance in Staphylococci: An evaluation of conditions for detection. Med Lab Sci 1989; 46: 2-5.
21. Annear DI. The effect of temperature on resistance of Staphylococcus aureus to methicillin and some other antibiotics. Med J Aust 1968; 1: 444-46.
22. Hartman BJ, Tomasz A. Expression of methicillin resistance in heterogeneous strains of Staphylococcus aureus. Antimicrob Agents Chemother 1986; 29(1): 85-92.
23. Kosmidis J, Ploychronopoulou-Karakatsanis C, Milona-Petropoulou D, Mavrogenis N, Xenaki-Kondyli M, Gargalianos P. Staphylococcal infections in hospital. The Greek experience. J Hosp infect 1988; 11 (Supplement A): 109-15.
24. NCCLS. Performance standards for antimicrobial disk susceptibility tests, tentative standard. 4th Ed. NCCLS Dcoument M2-T4. Villanova, PA: 1998.
25. Drew WL, Barry AL, O'Toole R, Sherris JC. Reliability of the Kirby-Bauer disc diffusion method for detecting methicillin-resistant strains of Staphylococcus aureus. Appl Microbiol 1972; 24(2): 240-47.
26. Pakistan Meterological Department. Weather report for scientific and technical purposes only. Lahore: Government of Pakistan, 1999.
27. Smith TL, Pearson ML, Wilcox KR, et al. Emergence of vancomycin resistance in Staphylococcus aureus. N Engl J Med 1999; 304(7): 493-501.
28. Hiramatsu K, Aritaka N, Hanaki H, et al. Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogenously resistant to vancomycin. Lancet 1997; 350: 1670-73.
29. Ploy MC, Grelaud C, Martin C, de Lumley L, Denis F. First clinical isolate of vancomycin-intermediate Staphylococcus aureus in a French hospital. Lancet 1998; 351: 1212.
30. Hewitt JH, Coe W, Parker MT. The detection of methicillin resistance in Staphylococcus aureus. J Med Microbiol 1969; 2: 443-56.
31. Hill AW, James AM. Effect of growth temperature on the surface properties of cells of Staphylococcus aureus with particular reference to methicillin-resistance. Microbios 1972; 6: 169-78.
32. Thornsberry C, Caruthers JQ, Baker CN. Effect of temperature on the in vitro susceptibility of Staphylococcus aureus to penicillinase-resistant penicillins. Antimicrob Agents Chemother 1973; 4(3): 263-69.