Selection of optimal combinations of loci by the Optimal Combination Finder computer program from a group of variable number tandem repeat loci for use in *Staphylococcus aureus* food poisoning case investigations

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Staphylococcus aureus is a pathogen that can be the cause of nosocomial infections, community-acquired diseases and food-borne disease outbreaks. A number of variable number tandem repeat loci have been reported by various groups for use in epidemiological studies and outbreak investigations. The aim of this study was to systematically evaluate a total of 18 commonly used loci with the same *S. aureus* population so that the properties of each locus could be compared and used for typing, and to develop a computer program enabling calculation of the Simpson index (SI) of all possible loci combinations so that an optimal combination of loci could be identified for multilocus variable-number tandem-repeat analysis (MLVA) typing. A collection of 160 *S. aureus* isolates from patients with sporadic food-borne illnesses, and pet animals, such as canine, feline and equine pets, with skin infections were used to assess the MLVA loci. The newly developed Optimal Combination Finder (OCF) computer program was used for the analysis and it was found that the minimal number of loci combinations that produced the same SI (0.999528) as that of the 18 loci combined was eight; SIRU05, SIRU07, SIRU13, SIRU15, SIRU21, *fnbB*, *sdrD* and *clfB*. This suggested that the optimal combination of eight loci could be used for the routine investigation and surveillance of future *S. aureus* outbreaks instead of using all 18 loci. In addition, the OCF software could be a useful tool for the development of typing schemes for other organisms.

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INTRODUCTION

Staphylococcus aureus is found on the skin, mucous membranes and hair of warm-blooded animals. Between 30 and 50% of the human population are carriers (Bannerman & Peacock, 2007). *S. aureus* can grow in a wide range of temperatures (7–48.5 °C, optimum 30–37 °C; Schmitt *et al.*, 1990), pH levels (pH 4.2–9.3, optimum pH 7–7.5) and NaCl concentrations (up to 15%, w/v, NaCl; Le Loir *et al.*, 2003). These properties enable *S. aureus* to grow in a wide variety of foods, making

it one of the leading pathogens with regard to food-borne diseases (Asao *et al.*, 2003; Cowell *et al.*, 2002; Fullerton & OzFoodNet Working Group, 2009). In addition, *S. aureus* has a combination of virulence factors including toxin production, invasiveness and antibiotic resistance, making it a significant cause of nosocomial and community-acquired infection (Coombs *et al.*, 2009; Hardy *et al.*, 2004; Vindel *et al.*, 2009).

A number of DNA-based typing methods have been developed for the investigation and epidemiological surveillance of *S. aureus* outbreaks and strain dissemination. Typing schemes using multilocus variable-number tandem-repeat analysis (MLVA) of *S. aureus* have been reported by various groups (Conceição *et al.*, 2009; Francois *et al.*, 2005; Gilbert *et al.*, 2006; Hardy *et al.*,

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Abbreviations: MLVA, multiple locus variable number tandem repeat analysis; OCF, Optimal Combination Finder; SI, Simpson index; SIRU, staphylococcal interspersed repeat unit.

2006; Melles *et al.*, 2009; Pourcel *et al.*, 2009; Sabat *et al.*, 2003; Schouls *et al.*, 2009). Comparisons of MLVA studies with other *S. aureus* typing methods have found that MLVA has advantages over pulsed-field gel electrophoresis (PFGE), *spa* typing and amplified fragment length polymorphism (AFLP) methods in reproducibility, discriminatory power and cost (Malachowa *et al.*, 2005; Melles *et al.*, 2009; Tenover *et al.*, 2007; Vindel *et al.*, 2009). MLVA is regarded as a well-recognized typing method (Lindstedt, 2005; van Belkum, 2007). MLVA provides genotypes expressed in strings of numbers corresponding to the number of repeats at each locus; therefore, this typing method produces results that are highly portable and easy to incorporate into an established database (Grissa *et al.*, 2008).

Reports of *S. aureus* MLVA in the literature are chiefly based on the following variable tandem repeats: staphylococcal interspersed repeat units (SIRUs), including SIRU01, -05, -07, -13, -15, -16, and -21 (Hardy *et al.*, 2006); intergenic and *S. aureus* repeats (STAR), such as 311 (Pourcel *et al.*, 2009); and tandem repeats within genes such as *clfA*, *clfB*, *sdnCDE*, *spa* and *sspA* (Sabat *et al.*, 2003). In the present study, a total of 18 MLVA loci were selected and assessed using a collection of *S. aureus* strains from our laboratory. These loci are frequently used and have been proven to be polymorphic in various reports. Authors validated these loci based on their own limited populations. Therefore, the present study aimed to compare these existing typing loci among strains of the same *S. aureus* population to identify optimum loci for use in future food-borne outbreak investigations.

As a large number of *S. aureus* MLVA loci have been described to date, the choice of the most appropriate combination of loci for typing is not obvious. The number of loci for typing must be reduced to a more practical number for routine use. To select an optimal combination of loci, ideally, the discriminatory ability of every possible combination of these loci should be determined. However, this approach is not feasible by manual calculation, as even for a five-locus combination, selected from 18 candidate loci, there are 8568 possible combinations. Therefore, a computer program was developed to quantify the ability of each possible combination of loci to discriminate between strains, making it possible to efficiently select the optimal combination of candidate loci. The aim of this study was to compare 18 commonly used MLVA markers in the same *S. aureus* population for assessment of the loci for future analysis, and to develop a computer program for the identification of an optimal combination of loci for use in future *S. aureus* epidemiological investigations and outbreak studies.

METHODS

***S. aureus* samples.** A total of 160 epidemiologically unrelated isolates were used in this study. Of the isolates, 38 were unassociated isolates from documented sporadic food poisoning cases in

Queensland from 2000 to 2008, which were stored at -80°C , and the remaining 122 were animal isolates collected from February to October 2009 and were obtained from Idexx Laboratories, Coorparoo, Queensland, a veterinary microbiology testing laboratory. The animal samples were from abscesses, skin lesions/wounds, nose discharge and milk of pets and zoo animals. The pet animal isolates comprised 63 canine, 21 feline, 19 equine, five bovine, three rabbit, one chicken and three goat isolates. Zoo animal isolates comprised four possum, one koala, one monkey and one wallaby isolates. Since pet and zoo animals have close contact with humans, isolates from pets and zoo animals were included to extend the *S. aureus* source representation for the MLVA marker assessment. Two reference strains of *S. aureus*, ATCC 6538 and NCTC 4163, were passaged using blood plates to assess the stability of the loci. Isolates at passages 1, 5, 10, 15 and 20 were tested for variability in the 18 loci.

To test the concordance of the MLVA markers in outbreak investigations, another 18 isolates from two known outbreaks were analysed (see Table 4). Detection of 19 enterotoxin genes was carried out for these outbreak isolates according to the method described by Hwang *et al.* (2007).

Choice of markers. The MLVA loci used by Hardy *et al.* (2006) and Sabat *et al.* (2003) were selected as they have been widely used in other studies. An additional five loci (*coa*, SAV1078, 311, *fnbA* and *fnbB*), used in a number of previous studies, were evaluated against the 13 sequenced genomes of *Staphylococcus aureus* that were publicly available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) at the time of this project. These loci were variable among the sequenced genomes of the 13 strains: COL, USA300F, MSSA476, MRSA252, RF122bov, Mu50, MW2, N315, NCTC8325, SubJH1, SubJH9, SubMu3 and Newman.

Primer design. Due to their high sequence similarity, the three genes *sdnC*, *sdnD* and *sdnE* are amplified in one reaction using the primers designed by Sabat *et al.* (2003); therefore, it was impossible to assign the amplified bands to *sdnC*, *sdnD* or *sdnE* in an Excel file for analysis. Because of this, efforts were made to design specific primers for amplification of each of the *sdnC*, *sdnD* and *sdnE* genes separately. To differentiate the amplification products further, forward primers for the *sdnC*, *sdnD* and *sdnE* genes were labelled with different coloured dyes, FAM, HEX and NED, respectively. The individually amplified products could be distinguished by their colours using an ABI Genetic Analyzer 3130. The products were sequenced to check the amplification specificity. This was also the case for the *fnbA* and *fnbB* genes. As the primers for the SIRU21 locus and *spa* genes targeted the same variable region, only the results of SIRU21 were presented here (Pourcel *et al.*, 2009; Sabat *et al.*, 2003). The primers used in this study are listed in Table 1.

DNA extraction. Genomic DNA was extracted using a MasterPure DNA extraction kit (Epicentre) following the manufacturer's instructions with a modification of the cell lysis step, which was performed with 50 U ml^{-1} lysostaphin (Sigma). The DNA was measured using a spectrophotometer (Gene Quant II, Pharmacia Biotec) at A_{260} and the DNA was made up to a concentration of $50\text{ ng }\mu\text{l}^{-1}$.

PCR conditions. To save time and reagents, multiplex PCR was applied wherever possible. The combinations were as follows: set 1: SIRU01, SIRU07 and SIRU16; set 2: SIRU05 and SIRU21; set 3: SIRU15 and *clfB*; set 4: *spa* and *sspA*; set 5: *fnbB* and *sdnE*; set 6: SIRU-13 and SAV1078; set 7: 311 and *fnbA*. Single PCRs were performed for *sdnC*, *sdnD*, *coa*, and *clfA* as multiplex PCR did not yield good results. A confirmation single PCR was carried out for the samples whose locus/loci were negative in the multiplex PCRs. Each PCR mixture contained $2\text{ }\mu\text{l}$ prepared template DNA, $0.4\text{ }\mu\text{M}$ each primer, $2.5\text{ }\mu\text{l}$

Table 1. Loci and primer sequences used in this study

Locus	Primer	Sequence (5'→3')	Repeat size	Reference
SIRU01	SIRU01_L SIRU01_R	FAM-CATGAGCAGTGCCTCCTTTA CGCCTTGTATCTTAAATTTGTTG	55	Hardy <i>et al.</i> (2006)
SIRU05	SIRU05_L SIRU05_R	FAM-AAAGCGTAAACACTCCGAACA CATCGGGTTCACCTCTTTCTT	60	Hardy <i>et al.</i> (2006)
SIRU07	SIRU07_L SIRU07_R	HEX-CGCAAAATTTATTTTCGTCGT TTGGCATCATTTAGCAGACG	56	Hardy <i>et al.</i> (2006)
SIRU13	N13-F N13-R	FAM-ATTACAGGTCAAACAATCCATGTAAATG CGAAATTTTCCACGTCGATTCACCTCC	64	This study
SIRU15	SIRU15_L SIRU15_R	TET-AATTATAGCATATTAGAGCCCCTTA ACGTAAAGGTCGCGACAAAA	131	Hardy <i>et al.</i> (2006)
SIRU16	SIRU16_L SIRU16_R	TET-TCAGCTTGTGACTCTTCTTTTCG AAGAACGTGTTGAAGGATTTTT	159	Hardy <i>et al.</i> (2006)
SIRU21	SIRU21_L SIRU21_R	FAM-AGCAGTAGTGCCGTTTGCTT GCTCAAGCACCAAAAGAGGA	24	Hardy <i>et al.</i> (2006)
<i>clfA</i>	ClfA-F ClfA-R	FAM-GATTCTGACCCAGGTTTCAGA CTGTATCTGGTAATGGTCTTT	18	Sabat <i>et al.</i> (2003)
<i>clfB</i>	ClfB-F ClfB-R	HEX-ATGGTGATTCAGCAGTAAATCC CATTATTTGGTGGTGTAACCTCTT	18	Sabat <i>et al.</i> (2003)
<i>sspA</i>	SspA-F SspA-R	HEX-ATCMATTTYGCMAAYGATGACCA TTGTCTGAATTATTGTTATCGCC	36	Sabat <i>et al.</i> (2003)
<i>sdrC</i>	sdrC-F sdrC-R	FAM-GATGCTGTAGATTCTGATGGACTAACT TTAGCTGTTTTATGCTGATCTTTAACC	18	This study
<i>sdrD</i>	sdrD-F sdrD-R	HEX-CCAACCTCARTAACCTCTGGAAATGATACT GTACTCATTGGTTTAAACAGGTGTGTGC	18	This study
<i>sdrE</i>	sdrE-F sdrE-R	NED-CAGCAGGTTACACGCCAACGGTTAA CAGGTGTATGTTTTCTGCATCCGAGTC	18	This study
<i>coa</i>	COA-F COA-R	FAM-AGGTATCCGTGAATACAACGATGGAAC ATGTCGCAGTACCATCTGCATGTGTTG	81	This study
SAV1078	SAV1078F SAV1078R	HEX-GTGCATAATGGCTTRYGAATTAT TGGGAGGAATTAATCATGTCTAATG	63	This study
311	311F 311R	HEX-AGGGTTAGAGCCCCGAGACATAAATTA TGACACTAATCATCTACTTAACAATATCG	55	This study
<i>fnbA</i>	fnbA-F fnbAB-R	FAM-AATGAAGCAATCAGAAAACACTCTAAT AATGAAGGTCAACAAACGATTGAAGAAG	42	This study
<i>fnbB</i>	fnbB-F	HEX-ACACTAATTAGAACGCCTTCATAGTGTC	42	This study

10 × buffer II (Applied Biosystems), 2.5 mM MgCl₂, 200 μM each dATP, dCTP, dGTP and dTTP (GE Healthcare), 1.25 μl DMSO (Sigma) and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR was performed in a GeneAmp 9700 PCR machine (Applied Biosystems) using the following cycling conditions: 95 °C for 10 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 7 min. After PCR amplification, the fragments were separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems). The sizes were determined by using a 50–1000 bp internal standard marker (MapMarker 1000, BioVentures) and Peak Scanner software version 1.0 (Applied Biosystems).

After analysing the results of the 18 loci, the optimal combination of loci was identified to be an eight-locus combination of SIRU05, SIRU21, SIRU13, SIRU15, *clfB*, *fnbB*, SIRU07 and *sdrD*. The eight loci were optimized and rearranged in the following multiplex PCRs: set 1: SIRU05, SIRU21 and SIRU13; set 2: SIRU15, *clfB* and *fnbB*; and set 3: SIRU07 and *sdrD*. A string of eight numbers starting in the same order as the eight loci was used to express the profile of an isolate.

The allele coding system. To calculate the number of repeats in an allele, the amplicon size in base pairs was recorded. The two flanking

regions were then subtracted from the amplicon length. The resulting figure divided by the repeat unit length was the number of repeats. For partial repeats, if the partial repeat was less than half the size of the repeat, they were included in the flanking regions as recommended by Hardy *et al.* (2004), and when the partial repeat was larger than half of the repeat it was rounded up to a full repeat. In addition, it has been observed that some strains have flanking regions but do not contain repeat units (Hardy *et al.*, 2004); therefore, an amplicon was still produced. In some strains, the flanking regions and the repeat unit were not present in the genomes; therefore, no PCR product was observed (Ikawaty *et al.*, 2008). To accommodate and differentiate between these different scenarios, a numerical coding system was used. The result was expressed as 0 if there was no amplicon (i.e. the locus was absent); 1 if the size of the amplicon corresponded with that of the flanking region (i.e. the locus is present but no repeat sequence was present); 2 if the amplicon length corresponded with the sum of the flanking region and one repeat, and so on. This numerical coding system was used for all 18 loci studied. The string of 18 numbers gave an allelic profile for each *S. aureus* isolate.

The development of the Optimal Combination Finder (OCF) program. The discriminatory power of a typing method is in its

Fig. 1. Comparison of the PCR products amplified by the previously designed *sdrCDE* primers (lanes 1, 5, 9) with the newly designed primers for the individual *sdrC* (lanes 2, 6, 10), *sdrD* (lanes 3, 7, 11) and *sdrE* (lanes 4, 8, 12) genes. Lanes: M, GeneRuler (Fermentas); 1–4, sample 09M1304; 5–8 sample 09M1305; 9–12, sample 09M1306. In sample 09M1304, the amplified PCR products of *sdrC*, *sdrD*, and *sdrE* (lanes 2–4) were similar sizes and the amplified PCR products of *sdrCDE* (lane 1) could not be separated well; therefore, only a single band was observed. In sample 09M1305, *sdrC* and *sdrD* (lanes 6 and 7) were a similar size, whereas *sdrE* produces a band of different size. As a result, only two bands were observed for *sdrCDE* (lane 5). In sample 1306, *sdrD* and *sdrE* (lanes 11 and 12) were a similar size, whereas *sdrC* was a different size. Therefore, only two bands were observed and the smallest of these (~600 bp) might have been a non-specific band. The newly designed primers for *sdrC*, *sdrD* and *sdrE* have improved the PCR specificity and made the use of the variability of these loci possible.

ability to distinguish between unrelated strains and is expressed as the Simpson index (SI) (Hunter & Gaston, 1988). This is dependent on the types defined by the test method and the relative frequencies of these types. In the present study, the discriminatory power of the MLVA method was calculated using a previously described formula (Hunter & Gaston, 1988). To facilitate the choice of an optimal combination of loci, the SI was calculated for every possible combination of loci using the Optimal Combination Finder (OCF) computer program, which was developed for this study. The program was written using Visual Basic (VBA) 2007 to derive every possible combination and was used in conjunction with Microsoft Excel using a series of cascading loops to calculate SI for every possible combination of loci. The SI results calculated by the OCF program were ranked in a list of loci combinations in order of highest to lowest. By systematically stepping through sets of 2–18 loci, the optimal combination of loci could be defined as the point at which the SI did not increase when more loci were selected into the combination. The optimal combination of loci consisted of the minimal number of loci producing the maximal SI so that minimal work load is required to obtain optimal results.

RESULTS

The VNTR loci

Three sets of loci with high sequence similarity, close locality and similar functionality were included in this study. These were *sdrC*, *sdrD* and *sdrE*; *clfA* and *clfB*; and

fnbA and *fnbB*. Attempts were made to design specific primers to amplify these genes individually. Fig. 1 shows that the original *sdrCDE* primers produced multiple bands and the newly designed primers could amplify the *sdrC*, *sdrD* and *sdrE* genes individually. In addition, the forward primers for amplification of the *sdrC*, *sdrD* and *sdrE* genes were labelled with FAM, HEX and NED, respectively, making it easier to differentiate between them in the ABI 3130 Genetic Analyzer. The amplified PCR products could, therefore, be assigned to their corresponding genes, making the information from these polymorphic loci useful in the analysis (Table 2).

For the 18 loci, the number of alleles observed was between three alleles at SIRU16 and 28 alleles at *clfB*. The discriminatory power, expressed in SI, for each individual locus is listed in Table 2. The highest SI value was 0.935 for *clfB*, and the lowest SI was 0.506 for SIRU01. The stability of the VNTR loci was tested by analysing the allele sizes on passages 1, 5, 10, 15 and 20 of *S. aureus* strains ATCC 6538 and NCTC 4163. No variations in allele size were observed, suggesting that the loci were stable within the passages tested (data not shown).

The choice of the MLVA panel

The discriminatory power of MLVA increases with the number of polymorphic loci included in the typing panel. Normally, loci are chosen based on their SI value and the individual loci with the highest SI are combined to form the MLVA testing panel. However, the best loci may not complement each other and may provide redundant information. For example, to make a three-locus combination out of the 18 loci studied, the conventional method would be to select the three individual loci with the highest SI (*clfB*, *clfA* and *sdrE*) to form the locus combination arbitrarily (Table 2), giving an SI of 0.99387. It is, however, hard to know if this is the optimal combination as the SIs of the remaining possible combinations from the 18 loci are not calculated and compared. From the 18 loci studied, there were a possible 816 kinds of three-locus combination. It is not logistical to manually calculate every combination. A computer program termed 'Optimal Combination Finder' (OCF) was developed to provide a solution to this problem. The program calculated the SI of 816 kinds of three-locus combination and ranked the SI values in order from the highest to the lowest. In this case, the optimal combination of three loci was *clfB*, *clfA* and SIRU21 with an SI of 0.99599 (Table 3), which was higher than that of our empirically selected combination of *clfB*, *clfA* and *sdrE* (SI 0.99387).

When a request was made to find the optimal combination of four loci, this program made a new calculation independently from the previous three-locus calculations. It calculated every possible combination of four loci from the 18 loci used in this study. The optimal combination of four loci was *clfB*, SIRU05, SIRU13 and SIRU21, as determined by the OFC program. A normal human

Table 2. Alleles and SI of the individual loci

Loci	Range of PCR products (bp)	No. of alleles observed	Null allele percentage	SI
<i>clfB</i>	552–1119	28	0.0 %	0.935
<i>clfA</i>	682–1158	23	1.2 %	0.896
<i>sdrE</i>	469–808	17	10.6 %	0.889
<i>sdrD</i>	789–1126	20	23.1 %	0.883
SIRU21	119–490	13	0.0 %	0.858
SIRU13	208–876	11	0.6 %	0.856
SAV1078	256–752	10	4.3 %	0.843
SIRU05	203–1045	13	28.7 %	0.839
<i>SspA</i>	67–216	7	0.0 %	0.786
<i>fnbA</i>	514–936	9	20.6 %	0.783
311	252–552	8	0.6 %	0.774
<i>fnbB</i>	469–808	8	0.6 %	0.742
SIRU07	246–417	5	13.1 %	0.716
<i>sdrC</i>	605–1133	16	50.0 %	0.710
<i>coa</i>	195–674	8	0.6 %	0.704
SIRU15	135–912	6	0.0 %	0.670
SIRU16	405–573	3	46.2 %	0.604
SIRU01	237–407	4	3.1 %	0.506

empirical choice would have been based on the assumption that the loci from the optimal three-locus combination would still perform well in a four-locus combination; therefore, the previous optimal three-locus combination *clfB*, *clfA* and SIRU21 would be kept and an extra locus would be added to form a four-locus combination. However, only two loci from the original three-locus combination, *clfB* and SIRU21, were included in the optimal four-locus combination as determined by the OFC program (Table 3). This suggests that the program overcame this human assumption and identified the optimal combination based on calculations of every possible combination.

Fig. 2 shows the SI values of the optimal locus combinations and the poorest locus combinations of 2–18 loci as identified by the OCF program. SI values based on a human empirical choice of loci for these combinations would most likely fall between these two curves and would probably require more empirical testing to achieve the same SI values as those of the optimal locus combinations.

A series of tests were carried out on combinations of 2–18 loci and it was found that a maximum SI of 0.999528 was observed with eight optimally selected loci: SIRU05, SIRU07, SIRU13, SIRU15, SIRU21, *fnbB* (or *fnbA*), *sdrD* and *clfB*. No increase of SI value was observed by the addition of extra loci to this set (Table 3). This means that the eight selected loci represent the minimal number of loci required to produce the maximum SI. In the optimal eight-locus combination, *fnbB* was selected over *fnbA* as *fnbB* had a lower null allele rate than that of *fnbA* (Table 2).

The discriminatory power of the loci reported by Hardy *et al.* (2004) and Sabat *et al.* (2003) for the samples in this study was assessed. Using the seven loci of Hardy *et al.* (2004), 140 genotypes with an SI of 0.9980 were observed, and using the five loci of Sabat *et al.* (2003), 140 genotypes

with an SI of 0.9971 were observed. These data suggest that the optimal eight-locus combination identified in the present study could produce more genotypes (154 genotypes observed) than that of either of the sets of loci reported by Hardy *et al.* (2004) and Sabat *et al.* (2003).

The case investigations

Stored isolates from two epidemiological investigations of *S. aureus* food poisoning outbreaks in Queensland were tested using the eight optimal MLVA loci (Table 4). The first outbreak investigation took place in 2000. The profiles of two isolates suspected to be from contaminated chicken salad were the same, except that for Chicken salad P252B00 sample A the *fnbB* locus was allele 5 and for Chicken salad P252B00 sample B the *fnbB* locus was allele 4. Interestingly, the isolates from patients, 00M1063-1, 00M1039-1 and 00M1039-2, had the same MLVA pattern as that of the food isolate Chicken salad P252B00 sample A. The profile of the other isolate from patient 1 (00M1063-2) was the same as that of isolate Chicken salad P252B00 sample B. In addition, all isolates in this case investigation had the same four enterotoxin genes, *sea*, *sek*, *seh* and *seq*, out of the 19 enterotoxin genes tested for (Hwang *et al.*, 2007). The MLVA profiles of isolates from the suspected food matched the profiles of isolates from patients, suggesting that the food contained different populations of *S. aureus* and subsequently the two different genotypes of the isolates were reflected in patient isolates.

The second outbreak investigation took place in 2008. The MLVA profiles from the isolates of suspected food and the nasal swab of a food catering staff member were the same, except that the MLVA profile of the isolates cream P891/08 and roast chicken P888/08 was allele 34 at the *sdrD* locus

Table 3. The highest five loci or loci combinations ranked after the computer program calculation

No. loci (no. possible combinations)*	The five loci combinations produced the best SI values in descending order								Genotypes	SI
1	<i>clfB</i>								28	0.93498
	<i>clfA</i>								23	0.89638
	<i>sdrE</i>								17	0.88939
	<i>sdrD</i>								20	0.88341
	SIRU21								13	0.85818
2 (153)	<i>clfA</i>		<i>clfB</i>						97	0.98954
	<i>sdrE</i>		<i>clfB</i>						81	0.98577
	SIRU21		<i>clfB</i>						79	0.98538
	SIRU05		<i>clfB</i>						81	0.98491
	SIRU13		<i>clfB</i>						80	0.98491
3 (816)	SIRU21		<i>clfA</i>		<i>clfB</i>				128	0.99599
	SAV1078		<i>clfA</i>		<i>clfB</i>				130	0.99583
	SIRU05		<i>clfA</i>		<i>clfB</i>				129	0.99576
	SAV1078		SIRU21		<i>clfA</i>				125	0.99552
	<i>sdrD</i>		<i>clfB</i>		<i>clfA</i>				125	0.99544
4 (3060)	SIRU21		SIRU05		SIRU13		<i>clfB</i>		143	0.99819
	SIRU21		SAV1078		<i>clfA</i>		<i>clfB</i>		144	0.99796
	SIRU21		SAV1078		<i>clfA</i>		<i>sdrD</i>		141	0.99796
	SIRU21		SIRU13		<i>clfA</i>		<i>clfB</i>		141	0.99788
	SIRU21		SAV1078		<i>sdrE</i>		<i>sdrD</i>		140	0.99788
5 (8568)	SIRU21		SIRU05		SIRU13		<i>sdrD</i> <i>clfB</i>		148	0.99889
	SIRU21		SIRU05		SIRU13		<i>clfA</i> <i>clfB</i>		149	0.99874
	SIRU21		SIRU05		SIRU15		SIRU13 <i>clfB</i>		148	0.99874
	SIRU21		SAV1078		<i>clfA</i>		<i>fnbB</i> <i>sdrD</i>		148	0.99874
	SIRU21		SIRU05		SIRU13		<i>fnbA</i> <i>clfB</i>		147	0.99866
6 (18 564)	SIRU21		SIRU05		SIRU15		SIRU13 <i>clfB</i> <i>sdrD</i>		151	0.99921
	SIRU07		SIRU21		SIRU05		SIRU13 <i>clfB</i> <i>sdrD</i>		151	0.99914
	SIRU21		SIRU05		SIRU13		<i>clfA</i> <i>clfB</i> <i>sdrD</i>		151	0.99914
	SIRU21		SIRU05		SIRU13		<i>fnbA</i> <i>clfB</i> <i>sdrD</i>		150	0.99914
	SIRU21		SIRU13		SAV1078		<i>fnbB</i> <i>clfA</i> <i>sdrD</i>		150	0.99914
7 (31 824)	SIRU07		SIRU21		SIRU05		SIRU15 SIRU13 <i>clfB</i> <i>sdrD</i>		153	0.99937
	SIRU21		SIRU05		SIRU13		SAV1078 <i>sdrD</i> <i>fnbB</i> <i>clfA</i>		152	0.99937
	SIRU21		SIRU05		SIRU15		SIRU13 <i>fnbB</i> <i>clfB</i> <i>sdrD</i>		152	0.99937
	SIRU21		SIRU05		SIRU15		SIRU13 <i>fnbA</i> <i>clfB</i> <i>sdrD</i>		152	0.99937
	SIRU01		SIRU21		SIRU05		SIRU15 SIRU13 <i>clfB</i> <i>sdrD</i>		152	0.99929
8 (43 758)	SIRU05		SIRU07		SIRU13		SIRU15 SIRU21 <i>clfB</i> <i>fnbB</i> <i>sdrD</i>		154	0.99953
	SIRU05		SIRU07		SIRU13		SIRU15 SIRU21 <i>clfB</i> <i>fnbA</i> <i>sdrD</i>		154	0.99953
	SIRU05		SIRU01		SIRU13		SIRU15 SIRU21 <i>clfB</i> <i>fnbB</i> <i>sdrD</i>		153	0.99945
	SIRU05		SIRU01		SIRU13		SIRU15 SIRU21 <i>clfB</i> <i>fnbA</i> <i>sdrD</i>		153	0.99945
	SIRU05		SIRU07		SIRU13		SAV1078 SIRU21 <i>clfA</i> <i>fnbB</i> <i>sdrD</i>		153	0.99945
9 (48 620)	SIRU01		SIRU07		SIRU21		SIRU05 SIRU15 SIRU13 <i>fnbB</i> <i>sdrD</i> <i>clfB</i>		154	0.99953
	SIRU01		SIRU07		SIRU21		SIRU05 SIRU15 SIRU13 <i>fnbA</i> <i>sdrD</i> <i>clfB</i>		154	0.99953
	SIRU07		SIRU16		SIRU21		SIRU05 SIRU15 SIRU13 <i>fnbB</i> <i>sdrD</i> <i>clfB</i>		154	0.99953
	SIRU07		SIRU16		SIRU21		SIRU05 SIRU15 SIRU13 <i>fnbA</i> <i>sdrD</i> <i>clfB</i>		154	0.99953
	SIRU07		<i>clfA</i>		SIRU21		SIRU05 SIRU15 SIRU13 <i>fnbB</i> <i>sdrD</i> <i>clfB</i>		154	0.99953

*Number of possible combinations for each set of loci are given in parentheses, i.e. there are 153 possible combinations if two out of the 18 loci are used.

(Table 4). Two MLVA profiles were observed among the seven patient isolates. They were the same as those from the food isolates, indicating that the *S. aureus* food poisoning outbreak in these seven patients was related to the consumption of the identified contaminated food. The

isolates in this case investigation had *sea*, *seb*, *sek* and *seq* enterotoxin genes. These two examples demonstrated good concordance between the MLVA profiles of the *S. aureus* isolates from patients and those from infectious food sources.

Fig. 2. SI curves of the optimal loci (▲) and poorest loci (■) combinations as determined by the OFC computer program.

DISCUSSION

In this study, the MLVA loci investigated were those most commonly used by various groups (Ikawaty *et al.*, 2008; Melles *et al.*, 2009; Pourcel *et al.*, 2009; Sabat *et al.*, 2003; Tenover *et al.*, 2007). The precise size of amplicons was acquired using an ABI 3130 Genetic Analyzer, making loci comparison data more informative and portable. In addition, efforts were made to design primers to individually amplify variable loci with high sequence similarities, including *sdrC*,

sdrD, and *sdrE*, and *fnbA* and *fnbB*, whereas previously designed primers amplified all three *sdrCDE* genes (Sabat *et al.*, 2003) and both *fnbAB* genes (Gilbert *et al.*, 2006) in single reactions. The new primers for amplification of each individual gene have made it possible to use the variable property of these loci in the MLVA (Fig. 1).

The nomenclature for *S. aureus* alleles is confusing as authors express a null allele of a locus in different ways. For example, Hardy *et al.* (2004) used 'x' and Ikawaty *et al.* (2008) used '999' to express a null allele, while Conceição *et al.* (2009) used '99' to express a null allele and '0' to express an amplicon that contained no tandem repeat unit but did contain the flanking sequences. A numerical nomenclature system was proposed to differentiate between no amplicon and amplicons containing no repeat units. The nomenclature system used in this study has made allele expression easier and more comprehensible and has enabled a computer program to analyse the data.

In the present study, the OFC computer program was developed to calculate the SI of a selected panel of loci, allowing investigators to gauge the efficacy of each combination. The OFC program was designed to calculate the minimal number of loci to get the maximal SI so that the optimal combination could be determined with a minimal work load. The maximum SI was achieved by using the optimal combination of eight loci (SIRU05, SIRU07, SIRU13, SIRU15, SIRU21, *clfB*, *fnbB* and *sdrD*) to get an SI of 0.999528, which was as good as that obtained with the 18 loci combined. Significant savings in time and resources

Table 4. Profiles of the eight optimal loci for MLVA of isolates from the two outbreak case investigations

Sample	Sample no.	SIRU05	SIRU07	SIRU13	SIRU15	SIRU21	<i>fnbB</i>	<i>sdrD</i>	<i>clfB</i>
Investigation 1									
Food	Chicken salad	0	3	5	2	8	5	30	40
	P252B00 sample A								
Patient 1	00M1063-1	0	3	5	2	8	5	30	40
Patient 2	00M1039-1	0	3	5	2	8	5	30	40
Patient 2	00M1039-2	0	3	5	2	8	5	30	40
Food	Chicken salad	0	3	5	2	8	4	30	40
	P252B00 sample B								
Patient 1	00M1063-2	0	3	5	2	8	4	30	40
Investigation 2									
Food	Cream P891/08	8	0	7	2	11	3	34	48
Food	Roast chicken P888/08	8	0	7	2	11	3	34	48
Patient	08M767	8	0	7	2	11	3	34	48
Patient	08M768	8	0	7	2	11	3	34	48
Food	Roast chicken P892/08	8	0	7	2	11	3	0	48
Staff	Nasal swab P903/08	8	0	7	2	11	3	0	48
Patient	08M766	8	0	7	2	11	3	0	48
Patient	08M770	8	0	7	2	11	3	0	48
Patient	08M771	8	0	7	2	11	3	0	48
Patient	08M775	8	0	7	2	11	3	0	48
Patient	08M784	8	0	7	2	11	3	0	48

are possible using this optimized MLVA typing scheme. Another feature of this method is that it enables the epidemiologist to make a choice regarding the number of loci used for an outbreak investigation, depending on the level of discrimination required. For example, if a discriminatory level SI of 0.9959 was sufficient, only the optimal combination of the three loci SIRU21, *clfA* and *clfB* would be required (Table 3).

The OFC program can analyse both MLVA and binary data. It is not limited to *S. aureus* and could be useful for developing typing systems for other organisms. The program can also be readily implemented on personal computers. A maximum number of loci was set arbitrarily at 30, considering the number of combinations created from one dataset. In addition, by setting an SI threshold, the program will ignore results that are less than the threshold, restricting the number of calculations that can be in excess of 65 536 combinations. This figure is based on the maximum number of rows in Excel 2003. A plan has been made to upgrade the program to an internet accessible web server so that the calculation speeds can be significantly improved and users from wider academic and research communities can access and use this program.

Generally, there is a high level of variation in *S. aureus* populations. In the present study, 154 MLVA genotypes were observed out of 160 isolates. The level of diversity observed here was similar to that identified by PFGE and a SIRU-based MLVA assay, achieving 97 and 81 genotypes, respectively, out of 104 isolates tested (Conceição *et al.*, 2009). Various combinations of MLVA loci chosen by researchers have produced SI > 0.95 in previous reports. Schouls *et al.* (2009) used eight loci including *sspA* (VNTR09_01), SIRU21 (VNTR24_01), *coa* (VNTR81_01) and SAV1078 (VNTR63_01), giving an SI of 0.985. Ikawaty *et al.* (2008) used the six SIRU loci of Sabat *et al.* (2003) producing an SI of 0.987. Pourcel *et al.* (2009) developed two panels of loci for MLVA typing, the first panel consisting of 10 loci, which produced an SI of 0.9946 and the second panel consisting of an additional four loci that gave an SI of 0.9965. Interestingly, seven of these loci were the same as those in the present study, including SIRU21 (=Sa0122), *coa* (=Sa0266), 311 (=Sa0311), SIRU13 (=Sa1291), SIRU15 (=Sa1756), SAV1078 (=Sa1132) and SIRU01 (=Sa387). In the present study, the eight optimal loci that were identified produced the maximum diversity, with an SI of 0.9995. This result was similar to, or higher than, those of previously published data (Hardy *et al.*, 2004; Pourcel *et al.*, 2009; Schouls *et al.*, 2009), the advantage being that the number of loci needed for typing has been reduced to a practical range, rather than 14 loci (Pourcel *et al.*, 2009).

In an epidemiological outbreak investigation, samples with a one locus difference are conventionally regarded as related isolates using MLVA typing. The allele difference at one locus was thought to be a mutation occurring during the epidemic period (Torpdahl *et al.*, 2007). In the two

outbreak investigations tested here, there were mixed bacterial populations in the infectious food sources with different genotypes at *fmbB* locus in the first investigation and at the *sdrD* locus in the second investigation (Table 4). Interestingly, the isolates from infected patients also had two MLVA profiles, which corresponded with the profiles from the food sources. This suggests that, in outbreak investigations, the suspected food source may have contained mixed populations of *S. aureus*. There is a need to analyse a certain number of randomly selected colonies from suspected food sources to establish a connection between these isolates and mixed MLVA profiles from patient isolates. Therefore, most likely links between outbreak cases and suspected infectious food sources can be established. Although the optimized panel of eight loci worked well in linking outbreak patient isolates with the suspected food isolates in the two case studies, it is worth mentioning that there may be limitations in using this panel as some of the isolates used in this study to assess the 18 loci were from pet and zoo animals.

In conclusion, after screening the widely used 18 loci, eight of them (SIRUs 05, -07, -13, -15 and -21 and *clfB*, *fmbB* and *sdrD*) were identified by the OFC as the minimal number of loci required for optimal results. As such, the number of loci needed to produce the maximal differentiation of isolates has been reduced to a practical manageable range. A high-throughput *S. aureus* typing scheme like this one can provide rapid turnaround in outbreak investigations. The OFC computer program has helped us to identify the minimal number and optimal combination of loci for MLVA of *S. aureus* isolates and it may be of further use in developing typing schemes for other organisms.

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