

Rapid detection and differentiation of the exfoliative toxin A-producing *Staphylococcus aureus* strains based on ϕ ETA prophage polymorphisms

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Abstract

The exfoliative toxin A (ETA) is encoded by the gene located on *Staphylococcus aureus* prophages. We have developed a single-reaction multiplex polymerase chain reaction (PCR) assay for rapid and specific detection of various ϕ ETA prophages of serogroup B responsible for dissemination of *eta* gene and ETA production in clinical strains. This PCR strategy enabled to classify the ETA-positive strains into 6 groups designated ETA-B1, ETA-B2, ETA-B3, ETA-B4, ETA-B5, and ETA-B6. The method was tested on a diverse set of 101 ETA and/or ETB-positive *S. aureus* strains isolated in 22 Czech maternity hospitals and 1 Slovak maternity hospital between 1998 and 2009. This novel PCR strategy is reliable in the rapid identification of yet undescribed ETA-converting B prophages and differentiation of the closely related ETA-positive strains, and it is a convenient tool for hospital epidermolytic infection control.

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1. Introduction

Exfoliative toxin (ET) produced by *Staphylococcus aureus* strains is the major causative agent of blistering skin disorders, the most severe of them being staphylococcal scalded skin syndrome (SSSS) that primarily affects neonates and young children (Ladhani et al., 1999; Plano, 2004). Serologically, this toxin isolated from human strains has been divided into 3 isoforms, the most frequently occurring ETA and ETB toxins (Kondo et al., 1975; Růžicková et al., 2003) and much less frequently occurring ETD toxin (Yamaguchi et al., 2002). A fourth ETC was isolated from a horse strain of *S. aureus* (Sato et al., 1994); however, it has not been associated with human disease. The *eta* gene encoding ETA is located on a prophage that is integrated into the *S. aureus* chromosome. To date, a few

ETA phages have been induced from bacterial cells and found capable of transferring the *eta* gene into a prophage-less strain (Endo et al., 2003; Yamaguchi et al., 2000; Yoshizawa et al., 2000).

Between 1998 and 1999, the ETA-producing *S. aureus* strains responsible for 2 outbreaks of pemphigus neonatorum in the Czech maternity hospitals were genotyped and examined for the content of prophages of serogroups A, B, and F. Analysis of the prophage carriage revealed the presence of at least 1 prophage in all strains (Růžicková et al., 2003). Prophages of serogroup B significantly predominated over the other prophages. Identification of several monolysogenic ETA-positive strains harboring single B prophage indicates that the *eta* gene is carried by a B phage. Because closely related strains can contain different ETA-converting prophages, their detection and characterization is very important for diagnostics of the strains responsible for outbreaks of SSSS or other forms of toxic epidermolytic diseases.

There have been several reports describing the use of molecular typing methods for characterization of ET-

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positive *S. aureus* strains (Dave et al., 1994; Mackenzie et al., 1995; Růžicková et al., 2003; Saiman et al., 1998) and polymerase chain reaction (PCR) for detection of 3 ET genes (Mehrotra et al., 2000; Růžicková et al., 2005). PCRs have been used to determine the genetic changes of strains lysogenized by ETA-converting phages (Endo et al., 2003; Yamaguchi et al., 2000), but rapid PCR genotyping of strains based on detection of the different *eta* gene-positive B prophages has not yet been described.

In this article, we report the novel multiplex PCR assay for detection of the 6 types of B prophages carrying the *eta* gene. The ability of this PCR assay was verified on a set of 101 ET-producing clinical *S. aureus* strains, which were isolated in 22 Czech maternity hospitals and 1 Slovak maternity hospital. Our objective was to make up a multiplex PCR method allowing a rapid genotyping of *S. aureus* strains containing different ETA-converting prophages.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A collection of 101 ET-positive *S. aureus* strains (72 isolates from skin blisters, 3 from stool of neonatal patients; 5 from expectant mothers; 14 of nurse hands; 7 from hospital equipment) were isolated in the 23 maternity hospitals in which cases of mass pemphigus neonatorum have occurred. These strains were collected by the National Reference Laboratory for Staphylococci, Prague, Czech Republic, in which they had been previously defined by virtue of their respective ET production by Reverse Passive Latex Agglutination Kit (Denka Seiken for Unipath, Tokyo, Japan). Seventy strains were ETA producers, 19 produced both ETA and ETB, and 12 of the strains synthesized only ETB. The following reference *S. aureus* strains were used: ETA-producing strains CCM 2330 (prophage ETA-B4 positive) and CCM 7057 (ETA-B1 positive), ETA- and ETB-producing strain CCM 7056 (ETA-B1 positive), and ETA- and ETD-producing strain CCM 2331 (ETA-B6 positive). The strains *S. aureus* NCTC 8325 and the prophageless *S. aureus* 1039 were used as the *eta* gene-negative controls. All the reference CCM strains were obtained from the Czech Collection of Microorganisms, Brno, Czech Republic. The strain NCTC 8325 was obtained from Prof P.A. Pattee, of the Iowa State University in Ames, IA, and the strain *S. aureus* 1039 from Dr Y. Yoshizawa (Jikei University School of Medicine, Tokyo, Japan). A collection of 30 methicillin-resistant *S. aureus* strains (all containing the *eta* gene-negative prophage of serogroup B) that emerged as dangerous nosocomial pathogens in high numbers at the burn department in a teaching hospital in Brno were examined. In addition, 5 previously genotyped monolysogenic (*seh* gene-positive) strains containing a prophage of serogroup B (Růžicková et al., 2008) were used.

For nucleic acid isolation, all the strains were subcultured in brain heart infusion broth (HiMedia, Mumbai, India) and incubated overnight with shaking at 37 °C.

2.2. DNA isolation from culture samples

DNA for PCRs was obtained from lysostaphin-treated cells, formerly pelleted from a 6-mL overnight culture with 10^8 to 10^9 CFU in 1 mL by centrifugation for 15 min at $8000 \times g$, and purified with the High Pure PCR Template Preparation Kit (Roche Diagnostics, Berlin, Germany), which was used according to the manufacturer's instructions.

2.3. Pulsed field gel electrophoresis

Genomic DNA was prepared as described previously (Pantůček et al., 1996). *Sma*I macrorestriction fragments were separated with a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA) under the following conditions: 1.2% agarose in $1 \times$ Tris-acetate-EDTA (TAE) electrophoresis buffer (0.04 mol/L Tris/acetate, 0.001 mol/L EDTA, pH 8.2) at 14 °C, 6 V cm^{-1} , pulse times of 1 to 55 s for 24 h. Pulsed field gel electrophoresis (PFGE) types were determined according to Růžicková et al. (2003).

2.4. PCR experiments

2.4.1. Lysogenic typing

Multiplex PCR detection of prophage content of serogroups A, B, Fa, and Fb in the strains under study was performed as described by Pantůček et al. (2004).

2.4.2. Primer design and multiplex PCR assay

Specific PCR primers ETA-66F and ETA-66R for the *eta* gene were derived from the sequence of phage ϕ ETA reported by Yamaguchi et al. (2000). Primers SAU1 and SAU2, to detect 217-bp *S. aureus* species-specific sequence derived from the methyltransferase gene (GenBank accession no. AJ132803), were reported previously (Pantůček et al., 2004; Štěpán et al., 2001). They were added to the multiplex PCR as an internal control. The novel 9 specific primers targeting the variable genomic regions of *eta* gene-positive B prophages (Table 1) were designed based on multiple alignment of entire genomic sequences of the following *eta* gene-positive phages: ϕ ETA (GenBank accession no. AP001553), ϕ ETA2 (AP008953), and the ϕ ETA3 (AP008954) showing sequence similarity from 46% to 63% (<http://www.ncbi.nlm.nih.gov/genomes/>). The conditions of the reactions were optimized according to Henegariu et al. (1997).

PCR amplification was performed as follows: 3 μL of DNA (50–100 ng) sample was added to 22 μL of PCR mixture consisting of 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 4 mmol/L MgCl_2 , and 200 $\mu\text{mol/L}$ each of dNTPs, 1 $\mu\text{mol/L}$ each of ETA1-02 and ETA1-06, 0.8 $\mu\text{mol/L}$ each of ETA3xis-1 and ETA3xis-2, 0.6 $\mu\text{mol/L}$ each of ETA2-11, ETA2-14, ETA3-14, ETA66F, and ETA66R, 0.2 $\mu\text{mol/L}$ each of SAU-1 and SAU-2 primers, and 1.25 U of *Taq* DNA polymerase (Invitrogen Life Technologies,

Table 1
Primers used for multiplex PCR assay

Primer name	Nucleotide sequence (5' → 3')	Location	Primer specificity	Product size (bp)	Amplicon specificity for the prophage types
ETA1-02	AAATTAGGTCTAGCTGCGTCAGT	1488–1501 ^a	ϕETA: <i>orf02</i> – <i>orf06</i>	1200	ETA-B1
ETA1-06	TAATGTCAATGGTTGCGTCTCT	2666–2688 ^a			
ETA2-14	CGTATTCTTCATCGCAGACA	6855–6876 ^b			
ETA3-14	TCTGATGCCATATCTAAGTC	7092–7111 ^a ; 6948–6967 ^c	ϕETA: <i>orf13</i> – <i>orf15</i>	537	ETA-B1, ETA-B4, ETA-B5
			ϕETA3: <i>orf11</i> – <i>orf14</i>	844	ETA-B3
ETA2-11	GGATACATTGCGATACGAGGCAG	6575–6597 ^a ; 6124–6147 ^{b,c}	ϕETA: <i>orf66</i> ^d	425	ETA-B1, ETA-B2, ETA-B3, ETA-B4, ETA-B5, ETA-B6
ETA-66F	ATACAGATGATAACGGTAATACT	42264–42286 ^a			
ETA-66R	ATAATTCCCAATACCAACAC	42669–42689 ^a			
ETA3xis-1	ACGTTTGTATTACCGTATA	1022–1041 ^c	ϕETA3: <i>orf01</i> – <i>orf02</i>	380	ETA-B2, ETA-B3, ETA-B4
ETA3xis-2	GACGAAATAGCTAAGCTGT	1383–1402 ^c			
SAU1	GACGGCTTTGATGGCTAGTGG	460940–460960 ^e	<i>S. aureus</i> ^f	217	All <i>S. aureus</i> isolates
SAU2	AGTTAATTCACGCCCTAGTG	461137–461156 ^e			

^a Location of primer sequence within ϕETA genome using the nucleotide numbering indicated in GenBank accession no. AP001553.

^b Location of primer sequence within genome of ϕETA2 using the nucleotide numbering indicated in GenBank accession no. AP008953.

^c Location of primer sequence within genome of ϕETA3 using the nucleotide numbering indicated in GenBank accession no. AP008954.

^d The *orf66* of ϕETA containing the *eta* gene sequence.

^e Location of primer sequence within NCTC 8325 genome using the nucleotide numbering indicated in GenBank accession no. NC_007795.

^f *S. aureus* internal control.

Carlsbad, CA). Thermal cycling parameters consisting of an initial denaturation step (3 min, 94 °C), 30 cycles of amplification including denaturation (45 s, 94 °C), annealing for 60 s at 56 °C, DNA chain extension for 90 s at 72 °C; and a final extension at 72 °C for 7 min were performed in a DNA thermal cycler (model Tgradient, Biometra, Germany).

After amplification, 10 µL of PCR samples was mixed with 3 µL of loading buffer (10% wt/vol Ficoll 400, 10 mmol/L Tris–HCl, pH 7.5, 50 mmol/L EDTA, and 0.25% bromophenol blue) and electrophoresed in a 2.5% (wt/vol) agarose gel (SERVA Electrophoresis, Heidelberg, Germany)

for 3.5 h at 5 V cm⁻¹ in 1 × TAE buffer (0.04 mol/L Tris–acetate, 1 mmol/L EDTA). Ethidium bromide (0.5 µg mL⁻¹ of TAE)-stained DNA amplicons were then visualized on a ultraviolet transilluminator at 302 nm. A 100-bp molecular weight marker (New England Biolabs, Ipswich, MA) was used to estimate the size of the PCR products. The prophage types were proposed according to 6 patterns of PCR products as shown in the Table 1 and Fig. 1.

3. Results and discussion

One hundred one clinical ETA- and ETB-positive isolates of *S. aureus* (of which 12 were only ETB producers) and the 6 reference strains were tested for presence of the *eta* gene encoding the ETA. Eighty-nine ETA-positive strains of which 72 isolates were the causative agents of skin blistering epidermolysis in neonates harbored *eta* gene-positive prophage of serogroup B.

We developed a novel multiplex PCR assay for detection of the DNA sequences unique to the *eta* gene-positive B prophages harbored by *S. aureus* strains. First of all, specificity of the primer pairs was verified on genomic sequences of phages ϕETA, ϕETA2, and ϕETA3 in silico and in single PCR reactions in the 4 reference ETA-positive strains: CCM 2330, CCM 2331, CCM 7056, and CCM 7057.

PCR primers designed for detection of the 6 various types of staphylococcal *eta* gene-positive B prophages were used in multiplex PCR assay to obtain amplicons differing in size and characteristic for each of the prophage types (Table 1; Fig. 1). Primers ETA1-02 and ETA1-06 were designed to amplify the 1200-bp sequence specific for the genomic

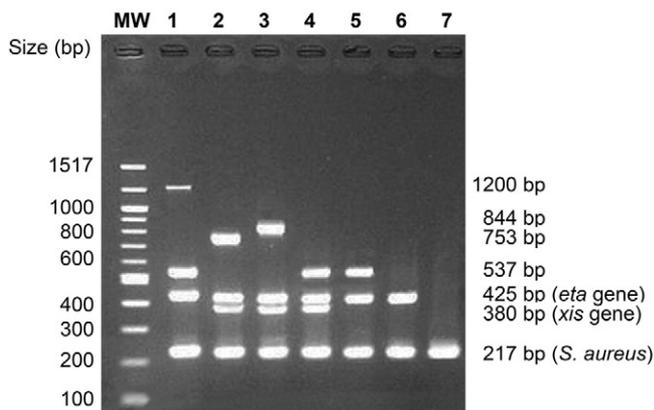


Fig. 1. Agarose gel electrophoresis of the multiplex PCR amplification products obtained from analysis of tested strains. MW = molecular weight marker 100 bp ladder (New England Biolabs). 217-bp amplicon represents species-specific sequence Sa 2052 in all *S. aureus* strains. Lane 1, ETA-B1; lane 2, ETA-B2; lane 3, ETA-B3; lane 4, ETA-B4; lane 5, ETA-B5; lane 6, ETA-B6 prophage type, respectively; lane 7, *S. aureus* 1039 (non-ETA producer). Amplicon specificity for the prophage types is shown in Table 1.

region containing 5 ORFs (the ORF2, ORF3, ORF4, and ORF5 encoding the hypothetic protein of unknown function and ORF6 encoding a repressor) of phage ϕ ETA (Yamaguchi et al., 2000). Primers ETA2-11 and ETA2-14 identified the 753-bp sequence of the ORF11, ORF12, ORF13, and ORF14 present in module for replication of the ϕ ETA2 (GenBank accession no. AP008953). Primers ETA2-11 and ETA3-14 amplified the respective 844-bp sequence of genomic DNA, corresponding to ORF11, ORF12, ORF13, and ORF14, located within replication module of ϕ ETA3 (AP008954), as well as the 537-bp sequences of the ORF13, ORF14, and ORF15 that occurred on the ϕ ETA. The 537-bp sequence was amplified in all strains carrying the B prophages of types ETA-B1, ETA-B4, and ETA-B5. The pair of primers ETA3xis-1 and ETA3xis-2, which was designed to amplify the 380-bp sequence located within a part of ORF1 and ORF2 of the ϕ ETA3, detected the genes for integrase (*int*) and excisionase (*xis*), respectively. This DNA amplicon was common to B prophages of types ETA-B2, ETA-B3, and ETA-B4.

The obtained PCR band patterns (Fig. 1) enabled to distinguish 6 B prophage types, and the ETA-positive *S. aureus* strains could be classified into 6 different groups (Table 2). PCR analysis of DNA from all strains known to produce ETA showed 2 amplicons: one specific for *S. aureus* (217 bp), and the second 425-bp band indicated the *eta* gene. The strains yielding additional PCR products of 1200 and 537 bp in size were classified into the ETA-B1 group and the strains showing the 753-bp together with 380-bp amplicon into the ETA-B2 group. The 844- and 380-bp PCR products were detected in the strains of the ETA-B3 group, and the 537- and 380-bp amplicons occurred in the strains assigned to ETA-B4 group. Strains generating the 537-bp PCR products were classified into the ETA-B5 group. Strains yielding only the 425- and 217-bp amplification products were affiliated with the ETA-B6 group. These results illustrate that more than 1 type of the *eta* gene-positive B prophages can be detected specifically in 1 reaction without any cross-reaction between primers. They also indicate that yet undescribed prophages, other than those of ETA-B1, ETA-B2, ETA-B3, ETA-B4, ETA-B5 and ETA-B6 types, can occur in clinical ETA-producing strains.

PCR assay results indicated that the ETA-B2 and ETA-B6 prophage types were closely associated with a single genotype. Genotyping based on PFGE and prophage content revealed that among the 89 ETA-positive strains tested in this study, the most frequently found were the following genotypes: G-7 and G-9 (20% and 18%, respectively) followed by G-1 (12%), G-4 (10%), and G-6 (8% strains). Despite the similarity in the *Sma*I macrorestriction patterns and prophage content, each of the ETA-producing strains examined was simply identified by its ETA-B-PCR profile.

As can be seen from Table 2, the strains of predominating genotype G-7 harbored prophages of types either ETA-B3 or ETA-B4, the strains of genotype G-6 contained prophages either ETA-B1 or ETA-B4, the strains of genotype G-9

Table 2

Analysis of B prophage type occurrence in ETA-positive *S. aureus* strains recovered in 20 Czech maternity hospitals and 1 Slovak maternity hospital

ETA-B prophage types	Genotype ^a	PFGE type ^b	Prophage carriage	Hospital/year of isolation (no. of strains)			
ETA-B1	G-1	ET1a	ABFb	Hosp 1/1998 (7)			
				Hosp 4/1999 (1)			
				Hosp 5/2001 (1); 2008 (1)			
ETA-B2	G-2	ET1b	ABFb	Hosp 7/2002 (1)			
				Hosp 8/2003 (1)			
				Hosp 1/1998 (1)			
ETA-B3	G-6	ET2	B	Hosp 8/2004 (1)			
				Hosp 2/1998 (5)			
				Hosp 15/2007 (5)			
ETA-B4	G-11	ET5	BFb	Hosp 21/2003 (5); 2006 (3); 2007 (2)			
				G-7	ET2	AB	Hosp 11/2003 (7)
							Hosp 12/2002 (4)
Hosp 9/2002 (3)							
ETA-B5	G-10	ET4	ABFa	Hosp 6/2001 (5)			
				G-7	ET2	AB	Hosp 8/2008 (1)
							Hosp 19/2008 (1)
Hosp 10/2008 (2)							
ETA-B6	G-10	ET4	ABFa	Hosp 3/1998 (1)			
				G-9	ET4	BFa	Hosp 1/1998 (1)
							Hosp 3/1998 (1)
ETA-B5	G-8	ET4	B				Hosp 14/2007 (1)
				G-3	ET1c	BFb	Hosp 7/2001 (1)
							Hosp 8/2001 (1)
Hosp 13/2004 (2)							
ETA-B6	G-4	ET1d	BFb	Hosp 14/2007 (4)			
				G-5	ET1f	BFb	Hosp 16/2008 (1)
							Hosp 20/2007 (2)
Hosp 17/2006 (2); 2007 (1)							
ETA-B6	G-9	ET4	BFa	Hosp 9/2003 (5); 2004 (2)			
				G-9	ET4	BFa	Hosp 18/2008 (2); 2009 (5)

^a Genotype based on PFGE and content of the prophages of serogroup A, B, Fa, and Fb.

^b The macrorestriction patterns of the PFGE types are shown in the dendrogram reported by Růžičková et al. (2003).

included ETA-B4 or ETA-B6 prophages, and the strains of genotype G-10 carried prophages of either ETA-B3 or ETA-B4 types. Analysis of the geographic distribution of genotyped *S. aureus* isolates revealed that the strains of genotype G-1, harboring prophages of type ETA-B1, occurred in 5 distant hospitals between 1998 and 2003. The strains containing prophages of type ETA-B4 were isolated in 7 hospitals. However, the strains classified into the groups ETA-B2 and ETA-B6 occurred only in 2 hospitals.

In addition to ETA-producing *S. aureus* strains containing the B prophage, we have analyzed several *eta* gene-negative strains (harboring B prophages) originated from other sources than impetigo to verify the specificity of developed PCR strategy. Amplification of DNA from the screened 30 methicillin-resistant *S. aureus* and 5 monolysogenic *seh* gene-positive strains (isolates from foodstuffs) resulted in production of the 217-bp PCR band typically indicative of *S. aureus* DNA Sa2052 sequence, but they did not produce any of the amplicons detected in the *eta* gene-positive B

prophages. No PCR product corresponding to B prophage sequences was obtained with the 12 ETB-producing strains harboring a prophage of serogroup F.

Bioinformatic analysis of genome sequenced *S. aureus* strains reveals that *eta* gene-negative prophages JH1, JH9, Mu3, Mu50, and phiPV83 (<http://www.ncbi.nlm.nih.gov/genomes/>) contain the 844-bp sequence, and genome of the sequenced phages $\phi 80\alpha$, $\phi 69$, and $\phi 11$ carry the 380-bp sequence. However, both the sequences corresponding to the PCR products obtained in the multiplex PCR (either 844-bp of ETA-B3 or 380-bp of ETA-B2, ETA-B3, and ETA-B4 prophage types) do not interfere with the developed multiplex PCR strategy in any case.

Having tested the developed PCR on a wide range of isolates from different localities and sources, we can recommend its use as a confirmatory test for the presence of ETA-converting B prophages. Our results indicate that the specific sequences localized in the lysogeny and replication modules of the different *eta* gene carrying prophages raise the possibility of establishment of a multiplex PCR assay. Polymorphisms of ETA phage sequences used for the development of a novel PCR assay enhanced the differentiation of ETA-producing *S. aureus* isolates. In combination with the simple DNA extraction procedure, the application of the single multiplex PCR, used in this work, should enable more samples to be simultaneously characterized in detail. In comparison with procedure based on PCR detection of the *eta*, *etb*, and *etd* genes previously reported by Růžičková et al. (2005) which enables only identification of the ET-encoding genes, this new approach offers classification of the closely related ETA-producing *S. aureus* strains.

This work has produced a multiplex PCR system that reliably and rapidly detects virulent *S. aureus* strains carrying different ETA-converting B prophages, which are the major mediators of the *eta* gene transfer between staphylococci. These features allow this procedure to be applied in clinical settings for hospital epidermolytic infection control. This efficient method could also facilitate the identification of additional yet undescribed ETA-converting phages.

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