Original Research

Identification of enterotoxigenic *Staphylococcus aureus* strains from meat and dairy products by multiplex PCR and reverse passive latex agglutination test in Nairobi, Kenya

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Abstract

Background: Foods of animal origin, especially meat and dairy products, are sometimes associated with food borne diseases. In many countries, *Staphylococcus aureus* is considered the second or third most common pathogen responsible for outbreaks of food poisoning. In Kenya enterotoxigenic staphylococcal food poisoning poses a potential health hazard to the consumers of meat and dairy products but little data is available about the strains involved in food poisoning.

Objective: The primary objective of this study were to investigate the occurrence of different strains of *Staphylococcus aureus* in meat and milk products and to determine the existence of gene coding, the classical staphylococcal enterotoxins.

Methodology: The survey was a cross-sectional descriptive study targeting meat, dairy products and meat processing plant. The study was confined in randomly selected meat and milk outlets in the Central Business District of Nairobi (CBDN) and its surroundings. Four hundred and twenty food samples of animal origin, comprising of meat and dairy products, were randomly sampled. Genes coding classical staphylococcal enterotoxins were profiled using multiplex Polymerase Chain Reaction (PCR) and the production by Reversed Passive Latex Agglutination (RPLA). Two hundred and fifty one samples of raw pork and packed pork products from a local processing factory were similarly tested.

Results: Data from a total 671 samples were examined and 37.4% of them were contaminated with *S. aureus* in which the contamination rate was higher in meat products than in the milk products. Enterotoxin production was detected in 74.5% of the isolated strains of *S. aureus*. Staphylococcal enterotoxin genes (*ses*) were detected in 77.3% of the total isolates. The most frequent gene was s*ea* (61.8%) followed by *see* (33.1%), *sed* (17.5%) and *sec* (15.9%) respectively. Staphylococcal enterotoxin B (*seb)* was the least occurring gene in the *S. aureus* isolates examined (13.9%). Genes occurring in pairs included *Sea/See* (21.2%), *Sea/Sed* (9.8%), *Sed/See* (2.1%), *Sea/Sec* (0.7%) and *Seb/Sec* (0.5%) among others. A relatively low number 4 (2.1%) of discrepancies between the results of multiplex PCR and RPLA were found where by the *sed* genes were expressed by PCR but the corresponding toxins were not detected by RPLA.

Conclusions: The study clearly indicated that meat and milk products marketed in and around Nairobi, Kenya were contaminated with enterotoxigenic *S. aureus* posing a high risk of food poisoning to the consumers. Equally, these data demonstrated that multiplex PCR and RPLA are useful methods for detection of enterotoxigenic potential of *S. aureus*. There is need for strict hygienic and preventive measures to the manufacturer, distributors and consumers of meat and milk products since the contamination of *S. aureus* is greater than other pathogenic bacteria previously reported.

Keywords: Staphylococcal enterotoxin, PCR, RPLA meat, Dairy products

Introduction

Foods of animal origin, especially milk and dairy products, are associated with food borne diseases [1,2]. In many countries, *S. aureus* is considered the second or third most common pathogen responsible for outbreaks of food poisoning [3]. Strains of *S. aureus* can produce one or more staphylococcal enterotoxins (SEs), which causes food poisoning in humans [4]. Due to a combination of toxin mediated virulence, invasiveness and antibiotic resistance *S. aureus* infections are difficult to control [5].

To date, 21 *staphylococcal enterotoxins* (SE) or enterotoxin-like proteins (SEls) have been identified [6]. On the basis of antigenic characters Staphylococcal enterotoxins, on the basis of antigenic characters are classified into five main

serological types characterized by the initials SEA, SEB, SEC , SED and SEE (enterotoxins "classic") [7,6]. However, in recent years, the existences of "new types" of SE (SElG, SElH, SEI, SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SER, SES, SET, SElU and SElV) have been reported [6]. The toxic shock staphylococcal toxin, (TSST-1) initially designated as SEF, lacks emetic activity [8]. The common feature of SEs is high stability and resistance towards most proteolytic enzymes, such as pepsin or trypsin, allowing protection of their activity in gastrointestinal tract [9]. The SE also retains their biological activities even after pasteurization [1].

Enterotoxigenic strains of *S. aureus* are estimated to account for about 25% of all isolated strains [10]. Data from various studies of food matrices gives different and sometimes higher values depending on the SE under consideration [11]. The predominance of Staphylococcal enterotoxin A (SEA) is well documented in different countries [12]. The SEA in low concentration of 0.6 ng/ml is the enterotoxin most commonly reported in foods, and is also considered as the main cause of SFP, probably due to its extraordinarily high resistance to proteolytic enzymes, followed by SEB, SEC and SED [10]. The fifth classical enterotoxin, SEE, has been infrequently reported in foods and food producing animals, and its involvement in Staphylococcal Food Poisoning (SFP) outbreaks has only been demonstrated in rare occasions [10,12]. Staphylococcal enterotoxins can be detected by immunoassay Enzyme Linked Immunosorbent Assay (ELISA), and latex agglutination but availability of these methods are limited to commercial tests for classical SEs [13]. The DNA-based approach (PCR assays) is thought to be an essential tool for investigating SE genes. Studies conducted elsewhere established the strains harbouring classical genes to be predominant [14]. Although several studies on staphylococcal enterotoxin genes (ses) of both classical and the new have been reported in other parts of the world currently, there is limited information regarding this in Kenya. The aims of this study was to investigate the occurrence of enterotoxigenic *S. aureus* isolates from meat and dairy products in Nairobi, Kenya, and to detect the genes encoding the classical SEs by multiplex PCR.

Materials and Methods

Meat and milk products samples collection: A total of 671 samples of meat and dairy products were used in this study. Out of these, 420 samples comprising of meat products (n=280) were purchased randomly from various butcheries and supermarkets (80 beef chunks, 40 pork, 60 fish, 40 sausages, 60 poultry carcasses). Likewise, dairy products (n=140), which includes 40 raw milk, from supermarket dispensers, 60 packets of yoghurt and 40 samples of pasteurized milk were purchased from, retail shops and supermarkets in Nairobi City and its surroundings were sampled between the June 2012 and May 2013. A total of 251 samples (79 finished pork products of 19 cooked salami, 20 fresh sausages, 20 uncooked hams, and 20 hot dogs, 172 raw pork) were randomly collected from a meat processing plant during processing stage were included in the study for comparison. All the samples were placed in sterile plastic bags and transported to the laboratory in cool boxes at 4^{o}C. Analysis was done immediately the samples arrived in the laboratory or within 4 hours of arrival.

Isolation and detection of Staphylococcus aureus: For each sample, 10 grams of the product was weighed and transferred into a sterile plastic bag and 90ml of 0.1% sterile peptone water was added. Each sample was homogenized for 2min in a stomacher blender (Elekta Ltd., Japan 400). Homogenates of 0.1mls of the sample were spread plated on Baird-Parker agar with egg York Tellurites Emulsion (Himedia M 043). For the dairy products (fresh milk collected from super market dispensers, yoghurt and packets of pasteurized milk), samples of approximately 10 ml were collected using sterile sample collection bottles and 0.1ml aseptically spread on Baird-Parker agar. After incubation at 37^{o}C, suspected *S. aureus* colonies with convex, black, shiny appearance with narrow white margin surrounded by clear zone were regarded as *S. aureus*. Five typical colonies with similar morphologies were isolated and cultured separately in blood Agar plates. These colonies were confirmed as *S aureus* by conducting Gram staining, coagulase test, catalase test, DNase and anaerobic utilization of glucose and mannitol [15]. All the isolated bacteria were kept frozen at -70^{o}C until the time of use.

*DNA extraction from bacteria***:** Total DNA was extracted from 5 ml of *S. aureus* culture grown at 35± 2ºC for 24 hours in Brain Heart Infusion (Oxoid) broth. DNA purification DNeasy blood and tissue kit (QIAGEN Group, Beckman Instrument, and Icl, USA) and lisozyme 10mg. mL^{-1} (Sigma Aldrich) were used for DNA isolation as per manufacturer's instructions. Enterotoxigenic *S. aureus* strains ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), ATCC 23235 (*sed*) and ATCC 27664 (*see*) were used as positive controls and *Staphylococcus xylosus* ATCC 29971 as negative control.

Multiplex PCR conditions: Primer mixes were prepared according to the master mixes of components [16] with slight modifications to the given instructions. The volume of this mix was adjusted to 50µl with sterile water. Evaporation of the reaction mixture was prevented by addition of 100µl of sterile mineral oil.

Amplification conditions: Amplification was carried out in a PCR 96 well Thermocycler (GeneAmp 9700 ABI) with the following thermal cycling profile: Initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 2 min, annealing at 57°C for 2 min, and extension at 72°C for 1 min), ending with a final extension at 72°C for 7 mins. The amplified PCR products were separated by electrophoresis (Major Science Mini Electrophoresis System CO., Ltd Taiwan) at 80 V for 70 min in 2% agarose gel (Loba Chemie V201504, India) and stained with ethidium bromide. Gels were visualized in a UV

transilluminator (Utra-Lum Electronic Paramount Ca USA) and images digitalized with a digital camera (Sony DCHX 400). Primer pairs used in this study and corresponding multiplex PCR are as described in Table 1.

Table 1: Nucleotide sequences, gene locations, and anticipated sizes of PCR products

Gene	Primer	Oligonucleotide sequence (5-3)	Location within gene	Size of amplified product (bp)
Sea	sea- sea-2	ggttatcaatgtgcgggtgg cggcacttttttctcttcgg	349–368 431–450	102
Seb	seb-1 seb-2	gtatggtggtgtaactgagc ccaaatagtgacgagttagg	666-685 810-829	164
Sec	sec-1 sec-2	agatgaagtagttgatgtgtatgg cacacttttagaatcaaccg	432-455 863-882	451
Sed	sed-1 sed-2	ccaataataggagaaaataaaag attggtatttttttttcgttc	492-514 750-769	451
See	see-1 see-2	aggttttttcacaggtcatcc ctttttttttcttcggtcaatc	237-257 425-445	290
femA	fema-1 fema-2	aaaaaagcacataacaagcg gataaagaagaaaccagcag	1444-1463 1556-1575	132

*Enterotoxin production detection by RPLA***:** Bacteria were centrifuged and supernatant assayed using Staphylococcal enterotoxin Reversed Passive Latex Agglutination (SET-RPLA) kit (Oxoid, England) according to manufacturer's instructions. The analysis was designed to detect four staphylococcal enterotoxins (SE's), one each for enterotoxins A (SEA), B (SEB), C (SEC) and D (SED). Micro titer plates were sealed with a plate sealer and shaken to mix the contents of the wells. Plates were incubated immediately at room temperature on a vibration-free surface and the agglutination reactions read after 22–24 hours according to the manufacturer's instructions. Reference strains of *S. aureus* were used for verification of SEs production using RPLA.

*Data analysis***:** Statistical package for social sciences (SPSS program version 11) was used to analyze the data. Analysis of variance of different variables were tested *Chi-square* (X^2) tests. The threshold for statistical significance was set at $p<0.05$.

Results

Isolation of S. aureus: Of the 671 samples examined, 37.4% were contaminated with *S. aureus*. Overall, 36.2% and 39.4% of the samples collected from, the food outlets and processing factory were contaminated with *S. aureus,* respectively. The proportions of contamination of animal products from the two sources were not significantly different (p=0.400). Analysis of *S. aureus* contamination by type of product revealed that significantly more contamination was observed in meat and meat products (40.7%) than in the dairy products (25.0%) with p=0.001 (Table 2).

Table 2: Distribution of *S. aureus* contamination

Characteristic	Total number of samples	*S. aureus*		p-value
		-ve (n=420)	+ve (n=251)	
Source				
Seller	420	268(63.8%)	152(36.2%)	
Processor	251	152(60.6%)	99(39.4%)	0.400
Type of animal product				
Meat products	531	315(59.3%)	216(40.7%)	
Dairy products	140	95(67.9%)	35(25.0%)	0.001

Detection of enterotoxigenic genes: Using multiplex PCR, the reaction with each individual primer pair resulted in the amplification of single products when DNA from each reference strain used as a template (Figure 1). Reference strains known for the production of *se* were used for the verification of this multiplex PCR (ATCC 25923-FEMA, ATCC 3565-SEA, ATCC 14458-SEB, ATCC 19095-SEC, ATCC 23235-SED, ATCC 27664-SEE). The sizes of the products obtained from control strains in multiplex PCR designs corresponded to the predicted sizes. Reproducibility was observed in all tested strains. Although there were variations with band intensity, their presence and sizes were the same. For providing an assurance against false negative results, *Staphylococcus aureus* ATCC 25923 known to have *femA* gene was included as an internal positive control. In order to determine the specificity of the primers, and detection of the genes with multiplex PCR f*emA*, and a mixer of each of *sea*, *seb*, *sec*, *sed* and *see* were amplified together.

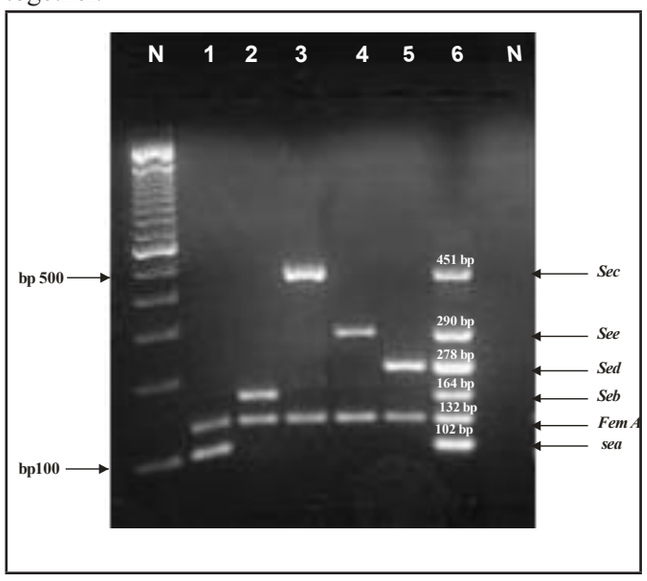

Figure: 1 Agarose gel electrophoresis patterns of multiplex PCR amplification products for Staphylococcal enterotoxin *genes* in standards reference strains. Lanes N, DNA molecular size marker (100-bp ladder; Thermo Scientific Fermentas™ lanes 1 to 6, PCR amplicons. Lanes: 1, *sea* plus *fem A*; 2, *seb* plus *femA*; 3, *sec* plus *femA*; 4, *see* plus *femA*; 5, *sed* plus *femA*; 6, *sea*, *seb*, *sec*, *sed*, *see*, and *femA* simultaneously; N, negative control.

Table 3 shows the distribution of genes coding for classical enterotoxins in *S. aureus* using the primers for enterotoxin A to E. Out of the 251 *S. aureus* isolates that were analysed, 76.9% were found to have one or more genes encoding for enterotoxins. The most frequent gene was s*ea* (61.8%) followed by *see* (33.1%), *sed* (17.5%) and *sec* (15.9%) respectively. Staphylococcal enterotoxin B (*seb)* was the least occurring gene in the *S. aureus* isolates examined (13.9%).

Table 3: Gene coding for *S. aureus* enterotoxins

Characteristic	Frequency	(%)
Genes coding for toxins (n=251)		
Absent	58	23.1
Presence of one or more genes	193	76.9
No. of genes coding for toxins (n=193)		
1	65	33.7
2	96	49.7
3	31	16.1
4	1	0.01
Specifications of the genes for enterotoxins (n=357)		
Sea	155	61.8
Seb	35	13.9
Sec	40	15.9
Sed	44	17.5
See	83	33.1

The specifications of the combinations of genes encoding for various exotoxins are listed in Table 4. Some isolates had only one gene encoding for enterotoxins, which included *Sea* (27.5%), *Seb* (2.6%), *Sed* (0.5%) and *See* (3.1%). Genes occurring in pairs included *Sea/See* (21.2%), *Sea/Sed* (9.8%), *Sed/See* (2.1%), *Sea/Sec* (0.7%) and *Seb/Sec* while only (0.5%) *Sea/Sec/Sed/See* coded for enterotoxin.

Table 4: Specifications of the combinations of genes encoding for enterotoxin

Genes	Frequency (n=193)	(%)
Sea	53	27.5
Seb	5	2.6
Sed	1	0.5
See	6	3.1
Sea/Seb	8	4.1
Sea/Sec	9	4.7
Sea/Sed	19	9.8
Sea/See	41	21.2
Seb/Sec	1	0.5
Seb/Sed	11	5.7
Seb/See	1	0.5
Sec/See	2	1.0
Sed/See	4	2.1
Sea/Seb/Sec	2	1.0
Sea/Seb/See	4	2.1
Sea/Sec/Sed	1	0.5
Sea/Sec/See	16	8.3
Sea/Sed/See	1	0.5
Seb/Sec/Sed	1	0.5
Seb/Sec/See	2	1.0
Sec/Sed/See	4	2.1
Sea/Sec/Sed/ See	1	0.5

Determination of SE using RPLA: Of the 251 isolates 187(74.5%) tested positive for the production of one or more SEs (RPLA (SEA to SED) as determined by the results of SET-RPLA (KITOXOID; Thermal scientific, UK) Staphylococcal enterotoxins most frequently detected from the 187 enterotoxigenic strains was SEA 90 (48.1%), followed by a combination of SEA and SEC 22 (11.8%). Sixteen strains (8.6%) were found to synthesize a combination of SEA and SEB while fifteen strains (8.0%) synthesized SEA and SED.

Additionally, nine strains (4.8%) synthesized SEB and SED. When comparing the results of multiplex PCR for isolated strains harboring genes of classical SEs (sea-see) and SET-RPLA (SEA-SED) for enterotoxin expression, the results showed 100% correlation particularly enterotoxin SEA, SEB, and SEC. The results indicated a minimal discrepancy 4 (2.1%) were PCR detected encoding genes for *sed* but enterotoxin production was not shown by the RPLA by the same strains.

Discussion

In this study, the overall occurrence of *S. aureus* in the analysed samples was 36.2% for food outlets and 39.4% in the processing factory. The proportions of contamination of the samples of animal products from the two sources were not significantly different (p=0.400). Significantly higher level of contamination was observed in meat and meat products (40.7%) as compared to dairy products (25.0%) (p=0.001). Such high contamination levels were similarly reported in other studies conducted in Turkey and USA [17-19], in milk in Zimbabwe (82%) and in Malaysia (60%) [20,21]. The findings of the current study differs from the results obtained in Egypt [22] in which the contamination rate of the raw milk was higher than that of the raw meat (58% vs. 18%). Lower rates than those of the current study were reported in Norway [2]. The considerable differences between their results and this study may be due to differences in geographic as well as study setting. The high contamination rate of *S. aureus* in meat and dairy products indicates contamination which could be introduced by food handlers who may be habouring the bacteria because 50% of the human population carry *S. aureus* as commensals. Other contamination sources are soil, water, dust and air during processing which may vary in different environmental condition. The midline, neck and hind portion of the carcass during slaughter were reported to be areas heavily contaminated with microorganisms [23, 24].

In the current study *Staphylococcus aureus* isolates were screened by multiplex PCR in order to detect the classical enterotoxin genes (*sea, seb, sec*, *sed* and *see*). The results demonstrated that 76.9% coded for one or more SE genes with 22 genotypes. A frequency close to this study was observed in Switzerland [25] where 172 samples of goat and sheep milk, 65.2% of 296 strains were positive for the of genes that encode enterotoxins. Similar findings were reported in Japan, 67.8% genes coding for one or more enterotoxins, were recorded [26] but such were lower than those observed in the current study. A previous study in Italy, found a higher frequency value of 67% of the *S. aureus* strains isolated from milk and dairy products positive for the toxin genes [27].

Results in our study indicated that of the 270 *S. aureus* isolates, gene coding for *sea* (61.8%) were the most frequent, followed by *see* (33.1%), *sed* (17.5%), and *sec* (15.9%) respectively. The least occurring gene in the *S. aureus* isolates examined was *seb (*13.9%). Similar results were reported in studies conducted elsewhere [5,12,25]. It was clear that 33.5% and 16.5% strains of *S. aureus* had one and three genes coding for enterotoxins, respectively. One strain (0.5%) encoding a combination of four enterotoxins (*Sea/ Sec/ Sed /See*) was realized. Recent studies have reported that most isolates harboured more than one toxin gene and 15 different toxinotypes were recorded with "sec" being the most frequent gene alone (28.6%), "sea, sed, ser, selj" (20%), "seg, sei" and "seh" (8.6%), which reverse of our study [28].

As observed in other studies, the enterotoxin type (SE) of 16 enterotoxigenic *staphylococci* isolates were found to produce SEA (9), SEA+SEB (3), SEA+SED (2), SEC+SED (1), and SEA+SEB+SEC (1) [29]. Of the 27 *S. aureus* isolated from 420 chicken nugget samples from the results showed that the most commonly detected genes were *sea* (25%), *sea* + *seg* (8.33%), *sec* (12.50%), *sea* + *sed* (12.50%), and *sea* + *sec* + *sej* (12.5%) but no *see* gene was detected [30]. The main cause of differences in the frequency of genes encoding strains in this study as well as other studies might be the origin of bacteria isolation, which could vary in animals, humans, foods or environment. This study overlooked new SEs that are being discovered which could have increased the percentages of potentially enterotoxigenic *S. aureus*. The role of the newly described SEs in staphylococcal food poisoning is still not clear in most parts of the world probably because of the limitation of the diagnostic kits for their detection. Further studies are needed to confirm the occurrence of these SEs and to assess the role they play in SFP.

The results obtained by multiplex PCR for harboring genes of classical SEs (*sea-see*) and SET-RPLA (SEA-SED) for toxin type were compared in this study. The frequency of detecting SEA, SEB, and SEC enterotoxin was consistent with their corresponding genes. The results showed a minimal discrepancy 4 (2.1%) for PCR detected *sed* genes but the RPLA did not detect the enterotoxin by these strains. *Staphylococcal enterotoxins* are similar in structural and biological properties but differ in amounts produced where SEB and SEC are expressed in greater quantities than SEA and SED [31]. Other studies conducted elsewhere indicated a small number of discrepancies (<5%) between the results using PCR and the RPLA assays [2, 32]. Markedly higher discrepancies of between 15 to 32% have been reported elsewhere [27, 33] between the results of multiplex PCR and RPLA assays, particularly on account of SEA. This discrepancy could be explained by the production of enterotoxin in a quantity that was below the limit of detection of the RPLA test or its non-expression.

Conclusions

This study clearly indicated that meat and milk products marketed in and around Nairobi, Kenya were contaminated with *S. aureus,* posing a high risk of food poisoning. *S. aureus* could be present in these products as a result of the animal suffering from disease condition and sheding the organism or due to unhygienic conditions during production, processing, storage on handling of these products. More than half of all detected isolates displayed the presence of *Staphylococcal enterotoxin genes* (ses) with *sea* being the most frequent gene. The results demonstrated a consistency of detecting enterotoxin production and their corresponding genes with a relatively low number of discrepancies between the results of multiplex PCR and RPLA. It is important to note that the PCR is only able to demonstrate the existence of enteroxoxin genes from the isolates but does not prove that the production of SEs proteins occurs.

Recommendations

More detailed investigations on the prevalence of newly discovered *staphylococcal enterotoxin gene* are needed in this country since contamination of meat and dairy products with new enterotoxigenic strains of this bacterium are increasingly being reported in many other parts of the world. The study suggests the need for strict hygienic and preventive measures to the manufacturer, distributors and consumers of meat and milk products since the contamination is greater than other bacteria pathogens previously reported.

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Conflict of interest statement

No conflict of interest exists between the authors or institutions with other third parties.

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