

Antimicrobial Susceptibility Testing of Staphylococci and Enterococci

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Staphylococcus aureus remain to be frequent cause of various infections including serious ones, while infections due to coagulase-negative staphylococci increased recently. Staphylococci are often resistant to various antimicrobial agents including methicillin (Chong and Lee, 1997). Recently, enterococci became frequently isolated nosocomial pathogens (Emori and Gaynes, 1993). Resistance of enterococci to high level aminoglycoside or to vancomycin is a serious problem in the United States (MMWR, 1993). Therefore, determination of the susceptibility of these organisms became important laboratory procedures. The most widely used susceptibility test methods include the NCCLS disk diffusion test and various commercial broth microdilution systems. Etest became popular for some special purposes. Recently molecular methods of detection of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) became feasible, but they may not be able to replace conventional routine tests (Uenal et al., 1992; Richard et al. 1994; Woodford et al., 1995).

Most of the phenotypic methicillin resistance of staphylococci are due to chromosome mediated PBP2' (PBP2a) production. Methicillin resistance due to hyperproduction of β -lactamase seems to be rare. MRSA is often considered to be heterogeneous in the expression of methicillin resistance, i.e., only small proportion of the cells show resistance. Therefore, accurate determination of methicillin resistance is difficult. The factors which enhance expression of methicillin resistance are: the presence of β -lactam antibiotics or 2-5% salt in the medium, growth at 30°C and extended incubation of more than 24 h (McDougal and Thornsberry, 1984; Chambers, 1988; Neuman et al., 1991). There are pitfalls in almost every test procedure, whether it be the disk diffusion test or commercial systems. I would like to present the difficulties we experienced in the detection of MRSA.

In Korea, MRSA was probably not detected in the 1960s (Park, 1969). But in the 1970s, MRSA started to be detected, i.e., 8% of *S. aureus* were methicillin resistant in 1974. In the 1970s, we used methicillin disk for the test as recommended by the NCCLS. We had difficulties in the quality control of methicillin disks, i.e., in 1980, 7.7% of the quality control results showed smaller methicillin zone than the NCCLS control limit and we had to discard 4 lots of the disks (Chong et al., 1980). Therefore, it was possible that some MSSAs were mistakenly reported as MRSA. In 1982, oxacillin disk became recommended by the NCCLS (M2-A2-S2).

Now the problem is missed detection of methicillin resistance. We started to follow other NCCLS recommendations: direct inoculum preparation and use of transmitted light for the zone observation. We began to use media dispensing instrument, PourMatic (New Brunswick)

in 1986, to have uniformly 4-mm-thick Mueller-Hinton agar. As to the incubation condition, we have been using 35°C incubator instead of 37°C but it was not always possible to read the plate after exactly 24 h incubation.

Chances of reporting false susceptible results depends on the prevalence of heterogeneous MRSA and test method used. Tomasz et al. (1991) classified MRSAs into 4 classes, depending on the characteristics of the population. It was shown by Hindler and Inderlied (1985) that there were two types of inhibition zone, definite and occult. The proportion of expression class 1 or 2, and occult type may depend on countries and may change in time. Our test in 1989-1990 showed that most of the isolates were definitive type and the zone diameter distributions showed clear separation of most of the strains (Doh et al., 1991). In a study (Table 1, 2), it was found that most of the MRSAs isolated in 1980 were class 2, while in 1994 it was class 4, i.e., heterogeneous type (Lee and Chong, 1996). This means that accurate detection of MRSA became easier for laboratories, while treatment of the patient became more difficult for clinicians. However, still there remain strains with intermediate susceptibility by the disk test.

Table 1. MIC of methicillin against MRSAs isolated in 1980 and 1994

Year and hospital	MIC of methicillin (µg/ml)		
	Range	50%	90%
1980 (hospital A)	16 - 1048	64	256
1994 (hospital A)	128 - 1048	512	1048
1995 (hospital B)	32 - 1024	512	1024

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Table 2. Coagulase types and expression classes of MRSA isolated in 1980 and 1994

Hospital	Year of isolation	Coagulase type (no.)	Number with expression class			
			1	2	3	4
A	1980	III (14)		11	3	
		I (1)			1	
	1994	II (6)			6	
		III (11)		7	4	
B	1994	IV (2)			2	
		II (5)			5	
		III (5)		3	2	
		IV (2)		1	1	
		VII (2)		1	1	
Total		(48)	0	11	15	22

Multiresistance is a clue to the possibility of methicillin resistance. Already in 1985, resistance rate of MRSA in Korea to gentamicin and tobramycin were 91% and 93%,

respectively (Chong, 1986). Sixty-nine percent of MRSAs isolated in 1994 were multiresistant to 4 antimicrobial agents, i.e., clindamycin, erythromycin, tetracycline and pefloxacin (Table 3; Lee and Chong, 1996).

Table 3. Multiresistance patterns of MSSA and MRSA isolated in 1994

Pattern	MSSA (376)		MRSA (738)	
	No.	%	No.	%
Ery, Tet, Pef, Cln ^a	3	0.8	510	69.1
Other 4 antimicrobials	35	9.3	140	19.0
Total	38	10.1	525	88.1

^a Ery, erythromycin; Tet, tetracycline; Pef, pefloxacin; Cln, clindamycin.

For equivocal isolates, use of the screening plate test with 4% NaCl and 6 µg/ml oxacillin and incubation at 35 °C for 24 h may resolve most of the problems (MacKenzie et al., 1995). In a laboratory proficiency testing program in Canada, with low-expression-class MRSAs, 16 of 76 (21%) disk tests showed incorrect results, while the rate by other methods were 1 of 104 (1%). All results by 30 laboratories, which used screening plate tests, were correct.

Because of the flexibility of drug selection, we have been using the disk test only, but lately started to use occasional Etests. It was reported (Murray, 1994) that, use of microdilution test significantly increased during the years 1982 to 1988 in US. In 1990, 30% of the tests were performed by the NCCLS disk method and remaining tests by broth microdilution method, although many laboratories returned back to the disk method (Woods and Washington, 1995). In Korea, laboratories using broth microdilution test have been increasing. Therefore, the broth microdilution test may have great impact on the detection of MRSA in Korea, too.

Thornsberry and McDougal (1983) reported successful detection of methicillin-resistant (heteroresistant) staphylococci when the broth was supplemented with 2% NaCl. Knapp et al. (1994) reported that all of the methicillin-resistant staphylococci were accurately detected with the Vitek software upgrades (6.1 and 7.1) of the GPS-SA card (bioMerieux). Woods et al. (1994) reported MicroScan rapid panel (Baxter) allowed reliable detection of MRSA in 3.5 to 15 h, and most in 4.5 to 7 h, but coagulase negative staphylococci did not grow well in the system. Struelens et al. (1995) reported that, compared to oxacillin agar screening method, the accuracy of Rapid ATB Staph test (bioMerieux) was 97% within 5 h of incubation. Occasional difficulties in the early detection of MRSA was also reported by Zambardi et al. (1996) with the C-MRSA-ID system (Becton-Dickinson). These results indicate that rapid tests, especially with coagulase-negative staphylococci, are prone to false susceptibility. Therefore, confirmatory test is required when equivocal results were obtained (Fig. 1). *mecA* gene detection may also resolve the problem, but it may not replace other routine tests. In the interpretation of the susceptibility test results, MRSA are considered resistant to all β-lactams. Therefore, it is required to test the susceptibilities only to penicillin G and oxacillin, among the many penicillins and cephalosporins.

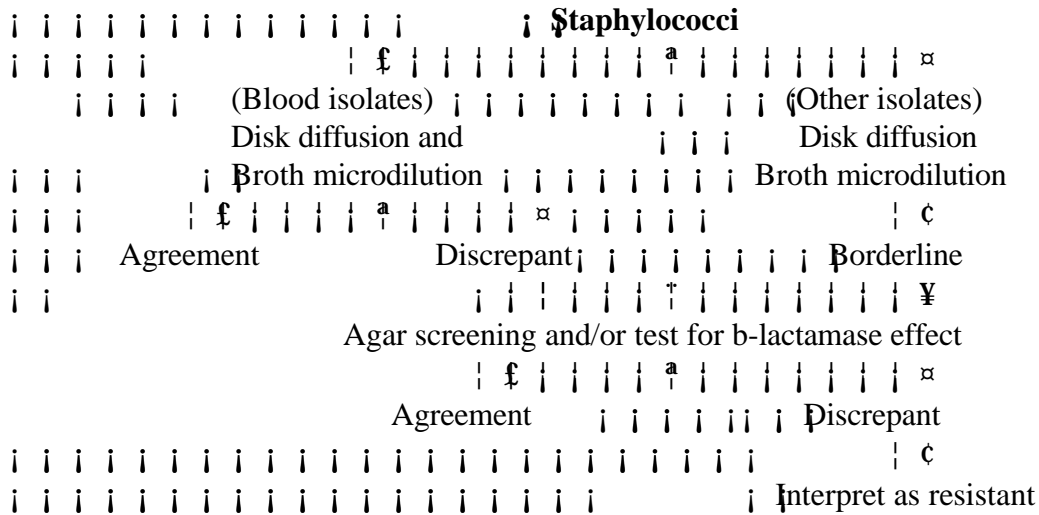


Fig. 1. Methicillin susceptibility testing of staphylococci (Chambers, 1988).

Enterococci became frequently isolated. The resistance of enterococci to high level aminoglycoside (HLR) and to vancomycin are current problem. Recent increase of HLR enterococci in Korea indicates importance of the test for the strains isolated from blood. Kim et al. (1992) reported the rate to be 60% to gentamicin, and 40% to streptomycin. In our study during the similar period, the rates to gentamicin were 20% for *E. faecalis* and 59% for *E. faecium* (Chong, 1993).

High level aminoglycoside resistance can be detected using broth medium or agar medium with 500 µg/ml of gentamicin and 1000 µg/ml (2000 µg/ml with agar medium) of streptomycin, and by disk diffusion test with 120 µg of gentamicin and 300 µg of streptomycin. The NCCLS methods of broth microdilution and agar dilution tests were shown to be very accurate when incubation was extended up to 48 h. But disk test was not very accurate especially with streptomycin and Mueller-Hinton agar, i.e., depending on the manufacturers of media and disks only 78-93% of the resistant strains were detected after 24 h incubation (Swenson et al., 1995). Yagupsky et al. (1990) reported practical way of testing HLR. We modified the procedure slightly, i.e., 2 ml of brain heart infusion with 500 µg/ml of gentamicin, 1000 µg/ml of streptomycin or no aminoglycoside was dispensed into small screw cap test tubes and stored in a freezer. Test strain is suspended to the turbidity of McFarland No. 0.5 tube and inoculated with a 1/1000-ml loop. Tubes are incubated at 35°C for up to 48 h.

Table 4. Recommended parameters for screening HLR to aminoglycosides among enterococci^a

Method	Agar dilution	Broth dilution	Disk diffusion
Medium	BHI	BHI	MHA
Inoculum(CFU or McFarland STD)	1 x 10 ⁶	1 x 10 ⁶	0.5
Concentration(§ µl or § µdisk)			
Gentamicin	500	500	120
Streptomycin	2,000	1,000	300
Incubation(h)	24 (48) ^b	24 (48) ^b	18-24
End point	Any growth > 1 colony	Any growth	6 mm = HLR 7-9 mm = inconclusive ≥10mm = susceptible

^a Modified from Swenson et al. (1995).

^b If streptomycin test is negative at 24 h, reincubate for additional 24 h.

VRE infection is rare in Korea at present, however, the situation may change (Park et al., 1992; Park, 1996; Peck et al., 1996). Vancomycin resistance is mediated by at least three types of genes. As some *vanB* and all *vanC* type strains show low level resistance to vancomycin, these types of resistance is difficult to detect. Disk test may fail to detect glycopeptide resistance because glycopeptides diffuse poorly from the disks.

¶ Tenover et al. (1995) compared various methods of VRE detection. Disk diffusion test showed 12% of minor error. Commercial nonautomated systems accurately detected VRE, but automated systems occasionally failed to detect low level resistance. ¶ It was reported that the sensitivity of 93% with the MicroScan Walk/Away (Baxter) could be increased to 99% when readings were performed manually (Wiley et al., 1992).

Wiley et al. (1992) reported agar screening test was reliable and Swenson et al. (1994) evaluated the agar screening test for the detection of VRE. The sensitivity and specificity were 100% and 96 to 99%, respectively. However, Tenover et al. (1995) showed that agar screening test with inocula of 10⁵ and 10⁶ CFU detected all of the VREs only when the microbiologists were trained for the reading.

It was reported that Etest was able to detect all level of vancomycin resistance and appeared to be more sensitive than disk diffusion test for detecting strains for which vancomycin MICs were 8-16 § µl/ml (Shulz and Sahn, 1993).

Table 5. Performance of screen test with BHI agar supplemented with 6 g/ml vancomycin^a

Species	Vancomycin MIC(μ g/ml)	Inoculum (CFU)	No.(%) correct results	
			24h	48h
<i>E. faecalis</i> , <i>E. faecium</i> , <i>E. raffinosus</i> , <i>Enterococcus</i> sp.	1-2	10^5	99	98
<i>E. caseliflavus</i>	2-4	$10^5, 10^6$	25	25
<i>E. gallinarum</i>	2-8	$10^5, 10^6$	100	100
<i>E. faecalis</i> , <i>E. faecium</i> , <i>E. gallinarum</i> , <i>E. raffinosus</i>	1-6	$10^5, 10^6$	100	100

^a Swenson et al., 1994.

Various selective media have been used to determine intestinal carriage of VRE (Van Horn et al., 1996). Bile esculin azide agar supplemented with 6 μ g/ml of vancomycin detected 85% of VRE after 24 h incubation. However, we experienced that it was very inefficient method to detect VRE. Among the specimens, 91% yielded growth, but only 1 specimen was positive for VRE. Landman et al. (1996) reported that among the various media and antimicrobial combinations, enterococcal broth supplemented with 64 μ g/ml of vancomycin and 60 μ g/ml of aztreonam was better. However, the isolates included strains for which vancomycin MICs were $\geq 2 \mu$ g/ml. In a study we used bile esculin azide broth supplemented with 64 μ g/ml of vancomycin and 60 μ g/ml of aztreonam. Among the 502 specimens 10 (1.9%) yielded VRE. This procedure require subculture onto agar plate and the limitation is detection of only high level resistant strains.

In summary, despite of improvements, some of the tests may fail to detect MRSA, and high-level aminoglycoside-resistant or glycopeptide-resistant enterococci. Use of appropriate methods with strict adherence to recommended procedure should be able to minimize test errors. Also confirmatory tests should be available to resolve equivocal results.

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