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Establishing the Use of Real-time PCR for Staphylococcal Enterotoxin

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Characteristics of Staphylococci

Staphylococci are spherical or elliptical bacteria with diameters of 0.4 – 1.2 m (average 0.8 m). They tend to form grape-like clusters seen under the microscope during growth, and thus are called Staphylococci. They are non-motile, gram-positive bacteria that do not form spores, although prolonged culture may render them gram-negative. They are widely distributed in the air, soil, water, foods, and on utensils, skin, hands, in the nasopharyngeal region, and nostrils of healthy individuals. Staphylococci possess several distinct biological characteristics: 1. they can ferment several types of sugar with acids instead of gas as the end products; 2. they are resistant to heat and aridity, as they can survive in arid environments for several months, and can only be killed after 30 minutes at 80 degrees C.; 3. they are salt-resistant and can live in media containing 10 – 15%

salt; 4. they once were very sensitive to sulfa drugs, but several strains are now resistant. The virulence of Staphylococci is determined by their multiple virulence factors that belong to three types: 1. toxins, including cytotoxin (, , . , leucocidin, hemolysin, exfoliative toxins, and lethal toxin), Staphylococcal toxic shock syndrome (STSS) toxin, and enterotoxin A, B, C, D, and E; 2. enzymes, including thrombin, plasmin, heat-stable nuclease, hyaluronidase, and lipase; 3. others, including capsule and peptidoglycan. 50% of *Staphylococcus aureus* strains can produce enterotoxins, which are heat-stable and relatively resistant to intestinal enzymes, and can remain intact after boiling for 30 minutes. Heating up to 100 ℃ for 2 hours is required to destroy enterotoxins, and therefore food poisoning can still occur after cooking. Enterotoxins tend to be produced while growing on carbohydrates and proteins, and therefore tend to cause food poisoning. For Staphylococcal enterotoxins to cause food poisoning, four factors are required: 1. massive contamination of the food by enterotoxin-producing Staphylococci; 2. placement of contaminated foods at optimal temperature for bacterial growth; 3. adequate incubation period; 4. components and character of the foods are suitable for bacteria growth and toxin production. Incubation period is about 1 – 7 hours after ingestion of enterotoxins. The most common symptoms, which can occur for 1 – 2 days and then abate, include drooling, nausea, vomiting, abdominal cramping, and diarrhea[1,2,3].

Staphylococcal enterotoxins (SEs) are a group of proteins with similar structure and molecular weight (22 – 30 kDa). Their isoelectric points are about 6.8 – 8.6. They are heat and cold-resistant proteins, and can remain intact at 100 degrees C. for 20 minutes or at 4 degrees C. for 67 days. At -70 degrees C. they can be preserved for an extended period. The molecules are formed by multiple -sheets and -sheets. They also contain a disulfide bond and large amount of lysine, aspartic acid, and tyrosine. According to serological characters, they can be

separated into SEA to SEJ, and SEC is further separated into SEC1, SEC2, and SEC3 that have 92 % similarity in their amino acid sequence. SEF is currently called toxic shock syndrome toxin-1 (TSST - 1). Each type of SE can cause food poisoning, and 53 % of cases are caused by SEA, which is also the most toxic species with a minimal toxic dosage around 1g. SED accounts for 5 % of cases, and SEC, SEB, and SEE are responsible for the rest. Several strains can produce multiple SEs, and of these, 18 % can produce SEA and SED, and 9 % can produce SEC and SED[3,4,5,6,7].

According to the statistics of the Department of Health, *S. aureus* is ranked as the second most common cause of food poisoning, and the SE-producing ability of *S. aureus* is an important index of virulence. Although food poisoning is not a notifiable disease, it still leads to social panic besides affecting the health of the populace. Therefore, the CDC has performed surveillance for food poisoning for several years, and accordingly, we have tried to establish using a PCR method for SEs and a method for screening.

Materials and methods

Sources of strains: 32 strains of *S. aureus*, including 8 strains from ATCC (ATCC 13567, 8095, 13566, 14458, 13566, 19095, 23235, and 27664), 11 strains from BCRC (BCRC 13962, 12657, 13963, 13826, 13828, 12657, 12656, 10783, 10789, 12156, and 12655), and 13 strains from clinical samples isolated from cases of food poisoning between 2000 and 2004. Strains containing SEs to be tested were selected. Negative reference strains were non-SE-producing *S. aureus*, *S. epidermidis*, *S. hominis*, and *E. faecalis*. Frozen bacteria were first cultured on selective Baird-Parker media, and confirmed single colonies were cultured in TSA (Difco, USA) and BHI broth (Difco, USA) at 37℃. for 18 – 20 hours.

Enterotoxin testing: BHI broth cultured over-night was centrifuged at 3,000 rpm for 20 minutes and the supernatant was then subjected to testing. ET-RPLA (Staphylococcal Enterotoxin Reversed Passive Latex Agglutination, ABCD) and ENTERTOX-F (Staphylococcal Enterotoxin Reversed Passive Latex Agglutination, ABCDE) kits (Denka Seiken Co. Ltd., Tokyo, Japan) were used according to protocols provided. Diluted supernatant was reacted with antisera to SEA-SEE and latex reagents in 96-well plates, and results were recorded after overnight incubation. Positive SEA to SEE controls and negative ones were included in each testing.

DNA extraction: chromosomal DNA was extracted with QIAamp DNA mini kit (Qiagen, Hilden, Germany). Bacteria were lysed in 180 l ATL buffer with 20 l protease K, and incubated in a 56℃.water bath for 3 hours. DNA extraction was performed according to the protocol provided. DNA was finally dissolved in 150 l AE buffer.

Real-time PCR: primers and probes used in real-time PCR are listed in table 1. Total reaction volume is 25 l. Real-time PCR for SEA - D gene detection was performed with SYBR Green PCR master mix (Applied Biosystems, USA), 600 nM primers, and 10 l DNA templates. Reactions took place in an ABI PRISM 7,000 sequence detection system, and the parameters were: 50℃ for 2 minutes, 95℃ for 10 minutes, 45cycles at 95℃ for 15 seconds and 60℃ for 1 minute.

Sensitivity test: bacteria were diluted serially from $1.5x10^8$ CFU/ml (McFaland No. 0.5) to $1.5x10^1$ (15) CFU /ml. Duplicated PCR was performed to each dilution point.

Specificity test: each test strain, clinical strain, and control strain was cross-reacted with primers and probes for SEA – E. At least two cross-reactions were done to each strain.

Results

We performed this study according to the results proposed by Klotz (2003) and Becker (1998)[6,7]. As shown by our results, specific PCR amplification reaction could be demonstrated when the Ct cutoff value was set at 35. Therefore, this criteria was used to perform SEA –E gene testing. Results of real-time PCR amplification reaction are shown in Fig. 1. Genes of the reference strains, including the control strains, were cross-reacted at least twice with other primer pairs and probes, and the results indicated that our reaction condition, primers and probes could effectively detect SE genes (table 2). Our reaction parameters also effectively detect clinical strains (table 3).

The results of sensitivity testing showed that real-time PCR could effectively detect SE genes (Ct cutoff of 35) above 1500 CFU, and therefore, a single colony of bacteria is sufficient to perform a test. The results of specificity test showed that real-time PCR has a specificity of more than 90 % for SEs. Control strains showed negative results whatever primers and probes were used.

Traditional SE detection methods require at least 4 days discriminating SE production and types. Nevertheless, real-time PCR can detect SEs from a single colony within a single day after DNA extraction.

Conclusion

Of all the traditional methods of SE detection, RPLA is the most widely used. Nevertheless, it takes at least 4 days of time-consuming manipulation to detect toxin production and types of the toxins. Reagents for RPLA are also not easily accessible. On the contrary, results of real-time PCR can be obtained within a single day, compatible with the timeframe for clinical application. However, real-time PCR can only show the existence of SE genes, which does not

necessarily indicate SE production. This may be the reason why real-time PCR and RPLA have revealed different results concerning SEB and SED in 19 reference strains.

According to the statistics of the Department of Health, an average of 105 outbreaks of food poisoning occurred each year between 1995 and 2001. On average, 1,440 individuals were affected each year. *S. aureus* is the second cause of food poisoning, and accounts for 26 outbreaks per year[8]. Accordingly, establishing a real-time RT-PCR method for SEs is necessary for disease prevention. The mission of the CDC is to rapidly terminate the spread of outbreaks and to protect the health of the population. Strengthening detection ability and application ranges are therefore necessary in order to effectively control outbreaks.

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Table 1. Primers and Taqman probes [a] used in real-time PCR for enterotoxins of *Staphylococcus aureus*

Gene	Primer or probe	Sequence （5'-3'）	Product size （bp）
SEA	SEA-F	AAAATACAGTACCTTTGGAAACGGTT	92
	SEA-R	TTTCCTGTAAATAACGTCTTGCTTGA	
	Probe	FAM-AACGAATAAGAAAAAATGTAACTGTTCAGGAGTTGGATC	
SEB	SEB-F	ACACCCAACGTTTTAGCAGAGAG	81
	SEB-R	CCATCAAACCAGTGAATTTACTCG	
	Probe	FAM-CAACCAGATCCTAAACCAGATGAGTTGCACA	
SEC1	SEC-F	AATAAAACGGTTGATTCTAAAAGTGTGAA	80
	SEC-R	ATCAAAATCGGATTAACATTATCCATTC	
	Probe	FAM-TAGAAGTCCACCTTACAACAA	
SED	SED-F	TGATTCTTCTGATGGGTCTAAAGTCTC	115
	SED-R	GAAGGTGCTCTGTGGATAATGTTTT	
	Probe	FAM-TATGATTTATTTGATGTTAAGGGTGATTTTCCCGAA	
SEE	SEE-F	CAGTACCTATAGATAAAGTTAAAACAAGC	178
	SEE-R	TAACTTACCGTGGACCCTTC	

[a] SEA-D [6] ；SEE [7]

Table 2. Results of detection of *Staphylococcus aureus* reference strains by RPLA and real-time PCR

Toxin phenotype	Reference strains	No. of strains	No. of positive strains detected by：		
			SET-PRLA	ENTERTOX-F	Real-time PCR
SEA	ATCC:13567,8095,13566 BCRC:13962,12657	5	5	5	5
SEB	ATCC:14458,13566 BCRC:13963	3	3	3	5
SEC1	ATCC:19095 BCRC:13826,13828	3	3	3	3
SED	ATCC:23235 BCRC:12657	2	2	2	3
SEE	ATCC:27664 BCRC:12656	2	- [#]	2	2
Nonproducer*	BCRC:10783,10789,12156 12655	4	0	0	0

* non-toxigenic S. aureus, S. epidermidis, S. hominis ,and E. faecalis

[#] SET-RPLA detects SEA-D

Table 3. Results of detection of *Staphylococcus aureus* clinical strains by RPLA and real-time PCR

Toxin phenotype	No. of strains	No. of positive strains detected by：		
		SET-PRLA	ENTERTOX-F	Real-time PCR
SEA	4	4	4	4
SEB	3	3	3	3
SEC1	3	3	3	3
SED	1	1	1	1
SEE	0	-[#]	0	0
Nonproducer*	2	0	0	0

* clinical isolates of non-toxigenic *S. aureus*, and *S. epidermidis*
[#] SET-RPLA detects SEA-D

Figure 1. Results of Staphylococcal enterotoxin (SE) gene detection by Real-time PCR