JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1994, p. 1866–1869
0095-1137/94/$04.00+0

Vol. 32, No. 8

Development of Enzyme-Labeled Oligonucleotide Probe for Detection of *mecA* gene in Methicillin-Resistant *Staphylococcus aureus*

MOTOMU SHIMAOKA,[1,2]* MYONSUN YOH,[1] AKIYA SEGAWA,[3] YUTAKA TAKARADA,[1,3] KOICHIRO YAMAMOTO,[1] AND TAKESHI HONDA[1]

Research Institute for Microbial Diseases, Osaka University,[1] Department of Anesthesiology, Osaka University Medical School,[2] Yamadaoka, Suita, Osaka 565, and Toyobo Research Center, Ohtsu, Shiga 520-2,[3] Japan

Received 13 January 1994/Returned for modification 1 March 1994/Accepted 6 May 1994

A DNA hybridization method with an enzyme-labeled oligonucleotide probe (*mecA*-ELONP) was developed to detect the methicillin-resistant gene (*mecA*) in methicillin-resistant *Staphylococcus aureus*. For rapid identification, bacterial colonies were transferred from agar plates directly onto nylon membranes. Lysis of cells, denaturation of DNA, and hybridization were performed on the membranes. These procedures required only 3 h for completion. The results obtained by this test closely corresponded with those obtained by determining the MICs of oxacillin against *S. aureus*. The results of the *mecA*-ELONP also correlated well with those of a commercially available PCR test. Thus, *mecA*-ELONP proved to be a reliable and convenient method for the rapid identification of methicillin-resistant *S. aureus*, which could be useful in clinical microbiology laboratories.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is highly resistant not only to β-lactam antibiotics but also to aminoglycosides and other chemotherapeutic drugs (6, 11). In some countries, MRSA has recently become a serious problem, especially as a cause of nosocomial infections (2). Appropriate treatment of MRSA infection requires the rapid and reliable identification of this pathogen. Conventional methods to identify MRSA are based on susceptibility (determination of the MIC) to oxacillin and/or methicillin (17). MIC determination is, however, uncertain in some strains, and results are often affected by culture conditions (3, 7, 13). These factors complicate the identification of MRSA.

The main mechanism of drug resistance in MRSA is explained by the production of a new penicillin-binding protein (PBP), designated PBP2′ or PBP2a, which has a low affinity for β-lactam antibiotics (16, 19, 28), in contrast to the four better-known PBPs. The gene which encodes PBP2′, *mecA*, has been cloned from the chromosomal DNA of MRSA and sequenced (12, 20, 22, 27). *mecA* has been distributed among MRSA but not methicillin-susceptible *S. aureus* (MSSA) (1, 15, 25).

DNA hybridization with fragments of *mecA* has recently been reported as a more reliable way to identify MRSA (1, 4, 9, 14). Although hybridization tests using radioisotope-labeled DNA fragments may be useful for research laboratories, radioisotope management in routine clinical laboratories is difficult. To avoid this problem, various enzyme-labeled oligonucleotide probes are under investigation (8, 18, 29). In this study, we have developed a DNA hybridization method with an enzyme-linked oligonucleotide probe to detect *mecA*, *mecA*-ELONP. We compare this method with conventional MIC determination and a commercially available *mecA* gene detection test based on PCR.

* Corresponding author. Mailing address: Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan. Phone: 81-6-879-8276. Fax: 81-6-879-8277.

MATERIALS AND METHODS

Bacterial strains. A total of 175 staphylococcal isolates (140 *S. aureus* isolates and 35 coagulase-negative staphylococci [CNS] isolates), all clinical isolates from several city hospitals in the Osaka prefecture, Japan, were used. All isolates were derived from individual patients; there was no duplication. *S. aureus* RIMD3109028 (MIC of oxacillin was 256 μg/ml) and RIMD3109036 (MIC of oxacillin was 1 μg/ml) were used as positive and negative controls, respectively, for the detection of the *mecA* gene. The presence of *mecA* in RIMD3109028 and its absence in RIMD3109036 were confirmed by PCR as described previously (15). These control strains were supplied from the Laboratory for Culture Collection, Osaka University.

Media and solutions. Heart infusion agar (Difco Laboratories, Detroit, Mich.) and Mueller-Hinton agar medium (Oxoid Ltd., Hampshire, England) were used for the growth of *S. aureus* and CNS. SSC (1× SSC was 0.15 M NaCl plus 0.015 M sodium citrate) buffer was prepared as previously described (21).

Susceptibility test. MICs were determined by the plate dilution method with Mueller-Hinton agar medium containing 2% NaCl inoculated with approximately 10^4 CFU of bacteria. The MIC was defined as the lowest concentration of antibiotics inhibiting visible bacterial growth on the agar plates after incubation for 24 h at 35°C. Methicillin resistance was determined when MICs of oxacillin were 4 μg/ml or higher (17).

Preparation of probes. Oligonucleotide probes with an internal deoxyuridine possessing a six-carbon spacer were prepared following a previously described phosphoramidate method utilizing a DNA synthesizer (8, 24). The probe was purified on a Mono-Q column (Pharmacia, Uppsala, Sweden).

The selected nucleotide sequence for this probe extended from nucleotides 1552 to 1576 after the initiation codon ATG of *mecA* (20). The sequence was 5′-XAGAGTAGCACTC GAATTAGGCAGT-3′ (X denotes a deoxyuridine to which a side arm is linked). A purified linker-armed oligonucleotide was covalently cross-linked with alkaline phosphatase as described previously (8, 24). Conjugates were then dissolved in conjugate buffer (30 mM Tris-HCl, 3 M NaCl, 1 mM $MgCl_2$,

0.1 mM $ZnCl_2$, 0.05% NaN_3 [pH 7.6]) at 10 μg of DNA per ml and stored in the dark at 4°C until use. Enzyme activity was found to be stable for at least 12 months.

Hybridization procedure. Following overnight growth at 37°C on heart infusion agar plates, the colonies, which included positive and negative control strains, were transferred onto nylon membranes (GeneScreen Plus; Dupont-NEN, Boston, Mass.) with sterile toothpicks. A filter paper (3MM; Whatman Paper Ltd., Maidstone, England) infused with 0.5 N NaOH–1% sodium *N*-lauroyl sarcosinate was preheated in a plastic tray in a 98°C water bath. The membrane was laid on the filter paper and similarly incubated for 10 min in the 98°C water bath to lyse the cells and to denature the DNA. To neutralize the alkaline solution, each membrane was initially laid on a filter paper wetted with 1 M Tris-HCl buffer (pH 7.4), left for 1 min at room temperature, subsequently transferred to a new filter paper suffused with the same buffer, and allowed to stand for 10 min at room temperature. Each membrane was then transferred to a tray containing 100 ml of 5× SSC, and the residual bacterial debris was rubbed off with a plastic sponge. After being rinsed with 5× SSC, the membrane was transferred to a plastic hybridization bag containing 1 ml of hybridization buffer (5× SSC with 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, 1% sodium dodecyl sulfate [SDS]) and 5 μl of the probe solution (for a 100-cm^2 membrane). The bag was incubated for 15 min at 55°C in a water bath. Then, each membrane was removed from the plastic bag, transferred to a tray containing 100 ml of 2× SSC–1% SDS preheated to 55°C in a water bath, and incubated with gentle shaking at the same temperature for 10 min. The membrane was transferred to a tray containing 100 ml of 1× SSC–0.5% Triton X-100 and left for 10 min at room temperature. Following this, each membrane was transferred to a plastic bag containing 7.5 ml of substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.1 M $MgCl_2$, 0.1 mM $ZnCl_2$, 0.05% NaN_3 [pH 8.5]), 33 μl of nitroblue tetrazolium (75 mg/ml in 70% dimethylformamide), and 25 μl of 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml in dimethylformamide) and incubated at 37°C for 60 min. Positive results were ascertained by the appearance of purple color on the membrane. The presence of *mecA* was determined visually in relation to the controls.

In order to determine the detection limit of the number of bacterial cells, serial dilutions of a broth culture were also prepared, and 2-μl portions of diluted broth culture were spotted onto a membrane. After being dried, the membrane was assayed as described above. Portions (100 μl) of diluted broth culture were also applied to heart infusion agar plates and incubated at 37°C for 24 h, colonies were counted, and detection limits were determined.

PCR. Enzyme detection by PCR (ED-PCR) (*mecA* gene identification test kit WAKUNAGA; Wakunaga Pharmaceutical Co., Ltd., Osaka, Japan) was done with staphylococci as reported previously (26). Briefly, by ED-PCR, *mecA* genes are amplified by PCR utilizing a biotin-labeled sense primer and a dinitrophenol-labeled anti-sense primer. This amplified DNA is transferred into streptavidin-coated microtiter wells. When the DNA is captured with streptavidin bound to the bottom of the well because of biotin-avidin affinity, the quantity of the captured DNA can be determined by enzyme-linked immunosorbent assay with alkaline phosphatase-labeled anti-dinitrophenol antibody.

RESULTS

Sensitivity and specificity of *mecA*-ELONP. The minimal population of bacterial cells required for detection of *mecA* by *mecA*-ELONP was 2×10^5 CFU (data not shown). Table 1 shows the results of *mecA*-ELONP and oxacillin susceptibility in 140 *S. aureus* and 35 CNS isolates from clinical sources. Among the 104 *S. aureus* isolates with MICs of oxacillin of ≥4 μg/ml (MRSA), 103 strains were positive for *mecA* and 1 strain was negative according to *mecA*-ELONP results. The MIC of the negative strain was 64 μg/ml. Five of 104 MRSA strains showed borderline resistance (MICs of oxacillin were 4 μg/ml). All five strains tested positive for *mecA* by *mecA*-ELONP. Of the 36 *S. aureus* isolates with MICs of oxacillin of ≤2 μg/ml (MSSA), 1 was positive and 35 strains were negative by *mecA*-ELONP testing. The MIC of the MSSA strain which tested positive by *mecA*-ELONP was 1 μg/ml. Thus, *mecA*-ELONP had a sensitivity of 99% and a specificity of 97% for MRSA. All 16 CNS isolates with MICs of oxacillin of ≥4 μg/ml were positive. Of the 19 CNS isolates with MICs of oxacillin of ≤2 μg/ml, 17 strains were negative but 2 were positive. The MICs of the two *mecA*-positive CNS isolates were 1 μg/ml.

TABLE 1. Detection of *mecA* in clinical isolates by *mecA*-ELONP

Organism	MIC of oxacillin (μg/ml)[a]	No. of strains		
		Tested	Positive	Negative
S. aureus				
MRSA	≥4	104	103	1
MSSA	≤2	36	1	35
CNS	≥4	16	16	0
	≤2	19	2	17

[a] Determined by the agar dilution method.

Comparison of *mecA*-ELONP with ED-PCR. We compared *mecA*-ELONP with ED-PCR in 102 *S. aureus* strains (81 were MRSA and 21 were MSSA) and 35 CNS isolates from clinical sources. As shown in Table 2, all the MRSA strains tested positive by ED-PCR. All but one of the MRSA isolates were positive by *mecA*-ELONP. All five borderline-resistant MRSA isolates were positive by both methods. Of the 21 MSSA strains, 1 tested positive by *mecA*-ELONP, whereas 2 were positive by ED-PCR. Two strains showed discrepant results between the two tests. Comparatively, *mecA*-ELONP had a sensitivity of 99% and a specificity of 95% for MRSA; ED-PCR had a sensitivity of 100% and a specificity of 90%. In testing for CNS, *mecA*-ELONP and ED-PCR gave the same results. However, both methods also detected *mecA* in two CNS strains with MICs of oxacillin of 1 μg/ml.

DISCUSSION

Outbreaks of nosocomial infections caused by MRSA have been reported worldwide (11). The multidrug resistance of

TABLE 2. Comparison of *mecA*-ELONP and ED-PCR

Organism	MIC of oxacillin (μg/ml)[a]	No. of strains tested	No. of strain with result indicated by:			
			mecA-ELONP		ED-PCR	
			Positive	Negative	Positive	Negative
S. aureus						
MRSA	≥4	81	80	1	81	0
MSSA	≤2	21	1	20	2	19
CNS	≥4	16	16	0	16	0
	≤2	19	2	17	2	17

[a] Determined by the agar dilution method.

MRSA has become a serious clinical problem, and the rapid and accurate identification of MRSA is important for chemotherapy against the pathogen. Unfortunately, the results of conventional susceptibility tests are often influenced by various culture conditions, such as incubation temperature, incubation time, the concentration of NaCl in the medium, and the size of the inoculum used in test media (3, 7, 13). Thus, it is difficult to determine the levels of methicillin resistance of borderline-resistant strains, for example, strains producing large amounts of β-lactamase (4, 13).

Recently, several methods for the detection of *mecA* of MRSA with DNA probes (1, 4, 9, 14) and PCR (15, 23, 25, 26) have been reported. These genetic methods are probably the most reliable tools for identifying MRSA, especially when susceptibility tests are ambiguous. Most of these methods, however, use cloned DNA fragment probes labeled with radioisotopes, hazardous materials which are laborious to prepare, need special handling procedures, and require facilities for safety. A nonradioactive DNA probe with cloned DNA for the detection of MRSA has also been reported recently (9), but this cloned DNA probe requires much time and labor to prepare and hybridize, making its use less practical in a clinical laboratory. In this study, we have developed an effective alkaline phosphatase-labeled oligonucleotide probe for identifying *mecA* in MRSA. The results of this *mecA*-ELONP corresponded closely with those obtained by susceptibility testing of *S. aureus*. Results were inconsistent, however, for one MRSA strain, an MSSA strain, and two methicillin-susceptible CNS strains. The MRSA that was not detected by *mecA*-ELONP actually showed moderate resistance to oxacillin (MIC of oxacillin of 64 μg/ml). This MIC was far higher than that of the borderline resistant strain (4 μg/ml); ED-PCR was able to detect *mecA* in this strain. Why the *mecA*-ELONP could not detect *mecA* in this strain is unclear, but the simplest and most acceptable explanation seems to be that a point mutation might have occurred in *mecA* at the hybridization site of this probe. On the other hand, a single strain of MSSA and two methicillin-susceptible CNS strains proved positive by both *mecA*-ELONP and ED-PCR. Three possibilities to account for these false-positive results are offered. First, *mecA* may not have been expressed in PBP2′ (5, 19), for example, because of a defective promoter. In this case, this strain would have *mecA* but not PBP2′ and so would not become methicillin resistant. We did not, however, assay PBPs, and further study will be needed to confirm this possibility. Secondly, the strains may have harbored *mecA* encoding for a malfunctional PBP2′. Thus, it would not have been able to show methicillin resistance. Finally, factors other than PBP2′ may be responsible for the phenotypic expression of methicillin resistance (4). For example, the *femA* gene has been reported to influence methicillin resistance in MRSA (10). Inactivation of *femA* decreases the glycine content of the cell wall of MRSA and reduces the phenotypic level of methicillin resistance even if PBP2′ exists.

Clinical microbiology laboratories often miss the identification of MRSA in borderline-resistant *S. aureus* (3, 13). Both *mecA*-ELONP and ED-PCR allowed us to detect *mecA* in all five MRSA strains which showed borderline resistance. These methods may be superior to MIC determinations to confirm borderline-resistant MRSA strains.

mecA-ELONP, as developed, correlated well with ED-PCR. Inconsistent results were returned for only 2 of 137 staphylococcal strains tested. One was the MRSA strain discussed above and the other was an MSSA, which was *mecA*-ELONP negative and ED-PCR positive. Although the reason for this discrepancy is not clear, a plausible explanation is partial deletion in *mecA* at the hybridization site of the *mecA*-ELONP probe in this strain. In this case, the ED-PCR result would be positive, although this strain may not produce active PBP2′.

The advantage of PCR is its high sensitivity derived from the amplification of target nucleotide sequences. ED-PCR has been reported to be sensitive to as few as 5×10^2 CFU (26), whereas the sensitivity of *mecA*-ELONP is 2×10^5 CFU. For the detection of smaller numbers of bacteria, the PCR is probably more useful, but when sufficient numbers of organisms are available, *mecA*-ELONP is more convenient because, other than a water bath, it does not require special equipment. In clinical laboratories, this test can be used to identify MRSA from colonies grown on agar plates. Even a small single colony may be sufficient to detect *mecA* by *mecA*-ELONP. Thus, the *mecA*-ELONP method is sensitive enough for practical purposes and safe to handle, and it can be stored for long periods (at least 1 year; data not shown). Furthermore, this probe produces results in only 3 h after suspicious-looking colonies on agar plates are tested.

We conclude that the *mecA*-ELONP developed in this study is a reliable and convenient method for the rapid identification of MRSA, which could be useful in clinical microbiology laboratories.

ACKNOWLEDGMENT

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. **Archer, G. L., and E. Pennell.** 1990. Detection of methicillin resistance in staphylococci by using a DNA probe. Antimicrob. Agents Chemother. **34:**1720–1724.
2. **Brumfitt, W., and J. Hamilton-Miller.** 1989. Methicillin-resistant *Staphylococcus aureus*. N. Engl. J. Med. **320:**1188–1196.
3. **Chambers, H. F., G. Archer, and M. Matsuhashi.** 1989. Low-level methicillin resistance in strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **33:**424–428.
4. **de Lancastre, H., A. M. S. Figueiredo, C. Urban, J. Rahal, and A. Tomasz.** 1991. Multiple mechanisms of methicillin resistance and improved methods for detection in clinical isolates of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **35:**632–639.
5. **Gerberding, J. L., C. Miick, H. H. Liu, and H. F. Chambers.** 1991. Comparison of conventional susceptibility tests with direct detection of penicillin-binding protein 2a in borderline oxacillin-resistant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **35:**2574–2579.
6. **Hackbarth, C. J., and H. F. Chambers.** 1989. Methicillin-resistant staphylococci: detection methods and treatment of infections. Antimicrob. Agents Chemother. **33:**995–999.
7. **Hartman, B. J., and A. Tomasz.** 1986. Expression of methicillin resistance in heterogeneous strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **29:**85–92.
8. **Jablonski, E., E. W. Moomaw, R. H. Tullis, and J. L. Ruth.** 1986. Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. Nucleic Acids Res. **14:**6115–6128.
9. **Ligozzi, M., G. M. Rossolini, E. A. Tonin, and R. Fontana.** 1991. Nonradioactive DNA probe for detection of gene for methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **35:**575–578.
10. **Maidhof, H., B. Reinicke, P. Blümel, B. Berger-Bächi, and H. Labischinski.** 1991. *femA*, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J. Bacteriol. **173:**3507–3513.
11. **Maple, P. A. C., J. M. T. Hamilton-Miller, and W. Brumfitt.** 1989. World-wide antibiotic resistance in methicillin-resistant *Staphylococcus aureus*. Lancet **i:**537–539.
12. **Matsuhashi, M., M. D. Song, F. Ishino, M. Wachi, M. Doi, M. Inoue, K. Ubukata, N. Yamashita, and M. Konno.** 1986. Molecular

cloning of the gene of a penicillin-binding protein supposed to cause high resistance to β-lactam antibiotics in *Staphylococcus aureus*. J. Bacteriol. **167:**975–980.
13. **McDougal, L. K., and C. Thornsberry.** 1986. The role of β-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. J. Clin. Microbiol. **23:**832–839.
14. **Monzon-Moreno, C., S. Aubert, A. Morvan, and N. E. Solh.** 1991. Usefulness of three probes in typing isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). J. Med. Microbiol. **35:** 80–88.
15. **Murakami, K., W. Minamide, K. Wada, E. Nakamura, H. Teraoka, and S. Watanabe.** 1991. Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. J. Clin. Microbiol. **29:**2240–2244.
16. **Murakami, K., K. Nomura, M. Doi, and T. Yoshida.** 1987. Production of low-affinity penicillin-binding protein by low- and high-resistance groups of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **31:**1307–1311.
17. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
18. **Nishibuchi, M., M. Arita, T. Honda, and T. Miwatani.** 1988. Evaluation of a nonisotopically labeled oligonucleotide probe to detect the heat-stable enterotoxin gene of *Escherichia coli* by the DNA colony hybridization test. J. Clin. Microbiol. **26:**784–786.
19. **Rossi, L., E. Tonin, Y. R. Cheng, and R. Fontana.** 1985. Regulation of penicillin-binding protein activity: description of a methicillin-inducible penicillin-binding protein in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **27:**828–831.
20. **Ryffel, C., W. Tesch, I. Birch-Machin, P. E. Reynolds, L. Barberis-Maino, F. H. Kayser, and B. Berger-Bächi.** 1990. Sequence comparison of *mecA* genes isolated from methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Gene **94:** 137–138.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Song, M. D., M. Wachi, M. Doi, F. Ishino, and M. Matsuhashi.** 1987. Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. FEBS Lett. **221:**167–171.
23. **Struelens, M. J., R. Bax, A. Deplano, W. G. V. Quint, and A. Van Belkum.** 1993. Concordant clonal delineation of methicillin-resistant *Staphylococcus aureus* by macrorestriction analysis and polymerase chain reaction genome fingerprinting. J. Clin. Microbiol. **31:**1964–1970.
24. **Tamatsukuri, S., K. Yamamoto, S. Shibata, F. Leano, T. Honda, and T. Miwatani.** 1991. Detection of a heat-labile enterotoxin gene in enterotoxigenic *Escherichia coli* by densitometric evaluation using highly specific enzyme-linked oligonucleotide probes. Eur. J. Microbiol. Infect. Dis. **10:**1048–1055.
25. **Tokue, Y., S. Shoji, K. Satoh, A. Watanabe, and M. Motomiya.** 1992. Comparison of a polymerase chain reaction assay and a conventional microbiologic method for detection of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36:**6–9.
26. **Ubukata, K., S. Nakagami, A. Nitta, A. Yamane, S. Kawakami, M. Sugiura, and M. Konno.** 1992. Rapid detection of the *mecA* gene in methicillin-resistant staphylococci by enzymatic detection of polymerase chain reaction products. J. Clin. Microbiol. **30:**1728–1733.
27. **Ubukata, K., R. Nonoguchi, M. Matsuhashi, and M. Konno.** 1989. Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene, which encodes a methicillin-resistant *S. aureus*-specific penicillin-binding protein. J. Bacteriol. **171:**2882–2885.
28. **Ubukata, K., N. Yamashita, and M. Konno.** 1985. Occurrence of a β-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. Antimicrob. Agents Chemother. **27:**851–857.
29. **Yoh, M., K. Miyagi, Y. Matsumoto, K. Hayashi, Y. Takarada, K. Yamamoto, and T. Honda.** 1993. Development of an enzyme-labeled oligonucleotide probe for the cholera toxin gene. J. Clin. Microbiol. **31:**1312–1314.