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Rapid and reliable identification of *Staphylococcus aureus* harbouring the enterotoxin gene cluster (*egc*) and quantitative detection in raw milk by real time PCR

Vincenzina Fusco [a],*, Grazia Marina Quero [a], Maria Morea [a], Giuseppe Blaiotta [b], Angelo Visconti [a]

[a] *National Research Council, Institute of Sciences of Food Production (CNR-ISPA), Bari, Italy*
[b] *Department of Food Science, School of Agriculture of the University of Naples Federico II, Portici, Italy*

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ABSTRACT

A TaqMan and a SYBR Green real time PCR (rt-PCR) were developed for the reliable identification and quantitative detection of *Staphylococcus* (*S.*) *aureus* strains harbouring the enterotoxin gene cluster (*egc*) regardless of its variants. Both approaches revealed 100% specificity against a panel of 70 reference strains, including 29 clinical and foodborne *S. aureus* strains harbouring all the *egc* variants to date known, 4 *egc*$^-$ *S. aureus* strains and 37 strains of phylogenetically closely and distantly related species. Standard curves made by 10 fold dilutions of either genomic DNA or cells from an *egc*$^+$ *S. aureus* log-phase broth culture showed a good linearity of response ($R^2 \geq 0.993$) for six orders of magnitude, with about 100% relative accuracy and a low inter-assay variability ($CV \leq 3.02$). The overall limit of quantification (LOQ) for both rt-PCR assays (about 100% PCR efficiency; running time 30 min) was 10 cfu or 10 genome equivalents per reaction mixture although 1 cfu or 1 genome equivalent was detected with a 33.33% probability. These performances were confirmed in raw milk artificially contaminated with log-phase broth cultures of either a single *egc*$^+$ *S. aureus* strain or a mixture of *S. aureus* strains harbouring all the *egc* variants to date known. Similar results were also obtained with a raw milk based standard curve of the *S. aureus egc*$^+$ mixture in the presence of 10^6 cfu/mL of *egc*$^-$ *S. aureus* strains harbouring some of the commonest enterotoxin genes associated to the staphylococcal food poisoning. Nonetheless, the TaqMan based approach resulted in a lower sensitivity (LOQ = 100 cfu equivalents per reaction mixture) than the SYBR Green based assay (LOQ = 10 cfu equivalents per reaction mixture). When applied to real milk samples, both PCR assays provided a good response with 100% diagnostic specificity and 96–107% relative accuracy, as compared to conventional culture-based PCR approaches. Due to the high specificity, the wide dynamic range of detection and the high sensitivity demonstrated even in a complex and potentially highly contaminated raw milk matrix, the SYBR Green rt-PCR assay is a useful diagnostic tool for quick, high throughput and reliable routine screening of *egc*$^+$ *S. aureus* isolates. Moreover, the SYBR Green based quantitative detection of these pathogens in raw milk could remarkably contribute to clarify their actual role in staphylococcal food poisoning and other clinical syndromes associated with the consumption of milk and milk-based products.

1. Introduction

Staphylococcus aureus is one of the commonest aetiological agents of bacterial diseases worldwide due to its ability to produce a broad range of exotoxins and other virulence factors. Among them, the staphylococcal enterotoxins (SEs) produced by some *S. aureus* strains are the main causal agents of one of the most widespread foodborne intoxications, the staphylococcal food poisoning (Balaban and Rasooly, 2000) and, together with toxic shock syndrome (TSS) toxin-1, are responsible for toxic shock syndrome, and staphylococcal scarlet fever (Holtfreter and Bröker, 2005). Apart from their emetic activity, all the aforementioned exotoxins share further biological characteristics such as pyrogenicity and superantigenicity enhancing the susceptibility to endotoxin shock (Dinges et al., 2000; Fraser and Proft, 2008; Llewelyn and Cohen, 2002). Moreover, these exotoxins are suspected to be involved in non-infectious diseases such as Kawasaki's disease and autoimmune diseases (Leung et al., 1995; Marrack and Kappler, 1990). Currently, named accordingly to whether or not they have been proved to cause emesis (Lina et al., 2004), 11 SEs (SEA-E, SEG-I, SER-T), and 10 enterotoxin-like (SEl) (SElJ-Q, SElU and SElV) *S. aureus* exotoxins are known. Their genes are often associated with mobile genetic elements such as prophages, transposons and plasmids usually referred to as pathogenicity islands (Novick and Subedi, 2007). Among the newly described exotoxin genes, *seg*, *sei*, *selm*, *seln*, and *selo* genes, encoding SEG, SEI, SElM, SElN and SElO, respectively, and the two pseudogenes *ψent*1 and *ψent*2 have been demonstrated to be part of the chromosomal operon

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* Corresponding author. Mailing address: National Research Council, Institute of Sciences of Food Production, via Amendola 122/O, 70121, Bari, Italy. Tel.: +39 080 5929322; fax: +39 080 5929374.
E-mail address: vincenzina.fusco@ispa.cnr.it (V. Fusco).

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named enterotoxin gene cluster (*egc*) (Jarraud et al., 2001). Letertre et al. (2003a) showed that an insertion of 15 bp in the *ψent*1 pseudogene of some *S. aureus* strains allows the translation of a putative 261-nucleotide open reading frame, named *selu* (Letertre et al., 2003a). Allelic variants of the *seg*, *sei*, *selm*, *seln*, *selo* and *selu* have been described so far (Abe et al., 2000; Blaiotta et al., 2004; 2006; Fernandez et al., 2006; Letertre et al., 2003a; Omoe et al., 2002). Thomas et al. (2006) demonstrated that a recombination between *selm* and *sei* gives the *selv* gene, whereas a limited deletion in the *ψent*1–*ψent*2 pseudogenes generates the *selu*2 gene. Holtfreter et al. (2004) proved that *S. aureus egc*-encoded superantigens are neutralised by human sera in a much less extent than classical staphylococcal enterotoxins or toxic shock syndrome toxin-1, suggesting a potential for increased severity of clinical diseases. During the last few years a high frequency of genes belonging to the *egc* has been ascertained in clinical, animal and foodborne *S. aureus* isolates regardless of the disease they caused (Bania et al., 2006; Becker et al., 2003; 2004; Chiang et al., 2008; Jarraud et al., 1999; 2001; Lawrynowicz-Paciorek et al., 2007; Mempel et al., 2003; Morgan et al., 2007; Naik et al., 2008; Shuiep et al., 2009; Smyth et al., 2005). These findings lead to hypothesise a potential significance of the egc^{+} *S. aureus* strains in public health and food safety. Although several variants of the *egc* genes have been discovered and methods to discriminate the various *egc* loci have been implemented, no suitable assays are to date available for the rapid detection of egc^{+} *S. aureus* strains.

Herein, we describe the development of a TaqMan and a SYBR Green rt-PCR based assay targeting the *egc* of *S. aureus*, regardless of its variants, for the rapid and reliable identification and quantitative detection of egc^{+} *S. aureus* strains. In addition, given the well recognised role of *S. aureus* as one of the commonest aetiological agent of clinical and sub-clinical mastitis (Barkema et al., 2006; Celik et al., 2009; Vanderhaeghen et al., 2010) and considering that milk and milk-based products contaminated with this pathogen are some of the food matrices more often involved in staphylococcal food poisoning (Asao et al., 2003; De Buyser et al., 2001; Jørgensen et al., 2005; Lindqvist et al., 2002; Ostyn et al., 2010; Soejima et al., 2007), we evaluated the effectiveness of our novel assays in artificially and naturally contaminated raw milk.

2. Materials and methods

2.1. Strains and growth conditions

A total of 70 strains, including 33 *S. aureus* strains (4 egc^{-} and 29 egc^{+} isolated from clinical and food samples) (Table 1), 25 reference type strains of other staphylococcal species (3 coagulase positive and 22 coagulase negative) as well as 12 strains belonging to species of other genera (Table 2) were analysed in this study. All the *S. aureus* strains were previously identified and characterised by molecular methods (Blaiotta et al., 2004; 2006).

Cultures were streaked on Baird Parker agar with egg yolk emulsion (BP-EY) (Oxoid, Milan, Italy) plates and grown at 37 °C for 24–48 h. Working cultures were obtained growing a single colony in 10 mL of Tryptone Soy Broth with 0.3% Yeast Extract (TSB-YE), at 37 °C overnight with shaking at 190 rpm. Viable counts were performed by spread-plating in triplicate on BP-EY and Tryptone Soy Agar with 0.3% Yeast Extract (TSA-YE) 10-fold dilutions in sterile quarter strength Ringer's solution (Oxoid) of each sample. Plates were incubated at 37 °C for 24–48 h.

2.2. DNA extraction

One millilitre of each working culture as well as 1 mL of raw milk samples were centrifuged at 8000×*g* for 10 min. Pellets were washed in TE (50 mM Tris HCl, 1 mM EDTA, pH 8) and resuspended in 100 µL of TE. Lysostaphin (final concentration 1U/100 µL) was added to each suspension and incubated at 37 °C for 30 min. Lysate solutions were then processed following the NucleoMag Tissue kit (Macherey-Nagel, Düren, Germany) manufacturer's instructions. For milk samples a pre-washing step with 1 vol of 2% Sodium Citrate was performed. Each mixture was vortexed and centrifuged for 15 min at 20,000 rpm. Pellets were then washed in TE and lysated as above described. DNAs were eluted in 100 µL elution buffer (Macherey-Nagel) and their quantity and quality were assessed spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA) and by agarose gel electrophoresis (Sambrook et al., 1989) with known amounts of lambda DNA, marker VI (Roche S.p.a., Milan, Italy) as standard.

Table 1
Origin, source and REA *egc* group (restriction endonuclease analysis of the *egc selm*–*seg* intergenic region), as performed by Blaiotta et al. (2006), of *S. aureus* strains analysed in this study.

Strain	Source[a]	Origin[b]	REA *egc* group[c]
DSM20231^{T}	DSM	Human pleural fluid	–
D4508	CNTS	————————————	–
ATCC14458	ATCC	Feces of child	–
ATCC27664 (FRI326)	CNTS	Chicken tetrazzini	–
A900322	CNTS	Patient with TSS	1
NCTC9393	CNTS	—————————	1
RIMD31092	CNTS	MRSA strain	1
BS4	DSAN	NTS (sample B)	1
DS18g	DSAN	NTS (sample D)	1
AS14g	DSAN	NTS (sample A)	1
AS27	DSAN	NTS (sample A)	1
SI1	DSAN	MCM (plant A)	1
LA14	DSAN	WBRM (plant A)	1
R1	DSAN	WBRM (plant A)	1
ATCC19095 (FRI137)	CNTS	Leg abscess	2
382 F	AFSSA	Unspecified (food)	3
ED-3	DSAN	RPM (sample ED)	4
ED4	DSAN	RPM (sample ED)	4
105	DSAT	SP (defeathering machine)	4
106	DSAT	SP (defeathering machine)	4
ATCC25923	ATCC	Clinical isolate	5
AB-8802	DSAN	RPM (sample AB)	6
109	DSAT	SP (defeathering machine)	7
7645a	IMM	HP (osteomyelitis)	7
OM56/2a	IMM	HP (osteomyelitis)	7
K4644/97	IMM	HP (blood cultures)	7
K6278/97	IMM	HP (blood cultures)	7
A2586/99	IMM	HP (nasal swabs)	7
A1048/98	IMM	HP (nasal swabs)	7
A2812/98	IMM	HP (nasal swabs)	7
A4178/98	IMM	HP (nasal swabs)	7
A652/99	IMM	HP (nasal swabs)	7
A900624	CNTS	—————————	nd

[a] ATCC, American Type Culture Collection, Rockville, Maryland, USA; DSM, Deutsche Sammlung von Mikroorganism, Braunschweig, Germany; CNTS, Centre Nationale des Toxemies a Staphylocoques, Faculté de Medecine Laennee, Lyon, France (Kindly provided by Prof. G. Lina and Dr. Vandenesch); DSAN, Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, Portici, Italy; DSAT, Dipartimento di Scienze degli Alimenti Università degli Studi di Teramo, Italy (Kindly provided by Prof. A. Ianieri); IMM, Institut für Medizinische Mikrobiologie, Universitätsklinikum Münster, Münster.

[b] NTS, "Napoli-Type" salami; WBRM, water buffalo raw milk; MCM, water buffalo Mozzarella cheese manufacturing (Natural Whey Cultures); RPM, raw poultry meat; SP, slaughterhouse for pigeon; HP, strains were isolated from different patients admitted to the University Hospital of Muenster (1997–1999).

2.3. Oligonucleotides for rt-PCR

Alignment of the *S. aureus egc* types was performed by using Bionumerics software (Applied Maths, Sint Martens-Latem, Belgium), version 5.1. Probe and primers were designed based on the DNA sequence ranging from the 2919 to 3960 nucleotide positions of the 6418 bp DNA fragment of *S. aureus* A900322 harbouring the *egc*

Table 2
Source and origin of non *S. aureus* strains used in this study.

Strain	Species	Origin[a]
21GT0	*Actinomyces viscosus*	ISPA
P3/22	*Aeromonas* spp.	ISPA
15RM	*Citrobacter* spp.	ISPA
72GT0	*Enterobacter* spp.	ISPA
29GT0	*Enterococcus faecalis*	ISPA
19C	*Klebsiella gibsonii*	ISPA
DSM20597^T	*Macrococcus caseolyticus*	DSM
73GT0	*Pantoea agglomerans*	ISPA
C34	*Pseudomonas* spp.	ISPA
MY262A	*Rhanella acquatilis*	ISPA
DSM20672^T	*S. arlettae*	DSM
DSM20326^T	*S. capitis* subsp. *capitis*	DSM
DSM20608^T	*S. capreae*	DSM
DSM20501^T	*S. carnosus* subsp. *carnosus*	DSM
DSM20454^T	*S. chromogenes*	DSM
DSM20260^T	*S. cohnii*	DSM
LMG22190^T	*S. delphinii*	LMG
DSM20044^T	*S. epidermidis*	DSM
DSM20674^T	*S. equorum* subsp. *equorum*	DSM
DSM15097^T	*S. equorum* subsp. *linens*	DSM
DSM20610^T	*S. gallinarum*	DSM
DSM20263^T	*S. haemolyticus*	DSM
ATCC29663^T	*S. intermedius*	ATCC
DSM20676^T	*S. klosii*	DSM
DSM20352^T	*S. lentus*	DSM
DSM4804^T	*S. lugduniensis*	DSM
DSM11674^T	*S. condimenti*	DSM
LMG22219^T	*S. pseudointermedius*	LMG
DSM20229^T	*S. saprophyticus*	DSM
DSM20345^T	*S. sciuri* subsp. *sciuri*	DSM
DSM15096^T	*S. succinus* subsp. *casei*	DSM
DSM14617^T	*S. succinus* subsp. *succinus*	DSM
DSM15615^T	*S. vitulinus*	DSM
DSM20316^T	*S. warnerii*	DSM
DSM20266^T	*S. xylosus*	DSM
128GT0	*Streptococcus bovis*	ISPA
DSM20196^T	*Weissella confusa*	ISPA

[a] ATCC, American Type Culture Collection, Rockville, Maryland, USA; DSM, Deutsche Sammlung von Mikroorganism, Braunschweig, Germany; BCCM/LMG Belgian Coordinated Collections of Microorganisms, Deutcheland Laboratorium voor Microbiologie, Universiteit Gent (UGent) K.L. Ledeganckstraat; ISPA, Institute of Sciences of Food Production, National Research Council, Bari, Italy.

cluster (GenBank accession number AF285760; Jarraud et al., 2001). Probe and primers were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, USA), with the software's default settings. Candidate primers and probe sequences were examined for specificity following BLAST (http://www.ncbi.nlm.nih.gov/blast/) and EMBL (http://www.ebi.ac.uk/embl/) nucleotide sequence database searches for DNA sequences. The set of primers *egc*AUf/*egc*AUr1 (Table 3) was synthesized by SIGMA (Milan, Italy) and used for amplifying, by both TaqMan and SYBR Green rt-PCRs, an 82 bp fragment of the *S. aureus egc*. The internal MGB TaqMan probe (Table 3) was synthesized by ABI PRISM Primers and TaqMan Probe Synthesis Service (Applied Biosystems). It contained the fluorescent reporter dye 6-carboxy-fluorescein (FAM) and a minor groove binder (MGB) group at the 3′ end along with a non-fluorescent quencher (NFQ). The MGB and the NFQ are added to the probe for increasing stability and specificity of probe hybridization and enhance fluorescent performance.

2.4. Rt-PCR optimisation

Rt-PCR reaction mixtures for the ABI PRISM 7500 fast rt-PCR system (Applied Biosystems) were optimised following the manufacturer's instructions (Applied Biosystems, technical guide), using 30 pg of genomic DNA of *S. aureus* NCTC9393 as template. In particular, for the SYBR Green rt-PCR assay primer concentrations of 50, 100, 150, 200 and 300 nM (each in triplicate) were tested. To optimise the TaqMan-based assay, primer concentrations of 50, 150, 300, 400, 600, and 900 nM, in all the possible combinations (each in triplicate) were tested, keeping the probe concentration of 250 nM per each reaction. Once it was determined, the optimal primer concentration was used to detect the optimal probe concentration in the range between 50 and 300 nM, with increments of 50 nM.

2.5. Calculation of the *S. aureus egc* copy number

The copy number of the *S. aureus egc* was determined considering that, based on the molecular weight of the 2.74 to 2.9 Mbp-sized genome of *S. aureus* (Ben Zakour et al., 2008) (http://www.ncbi.nlm.nih.

Table 3
Nucleotide sequences, target genes, expected amplicon sizes of PCR primers and probe used in this study and PCR/rt-PCR results obtained for the 14 egc^+ *S. aureus* wild strains isolated from raw milk samples.

Primers	Sequence (5′–3′)	References	Target gene	Product (bp)	PCR/rt-PCR results
SEO1	AGT CAA GTG TAG ACC CTA TT	Blaiotta et al. (2004)	*selo*	534	+
SEO2	TAT GCT CCG AAT GAG AAT GA				
SEM1	CCA ATT GAA GAC CAC CAA AG	Blaiotta et al. (2004)	*selm*	517	+
SEM2	CTT GTC CTG TTC CAG TAT CA				
SEG1	TGCTATCGACACACTACAACC	McLauchlin et al. (2000)	*seg*	704	+
SEG2	CCAGATTCAAATGCAGAACC				
SEI1	GACAACAAAACTGTCGAAACTG	McLauchlin et al. (2000)	*sei*	630	+
SEI2	CCATATTCTTTGCCTTTACCAG				
SEI1	above reported	McLauchlin et al. (2000)	*selm–seg* intergenic region	3375	+
SEG2					
PSE1	TGA TAA TTA GTT TTA ACA CTA AAA TGC G	Letertre et al. (2003a)	*sei–seln* intergenic region	1150	+
PSE4	CGT CTA ATT GCC ACG TTA TAT CAG T				
PSE2	TAA AAT AAA TGG CTC TAA AAT TTG ATG G	Letertre et al. (2003a)	*selu*	790	–
PSE4	above reported				
PSE2	above reported	Letertre et al. (2003a)	$selu_v$	142	–
PSE6	ATC CGC TGA AAA ATA GCA TTG AT				
[a]*egc*AUf	5′-CTTCATATGTGTTAAGTCTTGCAGCTT	This study	*sei–seln* intergenic region [a]3092–3118	82	+
*egc*AUr1	5′-TTCACTCGCTTTATTCAATTGTTCTG		3148–3173		
egc probe	5′-(6-FAM) ATGTTAAATGGCAATCCT (MGB)-3′		3126–3143		
SEV1	GCAGGATCCGATGTCGGAGTTTTGAATCTTAGG	Thomas et al. (2006)	*selv*	720	–
SEV2	TAACTGCAGTTAGTTACTATCTACATATGATATTTCGACATC				
SEN1	ATT GTT CTA CAT AGC TGC AA	Blaiotta et al. (2004)	*seln*	682	+
SEN2	TTG AAA AAA CTC TGC TCC CA				

[a] Primers and probe used for the SYBR and TaqMan rt-PCR assays developed in this study; primers and probe position is based on the full sequence of the *S. aureus* A900322 *egc*, accession number AF285760 (Jarraud et al., 2001).

gov/sites/entrez?db=Genome&itool=toolbar), 3.03 fg of DNA equals the mass of a single whole genome, and that the *egc* is present in a single copy.

2.6. Rt-PCR selectivity

The selectivity of our assays, defined by the MicroVal Protocol/ISO (International Organization for Standardization) 16140:2003 (Anonymous, 2003) as a measure of the degree of noninterference in the presence of non-target analytes, was evaluated in terms of inclusivity and exclusivity. Inclusivity is intended as the ability of the PCR method to detect the target analyte from a wide range of strains, whereas exclusivity is the lack of response from a relevant range of closely related, non-target strains (Hoorfar and Cook, 2002).

The inclusivity and exclusivity of the SYBR Green and TaqMan rt-PCR assays were assessed first *in silico*, by homology searches of our primers and TaqMan MGB probe, in the nucleotide databases and, then *in vitro*, by testing 3 ng of genomic DNA of the 70 strains reported in Tables 1 and 2. Melting curve analysis (carried out as reported below) was performed after SYBR Green rt-PCR. In addition, some amplicons were analysed by agarose (2% w/v) gel electrophoresis (Sambrook et al., 1989).

2.7. Standard curves

A DNA standard curve and a cell standard curve in broth were constructed. For the DNA standard curve, *S. aureus* NCTC9393 DNA was serially 10-fold diluted in deionised water, obtaining dilutions ranging from 30.3 to 3.03×10^{-6} ng of DNA corresponding to an *egc* copy number of 10^7–10^0. For the cell standard curve a log-phase NCTC9393 *S. aureus* broth culture (0.6 OD_{600nm}) was serially 10-fold diluted in TSB-YE. The number of cfu per dilution was determined by the plate count method using TSA-YE plates. DNA was isolated from 1 mL of each dilution and eluted in 100 µL of elution buffer. In both cases (DNA and cell standard curves) a 1 µL aliquot of DNA from each dilution, in triplicate, was subjected to rt-PCR, once each, on three different days.

2.8. Rt-PCR applicability in artificially contaminated raw milk

Aliquots (27 mL) of raw milk, purchased by a local farmer, were distributed in a series of 50 mL falcon tubes. Three millilitres of a pure culture of a log-phase (0.6 OD_{600nm}) *S. aureus* NCTC9393 were centrifuged at 8000×*g* for 10 min. Each pellet was washed with sterile quarter strength Ringer's solution (Oxoid) and resuspended in 3 mL of raw milk, which were then added to 27 mL of raw milk. Thereafter, further decimal dilutions were carried up to 10^{-9}.

The contaminating broth culture, the corresponding dilutions in raw milk and the uninoculated raw milk were plate counted in accordance with the standard reference culture method recommended by the International Organization for Standardization (Anonymous, 1999) on BP-EY agar after incubation at 37 °C for 24–48 h. In order to ascertain the absence of <5 cfu mL^{-1} of uninoculated raw milk (negative control), an enrichment step at 37 °C for 24 h in Giolitti–Cantoni broth (Oxoid) preceded the plating on BP-EY. Total viable count of uninoculated milk was determined by plating 10-fold dilutions in quarter strength Ringer's solution (Oxoid) on Plate Count Agar (PCA) (Oxoid) after incubation at 30 °C, aerobically, for 72 h. DNA was isolated from 1 mL of each dilution and eluted in 100 µL of elution buffer. A 1 µL aliquot of DNA from each dilution, in triplicate, was subjected to rt-PCR, once each, on three different days.

2.9. Detection in milk with a background of egc^{-} S. aureus strains

In order to assess the applicability of our methods also in simulated staphylococcal food poisoning conditions (i.e egc^{-} *S. aureus* concentration above 10^5 cfu mL^{-1}) we tested raw milk artificially contaminated with a mixture of egc^{+} *S. aureus* strains (namely FRI137, AB8802, A900322, and A900624) alone and in the presence of a mixture of egc^{-} *S. aureus* strains harbouring (and not) other enterotoxin genes (DSM20231^{T}, D4508, ATCC14458, and ATCC27664) (Tables 1 and 4). For this purpose, aliquots of raw milk (18 mL), purchased in a local dairy farm, were distributed in a series of 15 mL falcon tubes. One millilitre of a mixture containing 250 µL of a log-phase (0.6 OD_{600nm}) pure culture of each egc^{+} *S. aureus* strain was centrifuged at 8000×*g* for 10 min. The resulting pellet was washed with sterile quarter strength Ringer's solution (Oxoid), resuspended in 2 mL of milk, which were then added to 18 mL of milk. Thereafter, further decimal dilutions were carried up to 10^{-9}. Aliquots of 9 mL from each dilution contaminated with the egc^{+} *S. aureus* strains' mixture were transferred in a series of 15 mL falcon tubes. In parallel, 1 mL of a mixture containing 250 µL of a log-phase (0.6 OD_{600nm}) pure culture of each egc^{-} *S. aureus* strain was centrifuged at 8000×*g* for 10 min. The resulting pellet was washed and resuspended in 1 mL of sterile quarter strength Ringer's solution (Oxoid). Thereafter, 9 µL of this mixture were added to each falcon containing the egc^{+} *S. aureus* strains' mixture. Viable cell counts and DNA extraction of the contaminating broth culture mixtures, the corresponding dilutions in raw milk and the uncontaminated raw milk were carried out as reported below. A 1 µL aliquot of DNA from each dilution, in triplicate, was subjected to rt-PCR, once each, on three different days.

2.10. Detection of egc^{+} S. aureus strains in real milk samples by rt-PCR and standard reference culture methods coupled with rt-PCR

The optimised SYBR and TaqMan rt-PCR assays were performed on 1 µL of DNA isolated (as reported below) from 1 mL of each milk sample, in triplicate.

In parallel, further decimal dilutions were realized and used to plate count staphylococci and total mesophilic aerobic microorganisms on both BP with EY (Oxoid) following the ISO 6888-1 (Anonymous, 1999) and PCA as described above. A total of 130 typical, suspect and atypical presumptive *S. aureus* colonies (about ten per each sample) were randomly picked from BP-EY agar plates seeded with the highest sample dilutions and purified by repeated streaking on the same medium. Thereafter, isolates were subjected to DNA extraction and 1 µL aliquots, in triplicate, were screened for the presence of *egc* by our SYBR and TaqMan rt-PCR assays.

2.11. Conventional culture-dependent PCR based detection and egc characterisation

To validate our rt-PCR based *egc* detection protocol, DNA aliquots (10 ng) of all isolates were firstly subjected to a species-specific PCR targeting the thermostable nuclease gene (*nuc*A) (Brakstad et al., 1992). Once confirmed their presumptive identification, the resulting *S. aureus* isolates as well as reference *S. aureus* strains (at least one representative per each *egc* type group, Table 1) were screened for the presence and type of the *egc* operon by two PCR-RFLP (restriction

Table 4
Genotype of *S. aureus* strains used for the artificial contamination of milk.

Strain	Genotype	Reference
ATCC19095 (FRI137)	egc^{+}, sec^{+}, seh^{+}	Blaiotta et al. (2004)
AB8802	egc^{+}	Blaiotta et al. (2004)
A900322	egc^{+}, $selp^{+}$	Jarraud et al. (2001) Sergeev et al. (2004) Blaiotta et al. (2004)
A900624	egc^{+}	Thomas et al. (2006)
DSM20231^{T}	-	Blaiotta et al. (2004)
D4508	sea^{+}, seh^{+}	Blaiotta et al. (2004)
ATCC14458	seb^{+}, sek^{+}, $selq^{+}$	Sergeev et al. (2004)
ATCC27664 (FRI326)	see^{+}	Blaiotta et al. (2004)

fragment length polymorphism) based approaches. For this purpose, the 3375 bp *selm–seg* and the 1149 bp *sei-seln* intergenic regions of the *S. aureus egc* were amplified and enzymatically digested following the protocols reported by Blaiotta et al. (2006) and Collery et al. (2009), respectively.

In parallel, PCR detection of the *egc*-encoding genes in *S. aureus* isolated from raw milk was also performed, using primers listed in Table 3. PCRs were carried out in a 9700 Thermal Cycler (Applied Biosystems). Reaction mixtures and amplification conditions were previously described (Table 3). Amplification products were checked by agarose gel electrophoresis (2% w/v) in 1× TBE buffer, stained with 0.5 μg mL^{-1} of ethidium bromide (Sigma, Milan, Italy) and visualised by UV, using the Bio-Rad Chemidoc apparatus (Bio-Rad Laboratories Richmond CA, USA).

2.12. Rt-PCR

Rt-PCRs were performed using an ABI PRISM 7500 fast rt-PCR system (Applied Biosystems). The optimised SYBR Green PCR reaction mixture contained: 1× Fast SYBR Green master mix (Applied Biosystems), 100 nM each primer, 1 μL of DNA and deionised sterile water to a final volume of 20 μL. TaqMan PCR reaction mixture contained: 1× TaqMan Fast Universal Master Mix, no Amperase, 300 nM each primer, 250 nM *egc* probe, 1 μL of DNA and deionised sterile water to a final volume of 20 μL. The amplification conditions were the following: 20 s at 95 °C, 37 cycles at 95 °C for 3 s and 60 °C for 30 s for an overall run time of 30 min. After each run, by using the ABI PRISM 7500 Sequence Detection System (SDS) software version 1.4 (Applied Biosystems), baseline and threshold values were automatically set so that the threshold intersected amplification curves in the linear region of the semi log amplification plot, 10 units above the baseline. Thereafter, the cycle threshold (Ct) values were determined. In order to ensure comparability between data obtained from different experimental plates, the threshold value subsequently was manually set to the value corresponding to the arithmetic mean between the automatically determined thresholds annotated previously; then all data were reanalysed.

Following SYBR Green rt-PCR amplification, melting curve analysis was performed using the 7500 SDS software version 1.4 (Applied Biosystems). The PCR products were heated to 95 °C during 15 s, cooled at 60 °C for 20 s, and then slowly heated back to 95 °C at a rate of 0.2 °C/s. Fluorescence data were converted into melting peaks by the ABI 7500 SDS software, which removed background fluorescence and the effect of temperature on fluorescence. Plotting the negative derivate of the fluorescence over temperature versus the temperature ($-dF/dT$ versus T) generated peaks from which the melting temperatures (Tm) of the products were calculated. In each rt-PCR experiment, serial dilutions of standard DNA (ranging from 30 ng to 3 fg) and a negative control (water), both in triplicate, were included.

2.13. Data analysis

All statistical data analyses were performed using Microsoft® Excel. Means and standard deviations (SDs) of Ct and Tm values were calculated. The accuracy of each method was estimated, by linear regression analyses, as the coefficient of determination for cells and DNA standard curves obtained by plotting the mean Ct values versus log concentrations of either cfu or DNA (genomic equivalents) of *S. aureus* NCTC9393. The relative accuracy was expressed either as percentage of numbers of cfu/mL calculated by rt-PCR assay versus conventional culture plating technique combined with PCR, or as percentage of numbers of genome equivalents per 20 μL of reaction mixture calculated by rt-PCR assay versus spectrophotometric quantification. Estimated counts were calculated using the absolute quantification method by interpolation of sample mean Ct values in either the DNA standard curve or the egc^{+}/egc^{-} mix *S. aureus* cultures raw milk based standard regression curve. Reproducibility of results was assessed by estimations of mean values, SDs and inter-assay variation coefficients (from raw Ct values) for three independent repeat runs. The rt-PCR amplification efficiency (E) was calculated through the equation $E = -1 + 10^{(-1/s)}$, where s is the slope of the linear regression curve (Klein et al., 1999).

3. Results

3.1. Optimisation of rt-PCR

Optimal primer and probe concentrations, determined as the lowest Ct with the highest ΔRn (delta value of the normalized reporter signal minus the baseline signal) for the given target concentration of 30 pg, were 100 nM each primer for the SYBR Green rt-PCR assay, and 300 nM each primer and 250 nM *egc* probe for the TaqMan rt-PCR assay (data not shown).

3.2. Selectivity

Results of the homology searches of primers in nucleotide databases revealed no 100% identical sequences other than those reported for the *egc* bearing either *ψent*1 pseudogene, *selu*, $selu_{v}$, or *selu*2 genes of *S. aureus* deposited so far, confirming *in silico* the specificity of our assays on all the *egc* types to date known. The selectivity, i.e. the capacity of the optimised SYBR Green rt-PCR to discriminate between target (inclusivity) and non-target bacteria (exclusivity), was tested against a panel of bacterial DNA templates from the 70 strains reported in Tables 1 and 2. Only the egc^{+} *S. aureus* strains gave the expected increment of fluorescence, regardless of the variant of this operon, resulting in 100% inclusivity and exclusivity. The melting curve analysis of the PCR products performed after each SYBR Green rt-PCR run resulted in sharply defined melting curves with a narrow peak only when the target DNA was assayed, confirming that the fluorescence signal originated from specific PCR products rather than from primer–dimers or other artifacts (data not shown). The mean peak Tm of the amplicons obtained with 87 curves specific for the 29 egc^{+} *S. aureus* assayed (in triplicate) was 70.2 °C ± 0.6 °C (ranging from 69.4 to 71.1 °C). No amplification was observed in any of the negative controls containing either water or DNA from egc^{-} *S. aureus* or from all the other species and no peak was obtained for them in the melting curve (data not shown). A single fragment of the expected size (82 bp) was also confirmed when amplicons were visualised on a 2% agarose gel (data not shown). A further demonstration of the specificity of our rt-PCR assay resulted by the BLAST analysis (100% identity) of the *S. aureus* A900322 amplicon, after its purification, reamplification and sequencing. Our primers worked successfully even when combined with the TaqMan MGB probe. Also in this case 100% specificity was achieved and no amplification in any of the negative control samples was observed. Comparing the mean Ct values obtained by assaying 3 ng of each egc^{+} *S. aureus* genomic DNA with both approaches an average difference of approximately two Ct values (15.38 ± 0.146, and 17.70 ± 0.178, for SYBR Green and TaqMan, respectively) was observed, indicating a higher sensitivity of the SYBR Green assay.

3.3. Analytical sensitivity and quantification range of our rt-PCR assays

Ten-fold dilution series of *S. aureus* NCTC9393 pre-purified DNA (ranging from 30.3 ng to 3.03 fg, i.e. from 10^7 to 1 genome equivalent per 20 μL of reaction mixture) were amplified in the optimised rt-PCR conditions, with three replicates for each DNA concentration per run in three different runs. The resulting means and standard deviations of Ct values, the relevant coefficient of variations (CV) as well as the relative regression equations, obtained by either TaqMan or SYBR Green assays, are reported in Table 5. Positive amplification in all PCR

Table 5

SYBR and TaqMan rt-PCR performances of different amounts of *S. aureus* NCTC9393 DNA. Ct values are reported as means, standard deviations (SD) and coefficient of variations (CV) of nine replicates run in three different experiments. Relative accuracy (R%), PCR efficiencies, correlation coefficient and linear regression equations for the DNA standard curves obtained as reported in the "Materials and methods" section, are shown.

		SYBR Green			TaqMan		
		$R^2 = 0.9998$ $y = -3.4097x + 35.898$			$R^2 = 0.9994$ $y = -3.3856x + 38.218$		
Genome equivalents	[a]Signal ratio	Mean Ct ± SD	Inter-assay CV	[b,c]R%	Mean Ct value ± SD	Inter-assay CV	R%
10^7	9/9	12.22 ± 0.05	0.41	87.73	14.47 ± 0.047	0.32	103.14
10^6	9/9	15.33 ± 0.01	0.07	108.00	17.76 ± 0.023	0.13	110.19
10^5	9/9	18.72 ± 0.02	0.10	109.40	21.42 ± 0.071	0.33	91.50
10^4	9/9	22.21 ± 0.01	0.03	103.27	24.71 ± 0.070	0.28	97.47
10^3	9/9	25.68 ± 0.1	0.39	99.46	28.09 ± 0.092	0.33	98.00
10^2	9/9	29.14 ± 0.15	0.51	95.66	31.73 ± 0.049	0.15	82.68
10^1	9/9	32.52 ± 0.14	0.42	98.16	34.54 ± 0.051	0.15	122.04
10^0	3/9	34.84 ± 1.05	3.02	NA	37.09 ± 1.07	2.88	NA

[a] Signal ratio: positive signals/total reactions.
[b] The relative accuracy (R%) indicates the degree of correspondence between results obtained by spectrophotometry and those obtained by our SYBR and TaqMan rt-PCR assays.
[c] NA, not applicable.

replicates of each DNA dilution was achieved when 10 or more target molecules were present, while 1 target molecule could be detected with a 33% probability (Table 5). Therefore, the limit of detection (i.e. minimum 10-fold dilution in which there are some positive replicates) with both chemistries was one genome (3.03 fg) equivalent, while the limit of quantification (minimum 10-fold dilution quantifiable with a >95% probability) was 10 genome (30.03 fg) equivalents, with mean TaqMan Ct values slightly higher (approximately 2 units) than SYBR Green Ct values (Table 5). Specific melting peaks with mean Tm of 70.72 °C ± 0.215 were generated from amplicons obtained amplifying from 30.3 ng to 3.03 fg of pre-purified *S. aureus* DNA. The accuracy of each method, as determined by linear regression analysis of cells and DNA standard curves, was very high. Indeed, linear correlation coefficients (R^2) values ≥ 0.9994 resulting in a good linearity of response for six orders of magnitude (from 10^1 to 10^7 *S. aureus* cfu equivalents or DNA genomic equivalents) were obtained by both TaqMan and SYBR Green rt-PCR assays (Table 5). A high degree of correspondence between the quantitative results obtained with our methods in respect to the spectrophotometric approach, over a wide dynamic range (at least 6 logs) was reached. Indeed, the mean relative accuracy (%R), expressed as the deviation of the actual value (calculated by the spectrophotometer) with respect to the theoretical value (extrapolated by the regression curve) (Anonymous, 2003), was 100.24 ± 7.48 and 103.14 ± 12.7 for the SYBR and TaqMan rt-PCR, respectively (Table 5). The slopes of the standard curves were very close to the theoretical optimum of −3.32, resulting in a SYBR and a TaqMan PCR efficiency of 96% and 98%, respectively. To assess the precision and reproducibility of our assay, the DNA standard curve was assayed three times in three different days, with three replicates for each concentration per run. The relevant CV ranging from 0.03% to 3.02% (Table 5) indicates a high precision of our TaqMan and SYBR Green rt-PCR assays. The corresponding standard deviation was always below 0.25 log copies, a value that is recognised as the maximum allowed for analytical variability.

3.4. Performances of the SYBR and TaqMan rt-PCR based egc$^+$ *S. aureus* detection in pure broth culture

A cell standard curve was constructed using ten-fold dilutions of a log-phase broth culture (0.6 OD_{600nm}) of the NCTC9393 *S. aureus* strain. As shown in Fig. 1, linear regression analysis of C_T values and cfu equivalents per reactions yielded R^2 values and slopes similar to those obtained with purified genomic DNA, with a SYBR and TaqMan rt-PCR efficiency of 98% and 102%, respectively. The overall detection limit for both rt-PCR assays was 10 cfu equivalents per 20 µL of reaction mixture although 1 cfu equivalent was detected with both approaches with a 33.33% probability. Specific melting peaks with mean Tm of 70.57 °C ± 0.199 were generated by our SYBR Green rt-PCR assay from amplicons obtained amplifying 10^7–10^0 cfu equivalent of *S. aureus* NCTC9393 per 20 µL of reaction mixture.

3.5. Rt-PCR applicability in artificially contaminated raw milk

In order to determine the diagnostic sensitivity, defined as a measure of the degree to detect the target pathogen in the biological matrix, three standard curves in raw milk were constructed using log-phase broth cultures of either a single *egc*$^+$ *S. aureus* strain, a mix of *egc*$^+$ *S. aureus* strains and a mix of *egc*$^+$ and *egc*$^-$ *S. aureus* strains. One microlitre aliquots of DNA extracted from each dilution of the three artificially contaminated raw milk standard curves were subjected to our optimised TaqMan and SYBR Green rt-PCR assays. Three replicates for each DNA concentration per run, in three different runs, were performed. Total viable count of raw milk, as determined on PCA, was $1.02 \pm 0.7 \times 10^6$ cfu mL^{-1}. No typical neither suspect colonies of *S. aureus* were found on BP-EY agar plates even after the enrichment step, whereas $1.22 \pm 0.8 \times 10^5$ cfu of coagulase negative staphylococci per mL of uninoculated milk were plate counted. As reported in Fig. 2 (panel A), our SYBR Green rt-PCR assay allowed the quantitative detection of *S. aureus* (either as single culture or as a mix of strains harbouring all the *egc* variants to date known) in raw milk as well as in the broth culture, not only in the presence of a background

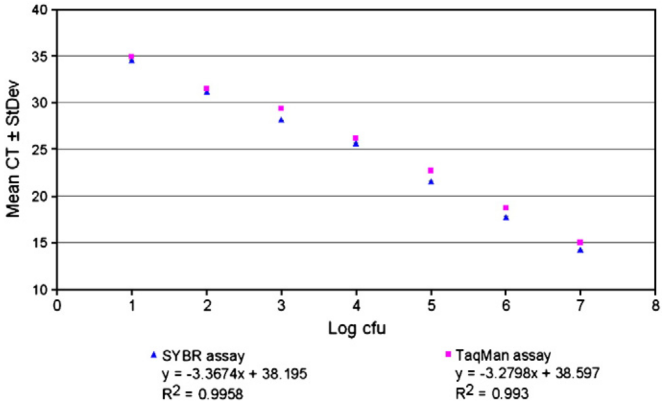

Fig. 1. TaqMan and SYBR rt-PCR based cell standard curves of *egc*$^+$ NCTC9393 *S. aureus* in log-phase broth culture. The main threshold cycle (Ct) ± standard deviation of nine replicates (three per run in three different runs) for each reaction is shown as compared to the log of cfu calculated per 20 µL of reaction mixture. The relative correlation coefficient (R^2) and linear regression equations are reported in the legend.

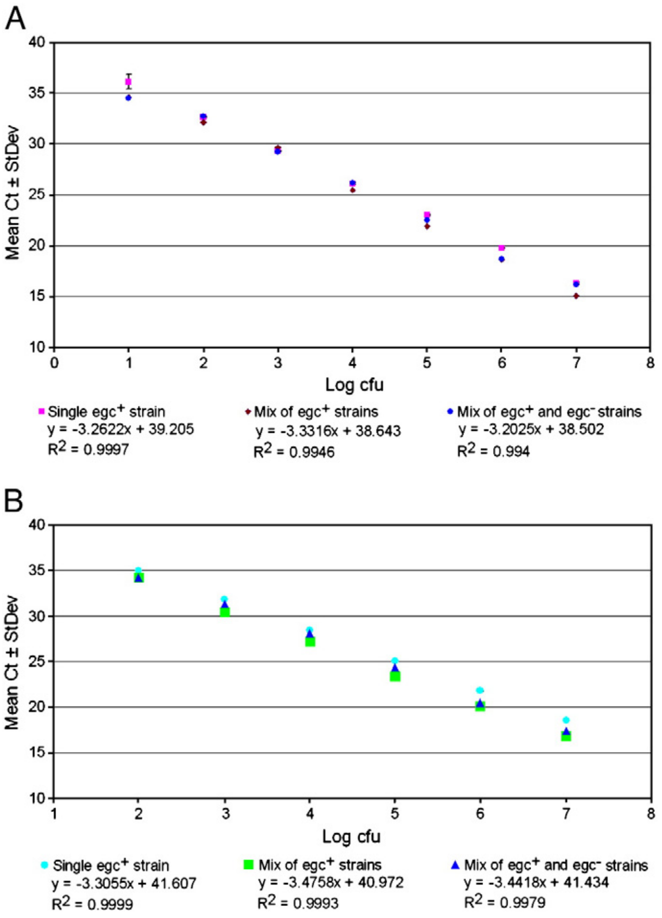

Fig. 2. Detection of *egc*$^{+}$ *S. aureus* in artificially contaminated raw milk, as single pure culture and as mix of 4 *egc*$^{+}$ strains either alone or in presence of high amount of egc-*S. aureus* strains by our SYBR Green (panel A) and TaqMan (panel B) rt-PCR assays. The main threshold cycle (Ct) ± standard deviation of nine replicates (three per run in three different runs) for each reaction is shown as compared to the log of cfu calculated per 20 μL of reaction mixture. The relative correlation coefficient (R^2) and linear regression equations are reported in the legend.

microflora but even when high levels of *egc*$^{-}$ *S. aureus* strains (10^6 cfu/mL of raw milk, as plate counted on BP-EY) were in the matrix. Indeed, the correlation coefficients revealed a good linearity of response for six orders of magnitude (from 1×10^1 to 1×10^7 *S. aureus* cfu equivalents per 20 μL of reaction mixture) with specific melting peaks Tm of 70.26 C° ± 0.48 and PCR efficiencies of 103, 100 and 105% for the single strain, the mix of *egc*$^{+}$ strains and the mix of *egc*$^{+}$ and *egc*$^{-}$ strains standard curve, respectively. As few as 10 cfu equivalents per 20 μL of amplification mixture (limit of quantification), i.e. 1000 cfu of *egc*$^{+}$ *S. aureus* (as single culture or as mix of *egc*$^{+}$ *S. aureus* strains harbouring all the variants to date known) per 1 mL of raw milk, could be detected with 100% probability by our optimised assay while 1 cfu equivalent per reaction mixture, i.e. 100 cfu/mL of raw milk, could be detected with a 33.33% probability (limit of detection). As reported in Fig. 2 (panel B), also with the TaqMan approach the raw milk standard curves of the single strain, the mix of *egc*$^{+}$ strains and the mix of *egc*$^{+}$ and *egc*$^{-}$ strains overlapped but revealed less sensitivity (LOQ = 10^2 cfu equivalents per reaction mixture of *egc*$^{+}$ *S. aureus* either singly, in mix and in mix with *egc*$^{-}$ *S. aureus* strains) as compared with the SYBR Green approach. Indeed, in all cases, linear correlation coefficients (R^2) values ≥ 0.9979 resulting in a good linearity of response for five orders of magnitude (from 1×10^2 to 1×10^7 *S. aureus* cfu equivalents) were obtained by our TaqMan rt-PCR assays (Fig. 2, panel B), with a PCR efficiency of 101, 94 and 95% for the single strain, the mix of *egc*$^{+}$ strains and the mix of *egc*$^{+}$ and *egc*$^{-}$ strains' standard curve, respectively.

The number of cycles in the rt-PCR assays herein developed was 40. This number was decreased to 37 cycles which were definitely employed in all the experiments with naturally contaminated milk samples to avoid the appearance of false positives (data not shown).

3.6. Detection and quantification of egc^{+} S. aureus in milk

The 13 raw milk samples contained on average 5.6 log cfu/mL of total viable mesophilic aerobic bacteria and 4.94 log cfu/mL of presumptive coagulase negative staphylococci, as plate counted on PCA and BP-EY, respectively. Eighty nine out of 130 isolates from typical, suspect and atypical *S. aureus* colonies were confirmed as *S. aureus* by species specific PCR targeting the *nuc*A gene. Seven out of the 13 raw milk samples (54%) resulted contaminated on average by 3.67 ± 0.31 log cfu/mL of *S. aureus*.

Fourteen out of 130 isolates tested positive for the presence of the *S. aureus egc*, as screened by our TaqMan and SYBR Green rt-PCR based identification of *egc*$^{+}$ *S. aureus* isolates resulting 100% effective as compared with the conventional PCR based approaches. As reported in Table 6, by means of our rt-PCR assays, counts ranging from 10^2 to 10^3 cfu/mL of *egc*$^{+}$ *S. aureus* were found in three out of 13 (23%) raw milk samples, with a high degree of correspondence to the quantitative results obtained by the reference culture-based method combined with the PCR based approach.

3.7. Characterisation of the egc of S. aureus wild strains isolated from milk

All the 14 *egc*$^{+}$ *S. aureus* isolates showed the REA-1 *egc* group pattern reported by Blaiotta et al. (2006). Based on the PCR-RFLP of

Table 6
Quantitative detection of *S. aureus* in milk.

Raw milk sample	[a]*S. aureus*	*egc*$^{+}$ *S. aureus* (log cfu/mL)					
		[b]Plating/PCR[a]	[c]Plating/rt-PCR[b]	[d]SYBR rt PCR	[e]R%	[d]TaqMan rt-PCR	[e]R%
1	3.64	2.70	2.70	2.59	95.79	2.74	101.64
8	4.00	3.84	3.84	3.95	102.90	3.95	102.99
13	3.84	2.88	2.88	2.89	100.31	3.08	107.02

[a] log cfu/mL of *S. aureus* in raw milk sample as determined by the reference culture-based method (ISO 6888-1) combined with conventional PCR (Brakstad et al., 1992) of DNA from typical and atypical colonies.

[b] log cfu/mL of *egc*$^{+}$ *S. aureus* in raw milk sample as determined by plating combined with conventional PCR (Blaiotta et al., 2006; Collery et al., 2009) of DNA from typical, suspect and atypical colonies.

[c] log cfu/mL of *egc*$^{+}$ *S. aureus* in raw milk sample as determined by plating combined with our rt-PCR assays of DNA from typical, suspect and atypical colonies.

[d] log cfu/mL of *egc*$^{+}$ *S. aureus* in raw milk sample as determined by our SYBR and TaqMan rt-PCR assays of DNA directly extracted from milk.

[e] The relative accuracy (R%), indicating the degree of correspondence between results obtained by the reference method (standard plating technique combined with either the conventional or rt-PCR) and those obtained by our SYBR and TaqMan rt-PCR assays, was expressed as percentage of numbers of log cfu/mL calculated by rt-PCR assay versus the reference method.

the egc *sei–seln* intergenic region described by Collery et al. (2009), these isolates were confirmed to harbour the *egc*1 type. These results were further corroborated by PCR detection of the *egc*-encoding genes of the 14 *egc*$^+$ *S. aureus* wild strains (Table 3).

4. Discussion

In the last decade, several efforts have been made to characterise the staphylococcal *egc*. PCR and sequencing of the *egc* encoded genes allowed individuating new *egc* enterotoxin-like open reading frames as well as allelic variants of already known *egc*-carried genes (Becker et al., 2004; Blaiotta et al., 2004; 2006; Jarraud et al., 2001; Letertre et al., 2003a; Thomas et al., 2006). These findings have been summarised by Collery et al. (2009), who, based on differences in the *egc* possession of individual genes/pseudogenes or nucleotide variations in the *egc*-encoding genes, distinguished at least four *egc loci* or subtypes (genes/pseudogenes given in transcriptional order): *egc*1 (*selo*, *selm*, *sei*, *ψent*1, *ψent*2, *seln*, and *seg*), *egc*2 (*selo*, *selm*, *sei*, *selu*, *seln*, and *seg*), *egc*3 ($selo_v$, $selm_v$, sei_v, $selu_v$, $seln_v$, and seg_v) and *egc*4 (*selo*, *selv*, *selu*2, *seln*, and *seg*). Simplex PCRs have been developed and used to detect specific gene sequences for *egc*-encoding genes/pseudogenes (Blaiotta et al., 2004; 2006; Letertre et al., 2003a; 2003b; McLauchlin et al., 2000; Omoe et al., 2002). Moreover, multiplex PCR, also in a real time format, for the simultaneous detection of some but not all the *egc*-harbouring genes have been reported (Bania et al., 2006; Letertre et al., 2003c; Loncarevic et al., 2005; Smyth et al., 2005; Zschöck et al., 2005). As a matter of fact, conventional PCR-based detection requires post-amplification confirmative analyses which, apart from the potential DNA carry-over, are time- and labour-consuming. Furthermore, due to differences either in the possession of individual genes or nucleotide variations, failure in detecting *egc*-encoding genes may occur resulting either in false negative results or a wrong typing of the *egc*. Alternatively, a specific intergenic region of the *egc* could be amplified and the relative amplicon subjected to a restriction endonuclease analysis, allowing the detection and discrimination of various *egc loci* (Blaiotta et al., 2004; 2006; Collery and Smyth, 2007; Collery et al., 2009). This restriction fragment length polymorphism (RFLP) PCR based approach requires additional multistep processing, further amplifying the aforementioned biases. Besides, also in this case, misprimings due to point mutations in the SEI1/SEG2 (McLauchlin et al., 2000) and PSE1/PSE4 (Letertre et al., 2003a) primer sets have been reported by Blaiotta et al. (2004, 2006), Collery and Smyth (2007) and Collery et al. (2009), so that three alternative primer sets were proposed by these authors, adding further PCRs and gel electrophoreses to the overall assay and making it even more time- and labour-consuming, therefore unsuitable for the rapid screening of large number of samples.

By contrast, rt-PCR assays are time saving (especially the “fast systems”) and require reduced handling, avoiding the risk of carry-over contaminations. Moreover, depending on the detection platform utilised, it is possible to process either 96 or 384 samples per run even in a multiplexing format.

Within this study we successfully developed a TaqMan and a SYBR Green rt-PCR targeting in a 82 bp highly conserved part of the PSE1/PSE4 (Letertre et al., 2003a) primer region, which has been widely recognised as the most conserved throughout the various *egc* loci (Blaiotta et al., 2006; Collery and Smyth, 2007; Collery et al., 2009). Both approaches showed very high selectivity for the detection of *egc*$^+$ *S. aureus* regardless of their origin and the *egc* type they hold, resulting in 100% inclusivity and exclusivity. In particular, we successfully tested the DNA of *egc*$^+$ *S. aureus* strains typed by Blaiotta et al. (2006) through REA-PCR of the polymorphic *egc selm–seg* intergenic region, also including the DNA of A900322, FRI137 and 382 F/AB-8802 *S. aureus* strains, later on classified by Collery et al. (2009) as harbouring the *egc* types *egc*1, *egc*2 and *egc*3, respectively. A900624 is the only *S. aureus* strain to date found to harbour the *egc locus egc*4 (*selo*, *selv*, *selu*2, *seln*, and *seg*) (Collery et al., 2009; Thomas et al., 2006). As it should be expected, since the *egc*4-encoded *seu*2 gene results from a simple deletion, namely one adenine at the 3′ end of the *ψent* pseudogene (Thomas et al., 2006), our primers and probe perfectly matched its homologous regions (GenBank accession number: EF030428; Thomas et al., 2006).

Combining our primers with the MGB probe in the optimised TaqMan rt-PCR assay the same specificity of the SYBR based assay was obtained but with a shift in the mean Ct of approximately 2 more units. This shift was confirmed also by comparing the DNA and cell standard curves obtained by our TaqMan and SYBR Green rt-PCR assays, given a PCR efficiency of about 100% for both approaches. This is probably due to the well known less sensitivity (but more specificity) of the TaqMan approach (Bustin and Nolan, 2004; Mackay, 2004).

The power of a well designed and optimised rt-PCR assay is proved by its ability to detect the target DNA in a vast excess of other non-target DNA (Mackay, 2004). Thus, raw milk is the ideal matrix to test the effectiveness of our rt-PCR assays due to its intrinsic complexity (in terms of composition and structure) and the likely presence of abundant background microflora affecting the efficiency of both the nucleic acid extraction and PCR amplification (Ercolini et al., 2004; Powell et al., 1994; Ramesh et al., 2002; Tamarapu et al., 2001; Wilson, 1997).

Our SYBR and TaqMan rt-PCR assays provided overlapping artificially contaminated raw milk based standard curves with R^2, slopes and PCR efficiency values similar to each other, indicating that both approaches potentially could be used to accurately quantify the *egc*$^+$ *S. aureus* population in a raw milk sample, regardless of the *egc* type and the background microflora present. Indeed, we successfully assayed a single *egc*$^+$ *S. aureus* strain raw milk culture as well as a mixed *S. aureus* strain raw milk culture harbouring all the *egc* types to date known, even in conditions simulating a staphylococcal food poisoning, i.e. in the presence of 10^6 *egc*$^-$ *S. aureus* strains harbouring some of the commonest enterotoxin genes associated to this syndrome. Therefore, our rt-PCR based approach may significantly contribute to shed light on the actual role of *egc*$^+$ *S. aureus* strains in the staphylococcal food poisoning and other clinical syndromes associated with the consumption of milk and milk based products.

Using our optimised rt-PCR conditions, we were able to quantitatively detect at least about 1×10^3 and 1×10^4 cfu of this pathogen per mL of raw milk (10 and 100 cfu equivalents of *egc*$^+$ *S. aureus* per reaction mixture) by our SYBR Green and TaqMan rt-PCR assay, respectively. To further lower these quantification limits, options available could be: i) performing the DNA extraction from a higher amount of sample and then concentrating it, running the risk of increasing the presence of inhibitors, that, on the other side, may negatively affect the sensitivity of our assay; ii) inserting an enrichment step, turning down the speed of our assays.

Both approaches performed well also when applied to real raw milk samples with excellent diagnostic specificity and quantification accuracy as compared with the reference culture-based method coupled with the conventional PCR approach. Moreover, our culture-independent rt-PCR based approaches, with an overall detection time on average of 6 h, were less time- and labour-consuming than the conventional method which requires plate counting, isolation, purification by streaking, biochemical confirmation of presumptive *S. aureus* isolates and finally (in the best case, i.e. if the colonies may be easily purified, after one week) PCR screening for the presence of the *egc*.

Three out of 13 samples resulted to contain from 10^2 to 10^3 cfu/mL of *S. aureus* harbouring the Collery et al. (2009) *egc* type1/Blaiotta et al. (2006) REA group-1, in accordance with several researchers who reported a high frequency of detection of enterotoxin genes belonging to the *egc* in raw milk and hypothesised a significant role of *egc*$^+$ *S. aureus* strains in staphylococcal food poisoning, in clinical and sub-clinical mastitis as well as in other clinical diseases (Bania et al., 2006; Becker et al., 2003; 2004; Chiang et al., 2008; Jarraud et al., 1999; 2001; Lawrynowicz-Paciorek et al., 2007; Mempel et al., 2003;

Morgan et al., 2007; Naik et al., 2008; Rall et al., 2008; Shuiep et al., 2009; Smyth et al., 2005; Zschöck et al., 2005).

In conclusion, we have successfully developed a TaqMan and a SYBR Green rt-PCR assay targeting a universal region of the *S. aureus egc*. Given their 100% selectivity against a vast panel of reference and wild *S. aureus* strains, both approaches are powerful diagnostic tools for a quick, reliable and high throughput routine screening of egc^{+} *S. aureus* isolates. Due to the wide dynamic ranges of detection and the high sensitivity demonstrated in a complex matrix such as raw milk with numerous background microflora, even in conditions mimicking a staphylococcal food poisoning, the SYBR Green approach allowed the precise and rapid quantitative detection of egc^{+} *S. aureus* but, unlike the TaqMan, without losing its high sensitivity. For these reasons, the MGB TaqMan probe is not considered essential. However, the TaqMan assay, unlike the SYBR approach, does not require melting curve analysis further reducing time to results, whereas the SYBR rt-PCR assay, in addition to being specific and more sensitive, is also less expensive and therefore more affordable for most laboratories.

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