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Exfoliative Toxin Detection Using Reversed Passive Latex Agglutination: Clinical and Epidemiologic Applications

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A rapid and simple method for detecting exfoliative toxin serotypes A and B from clinical isolates has been developed as a test kit (EXT-RPLA; Denka Seiken Co. Ltd., Niigata, Japan). This method is based on reversed passive latex agglutination. The detection limit of the EXT-RPLA observed for purified exfoliative toxin serotypes A and B was 1 ng/ml. We evaluated the clinical and epidemiologic uses of the EXT-RPLA. A total of 381 isolates of *Staphylococcus aureus*, 292 from various clinical specimens and 89 from the skin of dermatologic patients, were studied. The EXT-RPLA detected 19 exfoliative toxin producers, including 16 serotype A producers and 3 serotype B producers, but no double producers. The sensitivity and specificity of the EXT-RPLA were confirmed by the newborn mouse bioassay and a PCR assay for the structural genes for exfoliative toxin serotypes A and B (*eta* and *etb*, respectively). The overall positivity rate of exfoliative toxin producers was 5.0% (19 of 381), including 16 serotype A isolates and 3 serotype B isolates. Of the 89 isolates from the skin of dermatologic patients, 12 (13.5%) were positive for exfoliative toxin production. Only 2 (1.3%) of the 153 methicillin-resistant *S. aureus* isolates produced exfoliative toxin, while 17 (7.5%) of the 228 methicillin-sensitive isolates produced exfoliative toxin. The EXT-RPLA assay is a simple and reliable method for detecting exfoliative toxin, and we recommend its use for the rapid diagnosis of staphylococcal scalded skin syndrome. We also recommend its use for detection of this syndrome so that effective control measures can be taken against the spread of this syndrome.

Staphylococcus aureus produces a variety of extracellular protein toxins such as enterotoxin, toxic shock syndrome toxin 1, and exfoliative toxin (ET). ET causes staphylococcal scalded skin syndrome (SSSS) characterized by generalized exfoliative dermatitis (Ritter's disease and staphylococcal toxic epidermal necrolysis), staphylococcal scarlatiniform rash, and bullous impetigo (11, 12). Two serologically distinct forms of ET, ET type A (ETA) and ET type B (ETB), have been identified (7, 8). ETA and ETB have the same biologic activity, but differ in their genetic control of protein synthesis. The gene coding for ETA (*eta* gene) is chromosomal, while the gene for ETB (*etb* gene) is carried by a plasmid (9, 15, 25).

The definitive diagnosis of SSSS requires detection of ET from clinical isolates of *S. aureus*. Several methods for detecting ET have been developed: the newborn mouse bioassay (2, 12), Ouchterlony immunodiffusion (2), radial immunodiffusion (2, 26), electrosyneresis (19), slide latex agglutination (13), enzyme-linked immunosorbent assay (ELISA) (16), radioimmunologic assay (26), and molecular techniques such as a probe assay (19) and PCR (6, 21). These methods require expensive instruments, complicated procedures, and/or radioactive probes. Considering these limitations, a rapid, simple, and highly sensitive method for detecting ET is needed.

Recently, a new kit, EXT-RPLA (Denka Seiken Co. Ltd., Niigata, Japan), for detecting and identifying serotypes ETA and ETB has been developed. This kit uses reversed passive latex agglutination. In this study, we tested clinical isolates of *S. aureus* for ET production using the EXT-RPLA and evaluated the sensitivity and specificity of this method in comparison with the results of a biologic assay and gene detection by PCR. Moreover, on the basis of the data obtained by the EXT-RPLA, we also investigated the prevalence of ET-producing strains among clinical isolates of *S. aureus*.

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MATERIALS AND METHODS

Bacterial strains. A total of 381 strains of *S. aureus* were used in this study. Of the 381 isolates, 292 were collected by a nationwide nosocomial surveillance system, consisting of the 43 national university hospitals throughout Japan, between January 1990 and December 1993. These included 190 isolates from purulent wound exudates, 33 from sputa, 14 from nasal swabs, 12 from otorrhea, 9 from throat swabs, 7 from blood cultures, 7 from skin, 6 from urine, 4 from perineal swabs, 2 from eye swabs, 1 from ascites, 1 from pleural fluid, and 6 from unknown sites. All of the strains were tested for susceptibility to methicillin or oxacillin before this study. The ratio of methicillin-sensitive *S. aureus* (MSSA)/methicillin-resistant *S. aureus* (MRSA) was 153/139. Another group consisted of 89 isolates from the skin of 89 patients with dermatologic diseases such as eczema, skin tumors, furuncle (acute deep folliculitis), tylosis, panaritium, atheroma, bullous impetigo, atopic dermatitis, contact dermatitis, burns, and tinea pedis. The latter group of isolates was collected from patients in seven hospitals in Aichi Prefecture between December 1994 and April 1995. The ratio of MSSA/MRSA was 75/14. These isolates were stored at −80°C until testing.

Assay for ET production. ET production was assayed according to the instruction manual for the EXT-RPLA. Each culture was plated on a brain heart infusion (BHI) agar plate. Since the *etb* gene often coexists with the cadmium resistance gene on a plasmid (5, 15), a BHI agar plate containing $Cd(NO_3)_2$ at a concentration of 30 μg/ml was concurrently used as a selective medium for *etb* gene-carrying strains. When colonies were found on both plates, a colony growing on the cadmium-containing plate was inoculated into BHI broth. If no colonies were found on the cadmium-containing plates, a colony on the BHI plate (not containing cadmium) was inoculated into broth. After centrifugation of the broth culture, serial 10-fold dilutions (up to 10^{-4}) of the supernatants were added to triplicate wells of a V-shaped-bottom microtiter plate. Twenty-five microliters of each of the anti-ETA, anti-ETB, and negative control latex reagents was added to the wells. After thorough mixing, the plates were incubated for 18 h at room temperature. ET production by each strain was judged according to the macroscopic observation of latex agglutination in the wells.

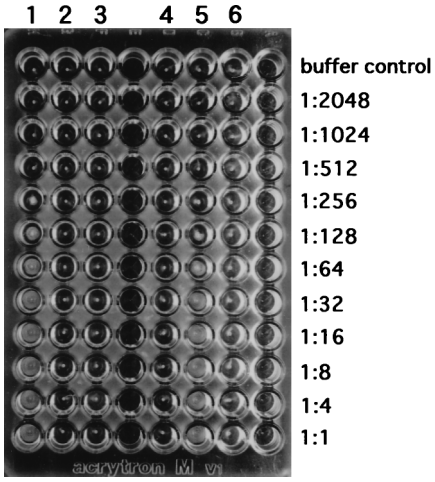

FIG. 1. Detection limits of the EXT-RPLA for purified ETA and ETB. Lane 1, various dilutions of purified ETA (100 ng/ml) and anti-ETA latex reagent; lane 2, ETA and anti-ETB latex; lane 3, ETA and negative control latex; lane 4, various dilutions of purified ETB (100 ng/ml) and anti-ETA latex reagent; lane 5, ETB and anti-ETB latex; lane 6, ETB and negative control latex. The latex agglutination (larger white-filled circle in the wells) is observed up to a 1:128 dilution of toxins in lane 1 and lane 5.

Biologic assay for ET. A 50-μl aliquot of culture supernatant was inoculated subcutaneously into a 1-day-old newborn mouse's back (ddY; Nihon SLC Co. Ltd., Hamamatsu, Japan). The skin of the mouse was gently rubbed at 6 h and the day after injection. If a positive Nikolsky's sign (extensive exfoliation of the skin) was observed, the isolate inoculated was judged to be an ET producer.

Nucleic acid isolation for the PCR method. *S. aureus* was pelleted from a culture by centrifugation at 15,850 × *g* for 5 min. The precipitated cells were washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA), incubated at 37°C for 1 h with 10 U of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) in 500 μl of TE buffer, and incubated at 55°C for 30 min with lysis solution (1% Tween 20, 1% Nonidet P-40). The DNA was subsequently extracted with phenol-chloroform and precipitated with ethanol. The DNA was dissolved in distilled H_2O and adjusted to a final concentration of 4 μg/ml.

PCR procedure for detection of the *eta* and *etb* genes. PCR was performed by a previously described method (6), with slight modifications. The PCR used 20 ng of the staphylococcal DNA and 50 pmol each of the sense and the antisense primers. Amplification was performed in a GeneAmp PCR System 9600R thermal cycler (Perkin-Elmer, Norwalk, Conn.) programmed for 30 cycles: 30 s (150 s for the first cycle) of denaturation at 94°C, 30 s of primer annealing at 57°C for the *eta* gene or 55°C for the *etb* gene, and 30 s of extension at 72°C. The amplification products were electrophoresed on a 2.0% agarose gel in TAE buffer (0.4 M Tris, 0.019 M acetic acid, 10 mM EDTA) and were visualized by staining with ethidium bromide.

Statistical analysis. The chi-square test with the Yates correction for continuity was used to test differences in the prevalence of ET-positive isolates among the MSSA and MRSA isolates and between isolates from various clinical specimens and those from the skin. Statistical analyses were performed by using Statview (version J-4.11; Abacus Concepts, Inc., Berkeley, Calif.).

RESULTS

Detection limits of the EXT-RPLA. Purified ETA and ETB were dissolved in phosphate-buffered saline containing 0.5% bovine serum albumin and were adjusted to a concentration of 100 ng/ml. Serial twofold dilutions of the preparations of 100 ng of ETA and ETB per ml were mixed and incubated with anti-ETA, anti-ETB, and negative control latex reagents in triplicate wells of V-shaped-bottom microtiter plates. Latex agglutination was seen up to 1:128 dilutions of both ETA and ETB. Neither nonspecific agglutination nor cross-reactivity was observed (Fig. 1). Thus, we determined that the lowest concentration of purified ET identified by the EXT-RPLA was approximately 1 ng/ml.

Reliability of the EXT-RPLA. Of the 381 *S. aureus* isolates tested, 19 were found to produce ET by the EXT-RPLA: 16 were ETA producers, 3 were ETB producers, and none was a double producer. To assess the reliability of the EXT-RPLA, all 19 ET producers and 53 randomly selected nonproducers, as determined by the EXT-RPLA, were examined for ET production by a biologic assay and for the *eta* and *etb* genes by PCR. Table 1 indicates that there was a complete concordance among the results obtained by the three methods. Both the sensitivity and specificity of the EXT-RPLA are 100%.

TABLE 1. Detection of ET-producing *S. aureus* isolates by the EXT-RPLA, bioassay, and PCR

Serotype of ET determined by EXT-RPLA (no. of strains)	No. of strains detected by the following method:				
	Bioassay		PCR		
	Positive	Negative	ETA	ETB	Negative
ETA (16)	16	0	16	0	0
ETB (3)	3	0	0	3	0
Non-ET producer (53)	0	53	0	0	53

Prevalence of ET determined by the EXT-RPLA. We investigated the prevalence of ET producers determined by the EXT-RPLA among *S. aureus* isolates, based on the source of isolation (Table 2) and susceptibility to methicillin (Table 3).

Of the 292 strains from various clinical specimens, 7 (2.4%) were ET-producing isolates. All seven of these strains were MSSA and produced serotype A. These seven ET-producing strains were isolated from five wound exudates, one otorrhea specimen, and one throat swab. Of the 89 strains isolated from the skin of dermatologic patients, 12 (13.5%) were ET positive: 9 for ETA and 3 for ETB. Isolates from the skin produced ET significantly more often than isolates from various other clinical specimens ($P < 0.0001$). Of the 12 ET-producing strains, 10 were MSSA and 2 were MRSA. These strains were isolated from 12 patients with the following diseases: 3 with bullous impetigo, 2 with atopic dermatitis, 2 with furuncle (acute deep folliculitis), and 1 each with atheroma, tylosis, panaritium, burn, and tinea pedis. With regard to susceptibility to methicillin, 17 (7.5%) of the 228 MSSA strains produced ET, while 2 (1.3%) of the 153 MRSA strains produced ET. MSSA isolates elaborated ET more frequently than MRSA ($P < 0.0138$).

The overall prevalence of ET producers was 5.0% (19 of 381), and ETA-producing strains (16 of 19; 84.2%) were much more frequently encountered than ETB-producing strains (3 of 19; 15.8%).

DISCUSSION

In this study, we demonstrated the excellent sensitivity of the EXT-RPLA for the detection of ET. The detection limit of the EXT-RPLA observed for both ETA and ETB was 1 ng/ml. Both the sensitivity and specificity of the EXT-RPLA were 100% when compared to the results of a biologic assay and gene detection by PCR.

Although the newborn mouse bioassay is a reference test, it

TABLE 2. ET serotypes and prevalence of ET producers according to the source of isolates

Source of isolates (no. of strains)	No. (%) of strains with the following toxin serotype:		
	ETA	ETB	Total
Various clinical specimens (292)	7 (2.4)	0	7 (2.4)
Skin swabs of dermatologic patients (89)	9 (10.1)	3 (3.4)	12 (13.5)

TABLE 3. ET serotypes and prevalence of ET producers according to susceptibilities to methicillin

Susceptibility to methicillin (no. of strains)	No. (%) of strains with the following toxin serotype:		
	ETA	ETB	Total
Sensitive (228)	15 (6.6)	2 (0.9)	17 (7.5)
Resistant (153)	1 (0.7)	1 (0.7)	2 (1.3)

is not easy to handle in clinical laboratories because it requires live newborn mice. Also, its sensitivity is 0.3 to 0.5 μg in 50 to 100 μl of test sample (2), and the assay does not allow for the identification of the two serotypes of ET. Among immunologic methods, electrosyneresis is well adapted to the epidemiologic study of ET-producing *S. aureus* isolates, since this method is rapid and can be used to test many isolates simultaneously. However, its sensitivity is relatively low (detection limit for ET protein, 5 μg/ml) (19). Another immunologic method, Ouchterlony immunodiffusion, is even less sensitive (50 to 60 μg/ml) (2, 13). Murono et al. (13) has reported a slide latex agglutination method also based on reversed passive latex agglutination, although the detection limit was 0.5 μg/ml. ELISA (16) and radioimmunoassay (26) for detecting ET have been shown to have high sensitivities. However, in the ELISAs, cross-reaction may occur between the ETB antigen and the ETA antibody preparation and between the ETA antigen and the ETB antibody preparation (16). Gene detection by molecular biology-based techniques is considered to be the most sensitive method. However, a positive result is indicative only of the presence of the targeted gene and does not indicate viability or the pathogenic toxic potential of the organism. Furthermore, molecular biology-based techniques require expensive instruments and reagents and/or radioactive probes.

Given these concerns, the EXT-RPLA may be a practical, sensitive, and reliable method which permits the rapid detection of ET in a few hundred samples at once by a simple procedure and which uses equipment that is standard for most laboratories. We therefore recommend this test kit for the rapid diagnosis of SSSS and for the detection of SSSS so that effective control measures against the spread of this syndrome can be taken, particularly in nurseries for neonates (18).

Epidemiologic investigations of ET-producing *S. aureus* strains in which clinical isolates are used have rarely been reported. In this study, 12 (13.5%) of the 89 dermatologic patients harbored ET-producing strains. This percentage was higher than that for the isolates from various clinical specimens. ET-producing *S. aureus* may easily colonize the skin of patients with dermatologic diseases. However, among the 12 patients, only 3 had bullous impetigo, and the other 9 had no clinical symptoms of SSSS. This may be related to the production of ET in vivo by *S. aureus* and the immune status of the host. Our data also demonstrate that MRSA elaborated ET less frequently than MSSA. Few investigators have reported a correlation between susceptibility to methicillin and ET production. Murono et al. (14) showed that among 61 ET-producing strains isolated from SSSS patients, no strains were resistant to methicillin. However, Richardson et al. (18) have recently reported an outbreak of SSSS caused by MRSA strains producing ETA in a nursery for newborns. Table 4 provides data from various countries on ET prevalence rates and the distribution of ET serotypes (1, 3, 4, 10, 13, 17). The ET positivity rate of 5% in our study was similar to those of other reports, except for the much higher percentages noted in two studies (3, 13) of isolates from SSSS patients. A preponderance of ETB-producing strains compared to those producing ETA or both ETA and ETB has been observed in Japan. This is in contrast to the situation for other countries, in which ETA producers are dominant. Our data for isolates in Japan suggest that ETA producers are much more common than ETB producers, as in other countries. Outbreaks of infection or colonization may affect the ET prevalence rates and the distributions of the two serotypes. Since we tested strains which had been collected from hospitals all over Japan, our data can be considered to be minimally affected by outbreaks. A previous report indicated that antibodies against ETA were present in 19% of the sera collected from 400 healthy Japanese, while antibodies against ETB were found in only 4% of the subjects (22). This suggests that people are much more frequently infected or colonized by ETA producers than by ETB producers in Japan. This may account for the preponderance of ETA producers in our study. On the contrary, we should bear in mind that the lower rate of ETB producers in our study and in other countries may be due to the spontaneous loss of the *etb* gene among the isolates tested. It is well documented that ETB production in *S. aureus* is unstable, since the plasmid carrying the *etb* gene is easy to eliminate by growth at high temperature or by treatment with ethidium bromide (20, 23, 24).

TABLE 4. Epidemiologic data for ET-producing *S. aureus* isolates from various countries

Country where strains were collected (no. of strains)	ET positivity rate (%)	Distribution of ET serotype (%)		
		ETA	ETB	ETA + ETB
Japan (418) (10)[a]	12.0	24	66	10
Japan (74)[b] (13)[a]	82.4	25	52	23
France (2,632) (17)[a]	6.2	88	4	8
Germany (944) (4)[a]	5.1	98[c]	0	0
Nigeria (194) (1)[a]	4.1	75	12.5	12.5
Britain and Ireland (116)[b] (3)[a]	70.0	46	16	38
Japan (current data) (381)	5.0	84	16	0

[a] The numbers in the second sets of parentheses are reference numbers.
[b] Study of isolates from SSSS patients.
[c] For the remaining 2% of isolates, serotypes were undetermined.

In summary, since the EXT-RPLA is reliable and requires neither complicated procedures nor specialized equipment, this method should be recommended for routine use in clinical laboratories. Moreover, this method may also be suitable for epidemiologic studies because of its capability to test many strains simultaneously. The epidemiologic status of ET-producing *S. aureus* strains has not been well studied. Further epidemiologic investigations by the EXT-RPLA with ET-producing *S. aureus* are needed.

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