BACTERIAL IDENTIFICATION USING *ssrA* ENCODING TRANSFER-MESSENGER RNA

Kayo Osawa[1,2], Katsumi Shigemura[2,3], Hiroki Shirai[1], Ayaka Kato[1], Yuma Okuya[1], Takumi Jikimoto[2], Soichi Arakawa[2,3], Masato Fujisawa[3] and Toshiro Shirakawa[1,3,4]

[1]Division of Infectious Diseases, Department of International Health, Kobe University Graduate School of Health Science; [2]Department of Infection Control and Prevention, Kobe University Hospital; [3]Department of Urology, Faculty of Medicine, Kobe University Graduate School of Medicine; [4]Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan

Abstract. Ribosomal DNA (rDNA) sequences are widely used for phylogenetic and bacterial identification. However, rDNA of different species often reveals similar or identical same sequences. This study employed the bacterial stable small RNA (*ssrA*) gene encoding transfer-messenger RNA (tmRNA) as a tool for identification of *Staphylococcus aureus*, *Enterococcus* spp, *Pseudomonas* spp and Enterobacteriaceae from clinical isolates as representative groups using PCR and species specific primers. The method correctly identified 11 standard strains and 99 clinical isolates. Quantitative PCR revealed a limit of detection of 10^{-5}μg of DNA for *S. aureus* and *Enterococcus* spp, and 10^{-6}μg for *Pseudomonas* spp and Enterobacteriaceae. Further studies with a greater number of bacteria especially from clinical samples will need to be undertaken before this bacterial molecular marker can be applied in a clinical setting.

Keywords: bacterial identification, *ssrA*, tmRNA gene, quantitative PCR

INTRODUCTION

Culture tests are in general the gold standard to identify causative bacteria, but this procedure is time-consuming and laborious (Buijtels *et al*, 2008) and there are some bacteria, such as *Mycobacterium leprae*, which are not easy to be cultured. As regards treatment, empirical antibiotic therapy prior to acquisition of culture results may result in possible use of unnecessary or inappropriate antibiotics and thereby leading to emergence or spread of antibiotic-resistant strains (Kollef, 2006). In this situation, molecular analysis based on nucleotide sequence of ribosomal DNA (rDNA), such as the 16S rDNA and 23S rDNA, has become a widely used method for identification of specific bacterial species (Woese, 1987; Amann *et al*, 1997; Ludwig and Schleifer, 1999).

However, the 16S rDNA sequences of closely related strains, subspecies or even different species, are often identical and therefore PCR primers used for amplifying 16S rDNA sequences spe-

Correspondence: Dr Katsumi Shigemura, Department of Urology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan.
Tel: +81 78 382 6155; Fax: +81 78 382 6169
E-mail: yutoshunta@hotmail.co.jp

cific to these bacteria cannot be used (Schönhuber *et al*, 2001). On the other hand, transfer-messenger RNA (tmRNA) gene, encoding both tRNA and mRNA, is widely distributed among eubacteria, and contains highly divergent sequences (Schönhuber *et al*, 2001; Takahashi *et al*, 2003; Hudson and Williams, 2015). The transcript from this gene (*ssrA*) plays at least two physiological roles in bacteria: 1) removing ribosomes, which have stalled on mRNAs, and 2) tagging the resulting truncated proteins for degradation (Corvaisier *et al*, 2003). Another advantage of using *ssrA* over 16S rDNA as molecular markers is that there is only about 350 bp in the former gene compared to 1,500 bp in the latter (Schönhuber *et al*, 2001; Dewhirst *et al*, 2012), thereby requiring shorter operating times for gel-electrophoresis or quantitative (q)PCR. The *ssrA* has been used in the identification of gram-positive bacteria (genera *Bacillus, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Staphylococcus*), *Listeria* (in food using qPCR) and *Caulobacter* spp (Keiler *et al*, 2000; Schönhuber *et al*, 2001; O'Grady *et al*, 2008).

In this study, we focused on using PCR amplification of species specific *ssrA* to identify gram-negative and gram-positive bacteria in clinical samples.

MATERIALS AND METHODS

Bacterial strains and clinical isolates

A total of 11 reference strains (*Staphylococcus aureus* ATCC 25923 and ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 49614, *Enterococcus faecalis* ATCC 33186, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp *enterica* serovar Typhimurium ATCC 14208, *Proteus vulgaris* ATCC 13315, and *Serratia marcescens* ATCC 8100, obtained from the American Type Culture Collection) and 99 clinical isolates obtained from such sources as urine and feces (provided by Kobe University Hospital, Kobe, Japan) were used in the study, even though we have no available data for exact distribution in the current situation (Table 1).

PCR assay

DNA extraction of strains was performed using Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, Tokyo, Japan) following an overnight culture in heart infusion broth (Eiken Chemical, Tokyo, Japan) according to the manufacturer's protocol. *S. aureus* ATCC 25923, *E. faecalis* ATCC 33186, *P. aeruginosa* ATCC 27835 and *E. coli* ATCC 25922 were used as quality controls. The primers used and amplicon sizes are listed in Table 2. PCR, conducted in a 25-μl volume, contained 5 μg of DNA, 0.625 U Takara Ex Taq™ (Takara Bio, Ohtsu, Japan), 1X Ex Taq buffer (Takara Bio), 0.2 mM dNTP mixture and 0.2 μM primer pair (Invitrogen, Carlsbad, CA). Thermocycling (conducted in TaKaRa PCR Thermal Cycler PERSONAL, Takara Bio) conditions were as follows: 94°C for 4 minutes; followed by 30 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 3 minutes. Amplicons were separated by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV light. Amplification of 16S or 23S rDNA were performed using 4 primer pairs targeting *Staphylococcus* 16S rDNA, *Enterococcus* 16S rDNA, *Pseudomonas* 16S rDNA and Enterobacteriaceae 23S rDNA as previously described (Matsuda *et al*, 2007).

qPCR assay

qPCR was performed to determine the sensitivity of the PCR assay. One μg

Table 1
Detection of bacteria by species specific *ssrA* amplification.

Bacterial strain		Number of *ssrA*-positive samples/total number of samples			
		S. aureus	*Enterococcus*	*Pseudomonas*	Enterobacteriacae
Staphylococcus aureus	ATCC 25923	(1/1)	(0/1)	(0/1)	(0/1)
	ATCC 29213	(1/1)	(0/1)	(0/1	(0/1)
	clinical strains (17 strains)	(17/17)	(0/17)	(0/17)	(0/17)
Staphylococcus epidermidis	ATCC 12228	(0/1)	(0/1)	(0/1)	(0/1)
	clinical strains (4 strains)	(0/4)	(0/4)	(0/4)	(0/4)
Staphylococcus lugdunensus	clinical strain (1 strain)	(0/1)	(0/1)	(0/1)	(0/1)
Streptococcus agalactiae	clinical strain (1 strain)	(0/1)	(0/1)	(0/1)	(0/1)
Streptococcus pyogenes	ATCC 49614	(0/1)	(0/1)	(0/1)	(0/1)
	clinical strains (2 strains)	(0/2)	(0/2)	(0/2)	(0/2)
Streptococcus pneumoniae	clinical strains (2 strains)	(0/2)	(0/2)	(0/2)	(0/2)
Enterococcus faecalis	ATCC 33186	(0/1)	(1/1)	(0/1)	(0/1)
	clinical strains (19 strains)	(0/19)	(19/19)	(0/19)	(0/19)
Enterococcus faecium	clinical strains (3 strains)	(0/3)	(3/3)	(0/3)	(0/3)
Bacillus subtilis	ATCC 6633	(0/1)	(0/1)	(0/1)	(0/1)
Bacillus cereus	clinical strain (1 strain)	(0/1)	(0/1)	(0/1)	(0/1)
Moraxella catarrhalis	clinical strain (1 strain)	(0/1)	(0/1)	(0/1)	(0/1)
Haemophilus influenzae	clinical strain (1 strain)	(0/1)	(0/1)	(0/1)	(0/1)
Acinetobacter baumannii	clinical strain (1 strain)	(0/1)	(0/1)	(0/1)	(0/1)
Pseudomonas aeruginosa	ATCC 27853	(0/1)	(0/1)	(1/1)	(0/1)
	clinical strains (19 strains)	(0/19)	(0/19)	(19/19)	(0/19)
Stenotrophomonas maltophilia	clinical strains (2 strains)	(0/2)	(0/2)	(0/2)	(0/2)
Escherichia coli	ATCC 25922	(0/1)	(0/1)	(0/1)	(1/1)
	clinical strains (12 strains)	(0/12)	(0/12)	(0/12)	(12/12)
Salmonella Typhimurium	ATCC 14208	(0/1)	(0/1)	(0/1)	(1/1)
Salmonella Enteritidis	clinical strain (1 strain)	(0/1)	(0/1)	(0/1)	(1/1)
Proteus vulgaris	ATCC 13315	(0/1)	(0/1)	(0/1)	(1/1)
Proteus mirabilis	clinical strains (5 strains)	(0/5)	(0/5)	(0/5)	(5/5)
Shigella sonnei	clinical strains (1 strain)	(0/1)	(0/1)	(0/1)	(1/1)
Serratia marcescens	ATCC 8100	(0/1)	(0/1)	(0/1)	(1/1)
	clinical strains (2 strains)	(0/2)	(0/2)	(0/2)	(2/2)
Klebsiella pneumoniae	clinical strains (4 strains)	(0/4)	(0/4)	(0/4)	(4/4)

Table 2
Primers used in the study.

Name	Target spp	Primer sequence (5' to 3')	Size (bp)
SAtm-F	*Staphylococcus aureus*	CGTCATCAACACACACAGTTTA	223
SAtm-R		GGTTTCGCATCATGAAAAGTG	
Entc-F	*Enterococcus* spp	GAGCTTGAATTGCGTTTCGT	240
Entc-R	(*Enterococcus faecalis*)	CCGAGTGACAGGCATTCTGTA	
Pre-F	*Pseudomonas* spp	TTGCCAACGACGACAACTAC	255
Pre-R	(*Pseudomonas aeruginosa*)	GCTCTCCGCTATCGGTT	
ENT2-F	Enterobacteriaceae	TTAGAGCCCTCTCTCCCTAGC	206
ENT2-R	(*Escherichia coli*)	CGTCCGAAATTCCTACATCC	

of DNAs from *S. aureus* ATCC 25923, *E. faecalis* ATCC 33186, *P. aeruginosa* ATCC 27835 and *E. coli* ATCC 25922 was serially diluted 10-fold and subjected to qPCR assay performed in a MyiQ real-time PCR system (Bio-Rad, Hercules, CA) using SsoFast EvaGreen Supermix (Bio-Rad), according to the manufacturer's recommendations. Thermocycling conditions were as follows: 94°C for 1 minute; followed by 40 cycles of 94°C for 20 seonds, 62°C for 20 seconds, and 72°C for 30 seconds. The melting curve was constructed from 55°C to 95°C with increments of 1°C/sec, followed by a hybridization step, on the purpose for absence of non-specific amplicons. Plots were generated of threshold cycle number (Ct) versus log bacterial DNA amount. Ct value for the non template negative control was 35. Three independent experiments were performed for each bacterial sample.

RESULTS

PCR-based amplification of *ssrA* in detecting gram-positive and gram-negative bacteria

Using species specific *ssrA* primers, amplicons of the expected sizes were obtained for both reference gram-positive [*S. aureus* ATCC 25923 (223 bp) (Fig 1A) and *Enterococcus* spp (240 bp) (Fig 1B)], and gram-negative [*Pseudomonas* spp (225 bp) (Fig 1C) and Enterobacteriaceae (206 bp) (Fig 1D)] bacteria. Among the 99 clinical isolates, all were detected correctly as regards the four species (Fig 1 and Table 1). This was confirmed using 16S and 23S rDNA primer sets (data not shown). The sets of *ssrA* primers were not capable of identifying phylogenetically-related species, but only *S. aureus* (using SAtm primer set), *E. faecalis* and *E. faecium* (Entc primer set), *P. aeruginosa* (Pre primer set), and *E. coli*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Proteus vulgaris*, *P. mirabilis*, *Shigella sonnei*, *S. marcescens* and *K. pneumoniae* (ENT2 primer set) (data not shown).

Sensitivity of *ssrA* detection

Based on the linearity and correlation coefficient ($R^2 > 0.980$) of curves of Ct versus known amounts of bacterial DNA, qPCR detection of species specific *ssrA* showed that the detection limit of *S. aureus* and *Enterococcus* spp was 10^{-5}μg, and that for *Pseudomonas* spp and Enterobacteriaceae was 10^{-6} μg (Fig 2).

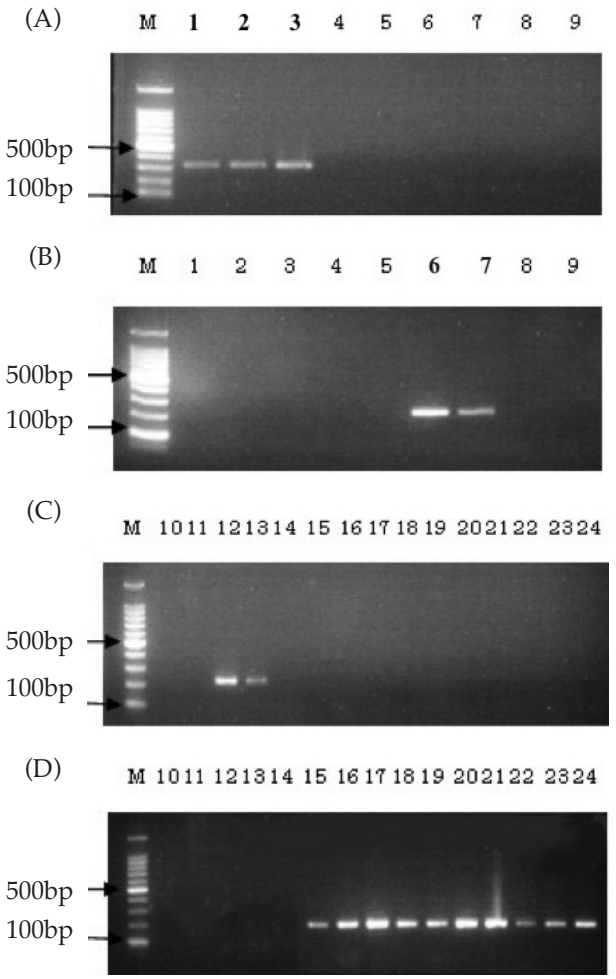

Fig 1–PCR amplification of *ssrA* of (A) *Staphylococcus aureus*, (B) *Enterococcus* spp, (C) *Pseudomonas* spp, (D) Enterobacteriaceae. PCR protocols and primers used are described in Materials and Methods. Lane M, DNA 100 bp size markers; lane 1, *S. aureus* ATCC 25923; lane 2, *S. aureus* clinical strain; lane 3, *S. epidermidis* ATCC 12228; lane 4, *Streptococcus agalactiae* clinical strain; lane 5, *S. pneumoniae* clinical strain; lane 6, *E. faecalis* clinical strain; lane 7, *E. faecium* clinical strain; lane 8, *Bacillus cereus* clinical strain; lane 9, *B. subtilis* ATCC 6633; lane 10, *Moraxella catarrhalis* clinical strain; lane 11, *Haemophilus influenzae* clinical strain); lane 12, *P. aeruginosa* ATCC 27853; lane 13, *P. aeruginosa* clinical strain; lane 14, *Stenotrophomonas maltophilia* clinical strain; lane 15, *Escherichia coli* ATCC 25922; lane 16, *E. coli* clinical strain; lane 17, *Salmonella* Enteritidis clinical strain; lane 18, *S.* Typhimurium ATCC 14208; lane 19, *Shigella sonnei* clinical strain; lane 20, *Klebsiella pneumoniae* clinical strain; lane 21, *Proteus vulgaris* ATCC 13315; lane 22, *P. mirabilis* clinical strain; lane 23, *Serratia marcescens* ATCC 8100; lane 24, *S. marcescens* clinical strain.

DISCUSSION

As sequences of bacterial rRNA genes are often the same and may not vary between strains and subspecies or even completely different species, this has made it difficult to distinguish individual bacteria precisely using PCR-based amplification

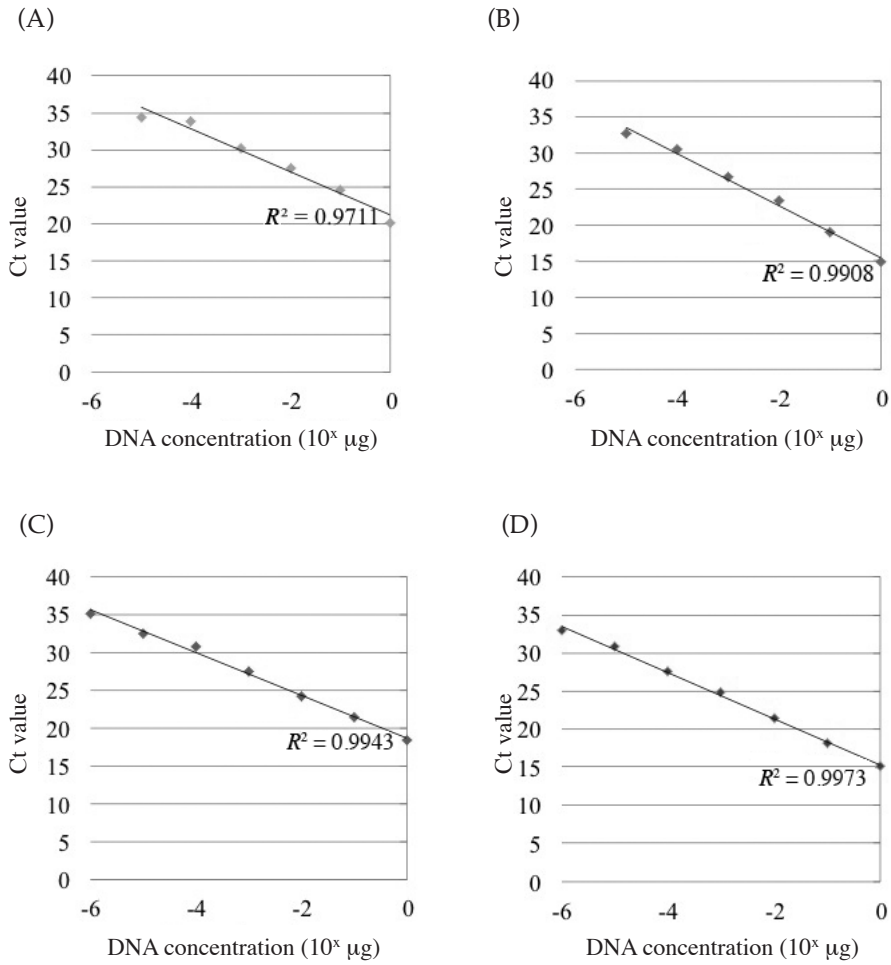

Fig 2–Determination of sensitivity of PCR-based *ssrA* assay for detection of (A) *S. aureus*, (B) *E. faecalis*, (C) *P. aeruginosa* and (D) *E. coli*. Experimental procedures for qPCR amplification of species specific *ssrA* are described in Materials and Methods. Ct value for non-template negative control was 35.

of 16S and/or 23S rDNA (Pérez Luz *et al*, 1998). Nevertheless, there are several new sensitive and specific detection systems for bacteria; for instance, Kubota *et al* (2010) using subgroup- or species-specific primer sets targeting 16S or 23S rDNA have identified such bacteria as *Enterococcus* and *Streptococcus* spp; Matsuda *et al* (2007; 2009) demonstrated the efficacy of rRNA-targeted RT-qPCR for bacterial identification using group- and species-specific primer sets targeting 16S rRNA of *Clostridium perfringens*, *Lactobacillus* spp, *Enterococcus* spp, and *Staphylococcus* spp; and Kurakawa *et al* (2012) showed detection of *Vibrio cholerae/mimicus*, *V. parahaemolyticus/alginolyticus* and *Campylobacter jejuni/coli* using specific primers targeting 16S or 23S rDNA. In addition, Goh *et al* (1997; 2000) have reported identification of *Staphylococcus* and *Enterococcus* spp using primers targeting chaperonin 60

(*cpn60*) gene, but the presence of *cpn60* in eukaryotes could compromise the assay.

However, employing *ssrA* as a molecular marker offers a number of advantages: (i) 350 bp in size, (ii) specific only to individual bacteria, even between subspecies, and (iii) present at high copy numbers (O'Grady *et al*, 2009). We have shown that PCR amplification of *ssrA* allowed ready identification of gram-positive and gram-negative bacteria from a variety of clinical sources and had a sensitivity as low as pg of bacterial DNA. Our data also suggest that *ssrA* has the potential to discriminate between closely related species observed especially in *S. aureus*, where we designed *ssrA* primers for specific bacteria, not for closely related species, according to the sequence alignment of *ssrA* in these bacteria (http://www.ncbi.nlm.nih.gov/nuccore/). Furthermore, qPCR-based detection is quicker and less laborious than conventional PCR and may be useful in a clinical setting especially for early detection of causative bacteria of life-threating infection. Barends *et al* (2010) have employed qPCR-based *ssrA* amplification to detect *Streptomyces coelicolor* with high sensitivity.

Regarding the limit of detection, Gordillo *et al* (2014) stated that in their qPCR-based detection of *E. coli* O157:H7 in meat products, it was 10^1 or 10^2 CFU/g for artificially contaminated meat products, and after a 4 hours enrichment period at 37°C, the detection limit decreased to about 1 CFU/g, even though we have no comparable data with them and it needs future studies for direct comparison.

We would like to emphasize that our study has a number of limitations. Firstly, the numbers and the kinds of bacteria studied were not comprehensive enough to reach definitive conclusions. Secondly, we did not use a comparative gene for detection, especially in the experiments regarding PCR sensitivity. Thirdly, this method did not discriminate each individual type of bacterium. These limitations should have to be overcome in future studies prior to clinical application.

In summary, we have identified *S. aureus*, *Enterococcus* spp, *Pseudomonas* spp and Enterobacteriaceae using species specific primers targeting *ssrA* and shown that the PCR assay was very sensitive based on qPCR analysis. Further studies may possibly lead to its clinical applications.

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