

Characterisation of *Staphylococcus aureus* strains isolated from food for human consumption

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Summary

An investigation was conducted to evaluate *Staphylococcus aureus* contamination in various types of food of animal origin. Of the 350 samples examined, 14.0% were found to be contaminated with *S. aureus*. Prevalence rates varied according to type, namely: 19.3% for fresh meat products, 13.3% for fresh cheeses, 3.6% for bakery products and 7.7% for deli products. The isolated *S. aureus* strains then underwent 16S rDNA polymerase chain reaction (PCR) followed by reverse latex agglutination tests to identify enterotoxigenic strains. The results were compared with data obtained by subjecting the same strains to tests for the genes coding for the *S. aureus* enterotoxins (SEs) *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei* and toxic shock syndrome toxin-1 (TSST-1). Reversed passive latex agglutination (RPLA) testing revealed that 16.3% of strains (8/49) produced enterotoxins, while on PCR, 48.97% (24/49) were found to carry one or more genes for the production of SEs, and were therefore potentially enterotoxigenic.

Keywords

Enterotoxin, Food, Italy, PCR, Polymerase chain reaction, Reversed latex agglutination test, Reversed passive latex agglutination, RPLA, Safety, *Staphylococcus aureus*.

Introduction

Staphylococcus aureus is one of the most commonly identified bacteria that causes food poisoning. Between 1993 and 1998, between 0.9% (the Netherlands) and 13.6% (France) of

food poisoning outbreaks recorded in Europe were caused by *S. aureus*. In Italy, 1.8% of cases reported in 1998 were attributed to this micro-organism. In the United States, it has been estimated that there are 185 000 cases of food poisoning caused by staphylococcal enterotoxins every year, resulting in 1 750 hospital admissions and two deaths annually (12, 29).

The large numbers of carriers (more than 30%-50% of the population), the contamination of food or one of its ingredients during handling, storage at unsuitable temperatures and the capacity of the micro-organism to develop in a wide range of pHs, free water concentrations and sodium chloride concentrations – and therefore a wide range of food products – are the main epidemiological features that create the ideal conditions for an outbreak of *S. aureus* food poisoning.

Investigations during outbreaks of food poisoning have revealed that meat and milk-based products are among the most frequently involved matrices (18, 30). Knowledge of the health conditions of animals – whether carriers or infected animals – destined for the production of food is therefore fundamental in establishing the contamination of food products (18). Raw milk is a vehicle that is widely recognised as transmitting microbial pathogens and high percentages of samples positive for *S. aureus* are found positive in milk. Contrasting data are reported in the literature: Oliver *et al.* (24) reported percentages of bulk tank milk contaminated by *S. aureus* that ranged from 27.4% to 37% in dairy farms but a lower level

of isolation rates was reported by D'Amico *et al.* (10).

The currently tolerated levels of *S. aureus* and its toxins in fresh cheeses and meat are established in European Commission (EC) Regulation 1441/2007 (8). Foods commonly associated with staphylococcal enterotoxins poisoning are meat (beef, pork and poultry) and meat products (ham, salami), salads, cream-filled bakery products and dairy products.

In Italy, a study conducted from 2000 to 2002 examined 9 869 samples of food of animal origin taken from retail outlets throughout the country. Of these, 19.7% were found to be contaminated with *S. aureus*. Here too, meat and cheese products were found to be the most frequently contaminated (23.1% and 20.7%, respectively). Other products, such as bakery products (3.5%), pasta (7.6%) and deli products (6.4%) played a secondary role (13).

Work surfaces and equipment used to prepare foods are an important source of indirect contamination. A study on the hygiene of working conditions in a catering facility revealed that 25% of swabs taken from work surfaces were contaminated with *S. aureus* and 71.7% of ready-to-eat products handled after heat treatment were contaminated (19).

The pathogenic action of this micro-organism is determined by the capacity of some strains to synthesise one or more *S. aureus* enterotoxins (SEs). Enterotoxigenic strains are estimated to account for about 25% of all isolated strains (18). However, data from various studies of food matrices give different and sometimes higher values, depending on the SE under consideration. In one study in Italy, 45.2% of strains isolated from meat products were found to produce enterotoxins as follows: staphylococcal enterotoxins C (SEC) (51.5%), staphylococcal enterotoxins A (SEA) (30.3%) and, to a lesser extent, staphylococcal enterotoxins B (SEB) and staphylococcal enterotoxins D (SED). A total of 59.9% of strains isolated from dairy products were found to produce enterotoxins, as follows: SEA (26.7%), SEC (28.1%), SED (25.8%), SE (A, D) (28.8%) (13).

SE production can begin at low bacterial concentrations (10^3 /g) after as little as 2 h incubation at 37°C. In humans, symptoms can occur after the ingestion of very small quantities of toxin (0.5 ng/ml) (2). One of the main properties of SE is heat resistance. This may be enhanced by the chemical and physical conditions in the food (pH, sodium chloride concentration) which can cause the biological activity to be maintained even after heat treatment used in industrial sterilisation (2, 18).

Some strains are able to produce one or more additional extracellular proteins, such as toxic shock syndrome toxin-1 (TSST-1) (11, 21, 23, 26, 27). In humans, this syndrome affects the immune system. The pathogenic action is triggered by the induction of a massive release of cytokines, hypersensitivity to the endotoxins produced by the body itself and vascular damage caused by both the liberation of vasoactive substances and the direct action of the toxin on the endothelium (11).

TSST-1 has been identified as the cause of lesions found in newborn babies dying of sudden infant death syndrome and in heart conditions diagnosed in subjects with Kawasaki syndrome, an autoimmune disease. In the United States, TSST-1 has been identified as the cause of an infantile form of acquired heart disease (11). Some authors have suggested that TSST-1 may play a role in the pathogenesis of bovine, ovine and caprine staphylococcal mastitis, as *S. aureus* strains carrying the toxic shock toxin (*tst*) gene and producing toxin have been identified in animals with both clinical and subclinical signs. The pathogenic action has not yet been established, although TSST-1 is thought to act as a 'superantigen' in the mammary immune system (30).

This study provides a summary of the evaluation of *S. aureus* contamination levels in various food products of animal origin and characterisation of non-epidemiologically related enterotoxigenic strains using a method based on polymerase chain reaction (PCR) to identify genes coding for the production of SE (17, 20) and TSST-1 (3, 4, 14, 28). The results were compared with data obtained from the application of the reversed passive latex

agglutination test (RPLA) for the detection of toxin produced *in vitro* by these strains.

Materials and methods

Sampling

Over a one-year period, 350 samples of meat, fresh cheeses, bakery products and deli products were examined. They were selected according to their availability and popularity of consumption. The products were sampled from retail outlets in the Abruzzi region of Italy and were refrigerated rapidly and transferred to the test laboratory.

Quantification of *Staphylococcus aureus*

Coagulase-positive *Staphylococcus* was quantified using the operating procedures reported in the second part of International Organization for Standardization (ISO) 6888:1999. After

incubation at 37°C, typical colonies were subcultured on trypticase soy broth (TSA, Biolife Italiana, Milan) and subjected to the Staphytest Plus latex test (Oxoid, Basingstoke) for the identification of *S. aureus*, according to method 995.12:2000 described by the Association of Official Analytical Chemists (AOAC). Biochemical identification was completed using the API Staph micromethod (BioMérieux, Marcy l'Étoile) in accordance with the manufacturer's instructions.

Identification of strains and detection of genes coding *Staphylococcus aureus* enterotoxins

Strains of *S. aureus* isolated from foods underwent further analysis with 16S rDNA PCR (14, 15). The oligonucleotide sequences of the primers used for the 16S rDNA region and genes coding for SEs are presented in Table I and are in line with the DNA sequences

Table I
Nucleotide sequences of the primers chosen to detect the genes coding for *Staphylococcus aureus* toxins and for 16S rDNA PCR

Gene ^(a)	Primer	Primus sequence	GenBank access no.	Location	bp	Reference strain	Ref.
<i>sea</i>	SEA1	TGGAAACGGTTAAAACGAA	M18970	490-590	120	3102TE	5
	SEA2	GAACCTTCCCATCAAAAACA		610-590			
<i>seb</i>	SEB1F2	GCAGAGAGTCAACCAGATCC	M11118	322-341	624	ATCC ^(b) 14458	11
	SEB2F2	TGGTGCAGGCATCATGTCAT		936-917			
<i>sec</i>	SEC1	GACATAAAAGCTAGGAATT	M28364	559-578	256	ATCC ^(b) 19095	7
	SEC2	AAATCGGATTAACATTATCC		815-796			
<i>sed</i>	SED1	CTAGTTGGTAATATCTCCT	M28521	332-353	317	ATCC ^(b) 23235	5
	SED2	TAATGCTATATCTTATAGGG		649-630			
<i>see</i>	SEE1	TAGATAAAGTAAAACAAGC	M21319	491-510	170	ATCC ^(b) 27664	9
	SEE2	TAACCTACCGTGGACCCTTC					
<i>tst</i>	TST-3	AAGCCCTTTGTGCTTCGG	J02615	51-69	444	ATCC ^(b) 33586	14, 20
	TST-6	ATCGAAGCTTTGGCCATACTTT		495-474			
<i>seg</i>	SEG1	CGT CTC CAC CTG TTG AAG G	AF064773	317-335	327	CCUG ^(c) 39423	17
	SEG2	CCA AGT GAT TGT CTA TTG TCG		FRI 572 644-624			
<i>seh</i>	SEH1	CAA CTG CTG ATT TAG CTC AG	U11702	245-264	360	ATCC ^(b) 51811	17
	SEH2	GTC GAA TGA ATC TCT AGG		603-583			
<i>sei</i>	SEI1	CAA CTC GAA TTT TCA ACA GGT AC	AF064774	325-347	465	CCUG ^(c) 39423	17
	SEI2	CAG GCA GTC CAT CTC CTG		790-773			
16S rDNA	5'-3' F	TAGATGGATCCGCGC	AF146368	145-159	916	NA	15
	5'-3' R	CTTAATGATGGCAACTAAGC		1061-1042			
Negative control						<i>S. epidermidis</i> ATCC 14990	-

bp base pair
NA not applicable

(a) nucleotide sequence: literature source
(b) American type culture collection, United States
(c) culture collection, University of Göteborg, Sweden

published on the basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/nucleotide).

Production and identification of enterotoxins

Strains identified and confirmed as *S. aureus* were tested for the production of the enterotoxins SEA, SEB, SEC, SED (SEA-SED). After inoculation in brain infusion broth (Oxoid) and incubation at 37°C for 24 h, the culture was examined using the staphylococcal enterotoxin test RPLA (SET-RPLA) (Oxoid) in accordance with the instructions provided by the manufacturer.

The gene sequences for *sea* (5), *seb* (16), *sec* (7), *sed* (5), *see* (9), *seg* (22), *seh* (25), *sel* (6), *tst* (4) were synthesised by the Eurobio Laboratories (Courtabeuf) and used without further purification.

A single PCR was used to identify the genes *sea*, *seb*, *sec*, *sed*, *see* and *tst* as described in the literature (1).

For *seh*, *seg*, *sei* and the 16S rDNA region, multiplex PCR was also performed with the following amplification cycles: denaturation for 1 min. at 94°C, primer annealing for 1 min at 55°C and primer extension for 2 min at 72°C. A total of 35 amplification cycles were performed using the thermal cycler GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, California).

For the detection of PCR products, each sample, consisting of 10 µl of amplified product in 2 µl of buffer (gel loading solution, concentration 1-6×, Sigma, St Louis, Missouri) underwent electrophoresis in agarose gel 2% (Agarose MP, Roche, Indianapolis, Indiana) at 100 V for 1 h. A 50-2 000 bp molecular ruler was used to estimate the weight of the fragments obtained (Amplisize™ Molecular Ruler, Biorad, Hercules, California).

The conditions applied in the extraction, amplification and PCR product detection phases were verified using reference *S. aureus* strains specific for the oligonucleotide sequences of the genes coding for the toxins under study.

Results

Table I gives the nucleotide sequences of the primers selected to detect the genes coding for *S. aureus* toxins and 16S rDNA sequence and the reference strains for the genes coding for the different toxins. Strains used to detect genes coding for *S. aureus* enterotoxins using PCR are presented in Table II.

Staphylococcus aureus contamination in food samples

A total of 49 (14%) of the 350 food samples examined were found to be contaminated with *S. aureus*. Of these, fresh meat products

Table II
Strains used for polymerase chain reaction detection of genes coding for *Staphylococcus aureus* enterotoxins (SEs) and for the production and identification of SEs with SET-RPLA

<i>Staphylococcus</i> reference strain	Enterotoxins identified with SET-RPLA	Genes coding for enterotoxins identified with polymerase chain reaction
<i>S. aureus</i> 310 2TE	SEA	<i>sea</i>
<i>S. aureus</i> ATCC 14458	SEB	<i>seb</i>
<i>S. aureus</i> ATCC 19095	SEC	<i>sec</i>
<i>S. aureus</i> ATCC 23235	SED	<i>sed</i>
<i>S. aureus</i> ATCC 27664	SEE	<i>see</i>
<i>S. aureus</i> ATCC 33586	–	<i>tst</i>
<i>S. aureus</i> ATCC 51811	–	<i>seh</i>
<i>S. aureus</i> CCUG 39423	–	<i>seg+sei</i>

SET-RPLA staphylococcal enterotoxins test – reversed passive latex agglutination
 SEA staphylococcal enterotoxins A
 SEB staphylococcal enterotoxins B
 SEC staphylococcal enterotoxins C
 SED staphylococcal enterotoxins D
 SEE staphylococcal enterotoxins E

accounted for 19.3% of positive samples (Table III), including fresh meat (contaminated in 30% of cases) and packed fresh meat products (16.7%) (Table III). Figure 1 gives the frequency of *S. aureus* contamination relative to the contamination level in fresh and packed fresh meat samples. In fresh meat, levels of between 5 colony-forming units (cfu)/g and 720 cfu/g were detected, while in packed meat products levels of between 30 and 2 900 cfu/g were observed.

A total of 13.3% of fresh cheese samples were contaminated, with 33.3% of sheep's milk cheeses and 4.8% of cow's milk cheeses (Table III). Contamination levels in fresh cheeses ranged between 750 cfu/g and 2 800 cfu/g. Table III gives the percentage of strains with genes coding for staphylococcal toxins. The distribution of contamination levels, with reference to the limits fixed for these products by legislation and the *S. aureus* concentrations needed to produce enterotoxins, are provided in Tables IV and V and in Figure 1.

Figure 2 shows the electrophoretic pattern in multiplex PCR for 16S rDNA and the genes *seg*, *sec* and *sei*.

Identification of enterotoxigenic strains

Of the 49 *S. aureus* strains isolated, 8 (16.3%) were found on SET-RPLA testing to be SE

producers. Thus, 2.3% of food samples were found to be contaminated with enterotoxigenic strains (one with toxin A and seven with toxin C).

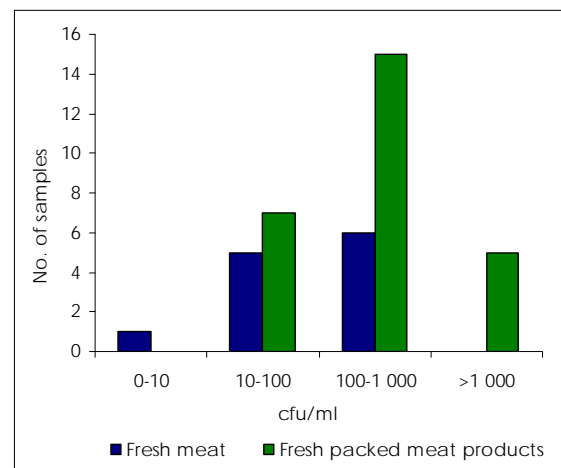


Figure 1
Staphylococcus aureus contamination levels in fresh meat samples

On PCR, 18 strains isolated from meat products were found to be positive for one or more genes coding for enterotoxins, as follows:

- 4 *sec*
- 1 *sei+sec*
- 1 *sec+seh*
- 9 *seg+sei*
- 1 *sec+sei+seg*
- 1 *sea+seg+sei*
- 1 *tst+seg+sei*.

Table III
Breakdown of meat products and fresh cheeses and positive test results

Product	No. of samples examined	No. of samples positive for <i>Staphylococcus aureus</i>	Percentage of isolation
Fresh meats (ground meat, poultry)	40	12	30.0
Meat-based products (packed fresh meat)	162	27	16.7
Total fresh meat products	202	39	19.3
Fresh sheep's milk cheese	9	3	33.3
Fresh cow's milk cheese	21	1	4.8
Total fresh cheeses	30	4	13.3
Bakery products (pastry cream)	55	2	3.6
Deli products (timbale)	13	1	7.7
Seafood (mussels)	50	3	6.0
Total products	350	49	14.0

Table IV
Distribution of levels of *Staphylococcus aureus* contamination found in positive milk and cheese samples (expressed in cfu/g for solid samples and cfu/ml for liquids)

Sample type	0-10 ^(a)	10-100 ^(b)	100-1 000	>1 000 ^(c)
Fresh sheep's milk cheese	0	0	2 (50%)	1 (25%)
Fresh cow's milk cheese	0	0	0	1 (25%)

(a) limit of acceptability 'm' for fresh cheeses according to EC/1441/2007
(b) limit of acceptability 'm' for fresh cheeses according to EC/1441/2007
(c) *S. aureus* contamination level capable of producing *Staphylococcus aureus* enterotoxins (2)

Table V
Distribution of levels of *Staphylococcus aureus* contamination found in fresh and packed meat samples (expressed in cfu/g)

Sample type	0-100 ^(a)	100-1 000 ^(b)	>1 000 ^(c)
Fresh meat	3 (11.1%)	19 (70.4%)	5 (18.5%)
Packed fresh meat	6 (50%)	6 (50%)	0

(a) limit of acceptability 'm' for ground meat according to EC/1441/2007
(b) limit of acceptability 'm' for ground meat according to EC/1441/2007
(c) *S. aureus* contamination level capable of producing SEs (2)

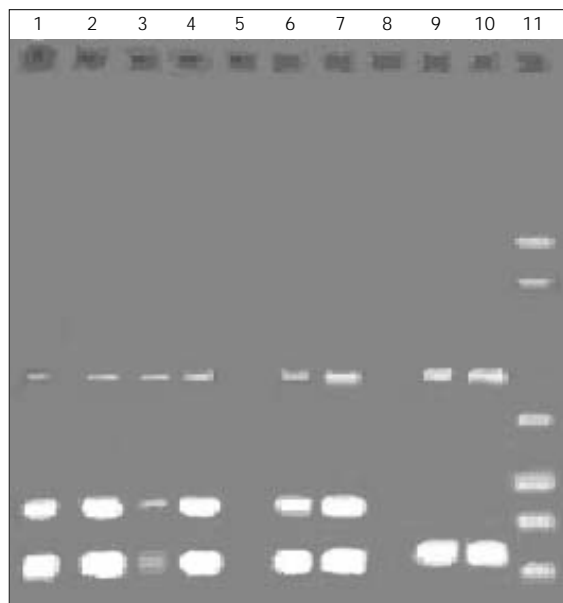


Figure 2
Electrophoretic pattern in agarose gel of products amplified with multiplex polymerase chain reaction for the genes *seg* (327 bp), *seh* (360 bp), *sei* (465 bp) and 16S rDNA (916 bp)
Lines 1 to 4: repeated and diluted samples of a *Staphylococcus aureus* strain isolated from meat products positive for *sei* and *seg*
Line 5: blank sample (negative control)
Lines 6 and 7: samples positive for *sei* and *seg* at different DNA concentrations from *S. aureus* CCUG 39423
Lines 8 to 10: repeated and diluted samples of a *S. aureus* strain isolated from molluscs positive for *sei* and *seg*
Line 11: molecular ruler

Table VI gives the percentage of strains carrying genes coding for staphylococcal toxins.

Only one of the four strains isolated from cheeses was found positive on PCR (*sea*).

Discussion and conclusions

Of the 350 samples taken from retail outlets and representing the most popular food products, 49 (14%) were contaminated with *S. aureus*. This level was slightly lower than those reported in another Italian study in 2000-2002, probably due to the fact that raw milk products was not included in our study (13). The foods most commonly contaminated with *S. aureus* were meat products and fresh cheeses (19.3% and 13.3%, respectively).

As the number of sample units specified by legislation were not taken at each sampling, the results were evaluated taking the lower limit 'm' (as defined by the reference standard) as the reference and products within this limit were considered 'satisfactory'. A total of 1 000 cfu/g or /ml (depending on whether the sample was solid or liquid) was proposed as the upper value for the contamination range considered, which corresponds to the concentration of *S. aureus* at which some authors have reported SE production (2). Only

Table VI
Percentage of strains carrying genes coding for staphylococcal toxins

Product	No. of strains examined	Toxigenic strains	Percentage positive of total examined
Fresh meat products	39	18	46.2
Fresh cheeses	4	1	33.3
Other products	6	5	83.3
Total	49	24	48.97

11.1% of the fresh meat samples examined were in conformity with these limits and therefore under 100 cfu/g, while 18.5% were found to have levels exceeding 1000 cfu/g which is considered capable of producing SE toxins (2). No samples of packed meat products were found to have levels above 1 000 cfu/g (Table V).

Although no samples of fresh cheese was found to be contaminated with levels above the limits specified by current legislation, 25% were found to have levels above 1 000 cfu/g, the level of *S. aureus* considered in the literature as capable of producing staphylococcal enterotoxins.

A total of 2.3% of the strains isolated were found to be enterotoxigenic which is similar to the level found nationwide (2.9%). The toxins detected were enterotoxin C (7/8) and A (1/8).

PCR testing revealed that 24 of the 49 strains isolated (48.9%) were potentially enterotoxigenic: of these, 18 were isolated from fresh meat products and one from fresh cheese. PCR detected not only genes from toxins A-D (*sea*, *seb*, *sec* and *sed*), but also the genes *seg*, *seh*, *sei* and *tst* coding for the production of enterotoxins G, H, I and TSST, which cannot be detected with SET-RPLA.

The 18 toxigenic strains isolated from meat products were found to be carriers of genes coding for SE. A total of 8 strains had at least one gene coding for SE (*sea* or *sec*), 11 had two genes coding for SE (*sei+sec*; *sec+seh*; *seg+sei*) and 3 strains had 3 genes coding for SE (*sea+seg+sei*; *sec+seg+sei*; *tst-seg-sei*). The toxigenic strain isolated from cheese by PCR was found positive for SEA. The other five toxigenic strains, deriving from bakery

products, deli products and molluscs, were found to be positive for *seg* and *sei* genes.

A comparison of the RPLA tests for the detection of enterotoxins A-D with the presence of the corresponding gene on PCR revealed total correspondence between results for 8 strains (16.3% of the total). Indeed, on RPLA, one strain was found to produce toxin A and seven toxin C; in these strains, PCR revealed genes SEA and SEC, respectively. However, PCR revealed a total of 10 strains (20.4%) as possessing the genes for enterotoxins A-D. This discrepancy could be explained by the production of enterotoxin in a quantity that was below the limit of detection of the RPLA test, which is 1 ng/ml, or its non-expression.

In summary, for the toxins detectable using RPLA, a good level of correlation was observed between the two methods, as also reported by other authors (30).

Although legislative bodies identify *S. aureus* to be an important risk factor, as indicated by the legislative provisions requiring its detection and quantification in various foods for human consumption, legislation on food hygiene requires testing for staphylococcal toxins only if the set limits are exceeded and for few product types. However, the *S. aureus* count may not be a reliable indicator of the presence of enterotoxins in the product, given that not all strains produce enterotoxins, the micro-organism may no longer be viable but have already produced enterotoxins that remain in the product or, yet again, the quantity of enterotoxin produced is below the detection limit of commercially available test methods.

Our study revealed the widespread presence of *S. aureus* strains that are carriers of genes coding for toxins other than those identifiable with traditional methods. The real significance of the presence of these toxins in foods and their possible impact as a cause of *S. aureus* food poisoning in humans is still unknown and further studies are required, using both traditional and molecular methods.

The use of innovative techniques for the identification of genes coding for the production of enterotoxin, along with traditional detection methods, would enable the identification of strains that carry genes that might, in suitable conditions, produce as yet unknown toxins potentially capable of producing sickness in humans.

The high percentage of positive samples of sheep milk cheese for staphylococcal enterotoxins draw attention to the need to improve the management of animals at the farm level, in particular through the implementation of specific monitoring programmes designed to reduce subclinical mastitis which is often the cause of milk contamination. An effective reduction of contamination levels could be achieved by improving sanitation and hygiene procedures.

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