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Real-Time Quantitative PCR of *Staphylococcus aureus* and Application in Restaurant Meals

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ABSTRACT

Staphylococcus aureus is considered the second most common pathogen to cause outbreaks of food poisoning, exceeded only by *Campylobacter*. Consumption of foods containing this microorganism is often identified as the cause of illness. In this study, a rapid, reliable, and sensitive real-time quantitative PCR was developed and compared with conventional culture methods. Real-time quantitative PCR was carried out by purifying DNA extracts of *S. aureus* with a *Staphylococcus* sample preparation kit and quantifying it in the LightCycler system with hybridization probes. The assay was linear from a range of 10 to 10^6 *S. aureus* cells ($r^2 > 0.997$). The PCR reaction presented an efficiency of >85%. Accuracy of the PCR-based assay, expressed as percent bias, was around 13%, and the precision, expressed as a percentage of the coefficient of variation, was 7 to 10%. Intraday and interday variability were studied at 10^2 CFU/g and was 12 and 14%, respectively. The proposed method was applied to the analysis of 77 samples of restaurant meals in Valencia (Spain). In 11.6% of samples *S. aureus* was detected by real-time quantitative PCR, as well as by the conventional microbiological method. An excellent correspondence between real-time quantitative PCR and microbiological numbers (CFU/g) was observed with deviations of <28%.

Restaurant meals are in demand because of the growing tendency to eat in places other than the home *(24)*. The foods provide a source of readily available, nutritious meals. However, questions have been raised about the safety and microbiological quality of these products *(1, 7, 9–12, 18–20, 21–23, 25–28)*. In Spain, *Staphylococcus aureus* is the second most common pathogen causing outbreaks of food poisoning, exceeded only by *Campylobacter*. Foods can be contaminated by improper holding temperatures and by food handlers who do not observe hygienic rules *(22)*. Quantification of this pathogenic microorganism presents useful information about systems contamination and the extent of downstream processing of foods and the necessity of comprehensive and correct handling to guarantee quality control before consumption *(14)*.

Rapid, sensitive, and infallible detection methods are required to determine in a routine way the pathogens in food *(17)*. The use of conventional methods for detecting staphylococci in foods consist of propagation in selective enrichment media followed by microbial and biochemical tests, which are cumbersome and time-consuming. That is why modern molecular biological techniques for the detection and differentiation of pathogens have gained in importance in food safety. The use of nucleic acid–based assay systems, like the real-time quantitative PCR LightCycler system, has led to improved surveillance. These methods are even capable of detecting nonviable and viable pathogenic microorganisms *(4, 5, 8, 13, 15, 16, 31)*. Hein et al. *(13)* applied the LightCycler system to the quantification of *S. aureus* in cheese, showing its suitability for fast and accurate quantification.

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The objective of this study was to develop a simple and rapid method of determining *S. aureus* by real-time quantitative PCR. The proposed procedure for detecting and identifying *S. aureus* was applied to samples of 77 restaurant meals from Valencia (Spain). Results were evaluated by comparison with conventional culture procedures.

MATERIALS AND METHODS

Bacterial strains. Type strains of *Staphylococcus aureus* (CECT 59, 82, 239, 240, 435), *S. epidermidis* (CECT 231, 232, 4184), *S. saprophyticus* (CECT 235), *S. hominis* (CECT 234), *S. capitis* (CECT 233), *Clostridium perfringens* (CECT 486), *Escherichia coli* (CECT 45), *Listeria monocytogenes* (CECT 911), *Salmonella choleraesuis* subsp. *choleraesuis* (CECT 409), and *Yersinia enterolitica* (CECT 500) were obtained from the Spanish Type Culture Collection (Burjassot, Spain). The culture was grown overnight in tryptic soya agar (TSA; Merck, Darmstadt, Germany) at 37°C.

Inoculated samples. Lettuce, egg, chicken, sausage, cheese, chocolate, and apple samples were purchased from different restaurants in Valencia (Spain). Each sample, in its original package, was placed in an individual sterile polyethylene bag and transferred to the laboratory in a cool box within 1 h. The cool box was maintained at 3 to 5°C with ice. On arrival at the laboratory, each sample was immediately placed with sterile disposable gloves in separate Stomacher bags with lateral filter. First, samples were analyzed by microbiological culture techniques described elsewhere *(25)*. Negative staphylococcus samples were selected to be inoculated. Samples were stored at 4°C until the moment of extraction and analyzed within 24 h.

Preparation of the staphylococcus suspension. Staphylococci were cultured each week on TSA. One loopful of the culture was inoculated into a flask with tryptone soya broth and incubated with shaking for 24 h at 37°C. The concentration of this stock

suspension was confirmed by serial dilutions in buffered peptone water. These dilutions were plated onto Baird-Parker agar and incubated for 24 h at 37°C.

Preparation of inoculated staphylococci. A food sample (100 g), trimmed and cut with a sterilized lancet, was placed into a sterilized plastic bag and sealed to facilitate inoculation. Each bag was inoculated with 1 ml of a different suspension of all studied staphylococci (10^1, 10^2, 10^3, 10^4, and 10^5 CFU/ml) then shaken gently 30 times to ensure distribution of the microorganisms in the product. Samples were stored overnight at 4°C to achieve attachment of *S. aureus* before quantification by microbiological culture techniques and PCR methods.

Preenrichment procedure. Inoculated samples (25 g) were placed in a Stomacher bag containing 250 ml of buffered peptone water and stomached for 2 min at 230 strokes per minute. After that, the liquid was poured through the lateral filter (to block the suspended food particles) into an erlenmeyer flask and incubated overnight at 37°C. A 1-ml suspension was withdrawn for the isolation of DNA by PCR assay. The remainder of the suspension was used for determination by microbiological culture techniques.

Microbiological culture techniques. Serial decimal dilutions in buffered peptone water were prepared. Samples (0.1 ml) of appropriate dilutions were spread on Giolitti-Cantoni broth agar with 3.5 ml/liter potassium tellurite solution (Oxoid, Hampshire, UK) and incubated at 37°C for 24 to 48 h. The positive samples were spread onto Baird-Parker agar supplemented with egg yolk emulsion (Oxoid) and kept at 37°C for 24 h. After incubation, colonies with typical *S. aureus* morphology (i.e., black, convex, with or without light halo on Baird-Parker agar) were subjected to Gram staining, examined microscopically, tested for catalase reaction, confirmed with an agglutination Staphytect Plus test (Oxoid), and counted.

PCR assay: DNA isolation. DNA of a 1-ml sample of the previous suspension was isolated with a High Pure PCR template preparation kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Briefly, the sample was centrifuged at 3,000 × *g* for 5 min to pellet bacteria. The pellet was suspended in 200 μl of phosphate-buffered saline, and 5 μl of lysozyme was added. The mixture was incubated at 37°C for 30 min to destroy the *Staphylococcus* cell wall. The supernatant was discarded, and the pellet was resuspended in 200 μl of binding buffer and 40 μl of proteinase K, mixed immediately, and incubated at 72°C for 10 min to cause cell lysis. After adding 100 μl of isopropanol, the mixture was placed into the upper reservoir of a combined filter tube–collection assembly and centrifuged at 8,000 × *g* for 1 min. The DNA was retained in the filter and the flow-through was discarded. The filter tube was twice washed with 500 μl of the washing buffer, which was eliminated by centrifugation at 8,000 × *g* for 1 min. Finally, the filter tube was inserted in a clean 1.5-ml reaction tube, treated with 200 μl of prewarmed (70°C) elution buffer, and centrifuged at 8,000 × *g* for 1 min. Aliquots of the eluted DNA were subjected to LightCycler PCR within 2 h or transferred to −80°C for long-term storage.

PCR assay: LightCycler PCR. The PCR reaction was performed in a total volume of 20 μl containing 5 μl of DNA extract, 15 μl of LightCycler DNA master hybridization mixture (*Taq* DNA polymerase, reaction buffer, dNTP mixture, primer, and hybridization probe mixture specific for *Staphylococcus* DNA, *Staphylococcus*-specific internal control, and nuclease-free PCR-grade H_2O; Roche Diagnostics). The reaction mix was loaded onto glass capillary tube and snap-sealed by a plastic cap (Idaho Technology).

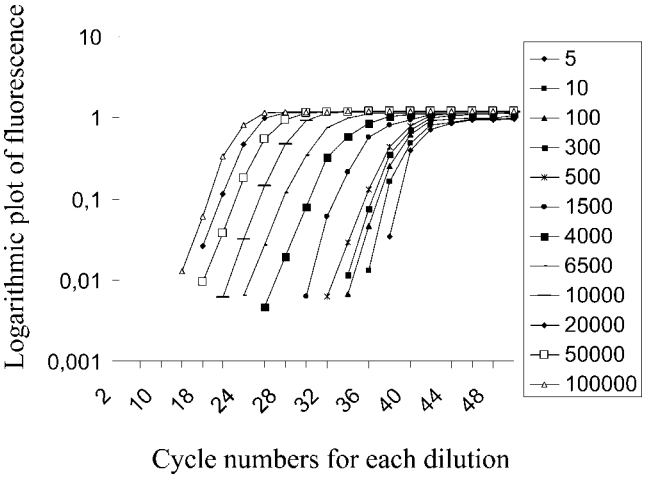

FIGURE 1. *Development of the fluorescence signal in the serially diluted control samples used for construction of the standard curves. Logarithmic plot of fluorescence versus cycle number for each dilution.*

The PCR LightCycler reaction was carried out as follows: one cycle of denaturation at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 10 s, 50°C for 15 s, and 72°C for 10s at a programmed temperature transition rate of 20°C/s. On completion of amplification, a melting curve was produced by holding the reaction at 95°C for 60 s then at 40°C for 60 s, followed by slow heating at a transition rate of 0.1°C/s to 80°C.

Fluorescence was monitored at regular intervals during the annealing phase and continuously throughout the melting phase. The cycle at which fluorescence exceeds background, called the threshold cycle (C_T), is inversely related to the initial bacterial copy number. Quantification of the bacterial load in each sample was obtained by interpolation of C_T values from the generated standard curve with the LightCycler software. The specificity of amplicon generation was verified by confirming the melting peak of the melting curve.

Real samples. Seventy-seven samples were taken from restaurants in Valencia (Spain) from September 2003 to March 2004. Samples were taken in the latest elaboration stages, placed in sterilized plastic bags, transported in ice chests to the laboratory, and cultured on the same day. The most consumed meal group during a week was selected and distinguished according to the major component—salad, desserts, egg, meat, milk, and pasta products—as shown in Table 1.

RESULTS

Standard curves for the proposed real-time PCR assay. PCR products were directly detected by monitoring the increase in fluorescence from the dye-labeled *S. aureus*–specific DNA probe, which resulted in an increase in fluorescein (FAM) fluorescence emission (F1) with a concomitant decrease in tetramethylrhodamine (TAMRA) emission (F2) that is measured by the ratio F1/F2.

After real-time PCR, logarithmic values of fluorescence (*y* axis) for each dilution were plotted against cycle number (*x* axis) (Fig. 1). Furthermore, a baseline was set just above the fluorescence background, and a crossing

TABLE 1. *Foods and their ingredients selected in restaurants from Valencia (Spain)*

Sample type	No. of samples	Ingredients
Salads		
Summer salad	5	Potato, onion, tomato, olive oil, salt
Russian-type salad	3	Mayonnaise, potato, carrot, tuna, peas
Valencian salad	3	Tomato, onion, lettuce, olive, tuna, hard-boiled egg
Egg products		
Spanish potato omelette	7	Potato, onion, egg, olive oil
Mushroom omelette	4	Potato, mushroom, egg, olive oil
Tuna omelette	4	Potato, tuna, egg, oil
Marrow omelette	4	Potato, marrow, egg, olive oil
Meat products		
Stew with veal	3	Veal, onion, oil, pepper
Breast of chicken	3	Chicken, egg, bread, oil
Spanish sausage	3	
Ham	3	
Sausage	3	
Milk products		
Fresh cheese	3	Cheese
Mature cheese	3	Cheese
Rice with milk	3	Rice, milk, sugar
Pasta products		
Spaghettini	3	Spaghettini, tomato salsa, onion, tuna
Lasagne	3	Cream, onion, minced meat, pasta
Ravioli	1	Pasta, cream, pepper, butter, cheese
Desserts		
Cream caramel	3	Egg, milk, caramel, sugar
Pudding	3	Egg, milk, caramel, sugar
Custard	5	Egg, milk, sugar, biscuit
Chocolate soufflé crepes	1	Egg, milk, flour, sugar, chocolate
Fruit salad	4	Orange, apple, kiwi, banana, sugar, orange juice
Total	77	

point was determined with the amplification curves obtained during the exponential phase of amplification.

There was a direct relationship between the cycle number corresponding to the crossing point and the log concentration of *S. aureus* cells initially present in the real-time PCR reaction.

Figure 2 illustrates one of the standard curves for the real-time PCR of *S. aureus* cells on the basis of the dilutions of DNA, showing a linear relationship between log CFU and C_T. These standard curves were log-linear for at least four orders of magnitude from 5 to 10^5 *S. aureus* cells. For the same batch of reagents, only two points of the linear standard regression curve were included each time that the analysis was conducted because there is an option in the LightCycler software that corrects the values of the standard curve according to the variation in the values of these two points ($r^2 > 0.997$, slope = 3.61). When new serial dilutions series from the same DNA purification was used in a separate PCR experiment, the slopes in the two runs were almost identical (3.54, 3.63). When DNA from a separate isolation was used, the variation in the slopes of the standard curves was no larger than that between different serial dilutions from the same DNA isolation (r^2 remained constant).

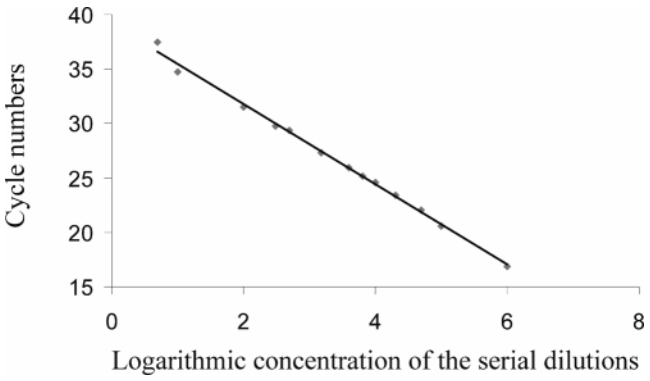

FIGURE 2. *Standard curve generated from the crossing points (cycle numbers) plotted against the logarithmic concentration of the serial dilutions.*

A standard curve was included each time new batches of reagents were used. However, the observed variations were as small as those reported for different DNA isolations.

The efficiency of the PCR reaction *(16)*,

$$E = 10^{+1/-m} - 1$$

TABLE 2. *Accuracy and precision of a proposed real-time quantitative PCR method*[a]

S. aureus cells (concn)	Accuracy (% bias)	Precision (% CV)
10	45	17
100	15	12
300	13	10
500	−10	10
1,500	+5	8
4,000	−4	8
6,500	−4	7
10,000	−5	8
20,000	−4	10
50,000	−9	8
100,000	18	11
1,000,000	25	12

[a] Values were obtained according to Anonymous *(3).*

where *m,* the slope of the standard curve, was typically >85%.

The number of *S. aureus* cells can be described by the equation

$$C_T = -3.6 \log \text{CFU} + 39.17$$

where

$$\text{CFU} = 10^{[(C_T - 39.17)/-3.6]}$$

Accuracy and precision of the proposed real-time PCR assay. When the assay was conducted with 3×10^2 to 5×10^4 *S. aureus* cells *(3)* (Table 2), the accuracy of the assay, expressed as percent bias, was around 13% and was calculated as % bias = [(mean of observed value − nominal value)/nominal value] × 100%. The precision, expressed as a percentage of the coefficient of variation (% CV), was 7 to 10% and was calculated as % CV = (SD/mean of observed value) × 100%, where SD is the standard deviation. On the basis of the precision of the assay, the limit of quantitation was estimated to be five *S. aureus* cells.

Intraday and interday variability of the proposed real-time PCR assay. The intraday variability, calculated by determining % CV of quadruplicate PCR amplifications, was 12% for 100 *S. aureus* cells, and the interday variability, calculated by determining the % CV of the assay on the basis of quadruplicate analysis of the same concentration performed on 4 days, was 14%.

Sensitivity of the proposed real-time PCR assay. The detection limit of the PCR assay was estimated to be 5 CFU for each bacterial culture without matrix-dependent effects.

Selectivity of the proposed real-time PCR assay. The selectivity was appropriate because the real-time PCR assay detected all isolates of *S. aureus* tested (5 including 240) and all other *Staphylococcus* spp. (four isolates) and did not distinguish between any of the other bacteria tested. In addition, the assay differentiated between *S. aureus* and other coagulase-negative *Staphylococcus* species because *S. aureus* formed a unique melting peak at about 62°C, whereas the other species amplified DNA and gave melting peaks at about 46 to 57°C.

TABLE 3. *Results of* S. aureus *detection in samples by real-time quantitative PCR and a conventional culture method*[a]

Sample	No. of positive samples		Positive ratio (%)	
	PCR	Culture	PCR	Culture
Salads	2/11	2/11	18.18	18.18
Egg products	2/19	2/19	10.52	10.52
Meat products	2/15	2/15	13.33	13.33
Milk products	1/9	1/9	11.11	11.11
Pasta products	0/7	0/7	0	0
Desserts	2/16	2/16	12.5	12.5

[a] According to the method of Soriano et al. *(21).*

Screening restaurant samples by real-time PCR and conventional microbiological cultures. A set of 77 meals from restaurants from Valencia (Spain) were screened for the presence of *S. aureus.* By means of proposed quantitative PCR, counts of *S. aureus* were found in 9 (11.6%) of the 77 samples, a result similar to that of the conventional microbiological procedure (Table 3). All culture-positive samples were also positive by real-time PCR. Quantitative results of *S. aureus* in these studied samples are presented in Table 4. Salads showed the highest concentration prevalence with 7,300 and 6,958 CFU/g for culture methods and PCR, respectively (Table 4). The pathogen was not isolated in pasta products by any of the procedures used.

DISCUSSION

Performance of the proposed real-time PCR assay. The proposed assay can be used daily and is an easy method for detecting *S. aureus* contamination. The high selectivity of this primer-probe set and its ability to differentiate *S. aureus* from other staphylococcus species by the melting curves has been demonstrated by the inoculation of different *Staphylococcus* and other bacterial species. The linearity and small variation of the standard curves, together with the constant efficiency of the PCR, confirmed that the assay was well suited to quantitative measurements of staphylococci in foods.

The limit of detection of this PCR assay achieved the requirements of legislation *(2).* The lack of matrix-dependent effects observed disagrees with the results reported by Yang et al. *(31),* which show a limit of detection for *C. jejuni* in chicken meat and milk that is higher than in the standard curves because of the presence of inhibitory substances in the samples. The dissimilarity could be explain because the DNA extraction step is quite different. The procedure used in this study was more selective because it included several specific clean-up steps that reduced other components in the final extract.

More in accordance with this study, Hein et al. *(13)* assay two real-time quantitative PCR approaches for quantifying *S. aureus* cells in artificially contaminated cheese of

TABLE 4. *Occurrence of* S. aureus *in restaurant meals from Valencia (Spain)*

Sample type	PCR method		Microbiological method[a]	
	Incidence	CFU/g	Incidence	CFU/g
Salads	2/11		2/11	
Summer salad	1/5	615	1/5	560
Russian-type salad	1/3	6,958	1/3	7,300
Valencian salad	0/3		0/3	—
Egg products	2/19		2/19	
Spanish potato omelette	1/7	619	1/7	560
Mushroom omelette	0/4		0/4	
Tuna omelette	1/4	1,075	1/4	1,000
Marrow omelette	0/4		0/4	
Meat products	2/15		2/15	
Stew with veal	0/3		0/3	
Breast of chicken	0/3		0/3	
Spanish sausage	2/3	133, 3,513	2/3	115, 3,700
Ham	0/3		0/3	
Sausage	0/3		0/3	
Milk products	1/9		1/9	
Fresh cheese	1/3	251	1/3	220
Mature cheese	0/3	—	0/3	—
Rice with milk	0/3	—	0/3	—
Pasta products	0/7		0/7	
Spaghettini	0/3	—	0/3	—
Lasagne	0/3	—	0/3	—
Ravioli	0/1	—	0/1	—
Desserts	2/16		2/16	
Cream caramel	0/3	—	0/3	—
Pudding	1/3	636	1/3	580
Custard	0/5	—	0/5	—
Chocolate soufflé crepes	0/1		0/1	—
Fruit salad	1/4	275	1/4	250
Total	9/77		9/77	

[a] According to the method of Soriano et al. *(21).*

different types, demonstrating that matrix-dependent effects were observed only for Camembert, the composition of which is very complex and with high fat content.

Screening restaurant samples by real-time PCR and conventional microbiological culture. A set of 11 salads, 19 egg products, 15 meat products, nine milk products, five pasta products, and 16 desserts were screened for the presence of *S. aureus.* The results from the two methods showed that salads, egg products, meat products, milk products, and desserts are contaminated with *S. aureus.*

Although the number of samples for each food category is too small to come up with any meaningful interpretations of why certain food items are more highly contaminated than others, some remarks can be made by comparing the data obtained in this study with those reported by other authors.

Staphylococci were not detected in the pasta products analyzed in this study. However, in a study carried out by Trovatelli et al. *(29),* 6 (29%) of 60 lots of ''homemade'' pasta exceeded the legislated standard.

Staphylococcus aureus has been a widely detected in Spanish sausage. The occurrence of enterotoxigenic staphylococci is normally associated with meat products *(22),* as reflected in previous studies *(26, 27).*

Staphylococcus aureus counts were higher in salad products in this study than has been reported by other authors *(12, 18, 25, 27)* and lower than established by Bocklehurst et al. *(7).* Russian-type salad has the highest value of *S. aureus* compared with other salads.

Egg and dessert products have egg as an ingredient, which is considered a traditional vehicle of illness transmission. This microorganism has been widely detected in these products *(1, 21, 23, 26, 27).* Eisenberg et al. *(10)* presented a case of known staphylococcal food poisoning from the consumption of an omelette aboard a commercial aircraft.

Staphylococcal food poisoning outbreaks have been well documented from dairy products *(9).* Eleftheriadou et al. *(11)* studied this microorganism in several foods from the Republic of Cyprus, fresh cheese being the most common vehicle. This product has water activity (a_w) higher than mature cheese and a salt content lower than other types of cheese.

Illness requires staphylococci at 10^5 CFU/g *(30).* In our

study, the positive samples had values lower than this guideline.

Comparison with the conventional method. Three advantages were observed with the proposed method. First, the data indicated 100% correlation between real-time quantitative PCR and the conventional microbiological method. Second, real-time quantitative PCR provides results immediately after completion of the amplification reaction and does not require further processing of the sample or opening of the test tubes, which greatly reduces the risk of carryover contamination. Finally, the universal real-time quantitative PCR assay is a rapid way to screen the isolated strains.

Real-time quantitative PCR can be applied successfully to the determination of *S. aureus* in food because of good correlation of the results with those obtained by conventional microbiological methods. The most impressive advantages of this system are speed and sensitivity.

The application of this method in real samples from restaurants indicated that the existing food safety assurance system, which includes a hazard analysis critical control point (HACCP) system, often fails to produce meals free of pathogenic staphylococci, indicating a need to re-evaluate HACCP.

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