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SUMMARY

Fish provide important protein to human population. The procedures to preserve and maintain quality of fish from fishing until consumption can play a role in contamination with pathogens. Consumption of contaminated sea food products such as fish may lead to food poisoning. Knowledge about the spectrum of fish bacterial contaminants may assist in prevention of contamination and control food poisoning incidences. The present study aimed at characterizing and estimating prevalence of *Staphylococcus aureus* in fresh Indian Mackerel Fish (*Rastrelliger kanagurta*) from landing sites in Unguja Island. A total of 400 Indian Mackerel Fish were collected from landing sites in Unguja Island and from each fish two samples, skin swab and muscle, were collected. The primary culture was obtained from Mannitol salt agar, Nutrient and Blood agar followed by Gram staining, catalase coagulase tests. PCR targeting 16S rRNA, *nuc*, *mec*A, *pvl*, *spa* and enterotoxin genes was run to genetically characterize isolates and identify *S. aureus*. The result indicates that there was growth of bacteria in 359 (89.75%) fish skin swabs and 102 (25.5%) in fish muscle samples. Based on biochemical tests, 27 isolates (6.75%) were confirmed to be Staphylococcus bacteria. Of the 27 isolates, seven (1.75%) were confirmed *S. aureus* based on PCR. All 27 isolates confirmed to be positive in 16Sr RNA gene, two isolates demonstrated *mec*A gene and one had SEB and SEC. Detection of *S. aureus* in fresh Indian Mackerel Fish at landing sites poses a contamination risk to other critical points along the value chain and threatens public health.

Key words: *S. aureus*, Indian Mackerel Fish, landing sites, public health, Unguja

INTRODUCTION

Fish is a vital source of food for people and contributes about 60% of the world's supply of protein. It has high consumer preference due to its inherent nutrient values, taste and easy digestibility. It is one of the most important sources of animal protein available in the tropics and has widely been accepted as good sources of protein and other elements for maintenance of health body (Eyo 2006). Sixty percent of developing countries derive 30% of their annual protein from fish (Abisoye et al. 2011). In Africa, fish supplies 17% of protein and is one of the cheapest sources of protein (Clucas and Ward 1996). In Tanzania, fishery products accounted for 20.7 percent of the total animal protein intake and per capita fish consumption is 5.7 kilogram (Breuil and Grima 2014)

Fish is a high perishable food item and the biological degradation is faster than vegetables. Therefore, it has to be handled, stored and marketed with extreme care in minimum possible time. Best hygiene has to be maintained in fish handling areas for prevention of contamination with harmful agent since bad handling and unhygienic practices may cause illness to the consumers (Montville, Chen, and Schaffner 2001).

Unlike other animal products, quality of fish is often more difficult to control due to variations of fishes in species, sex, age, habitants and action of autolytic as well as hydrolytic enzymes of microorganisms on the fish muscles (Vázquez-Sánchez et al. 2012).

Food safety is matter of concern in both developed and developing countries. Poor sanitations in developing countries increase risk to food borne diseases. Fishery products which are of great importance for human nutrition and provide clear health benefits, can also act as a source of various food borne diseases (Darlington and Stone 2001). One fourth the world's food supply and 30% of landed fish are lost through spoilage. Although the safety of the food has dramatically improved, overall progress in uneven and food borne out breaks from microbial contamination. e.g. *Escherichia coli, Staphylococcus aureus* has increased (DeWaal and Robert 2005). Possible sources of bacteria are fish skin, shells tissues and processing equipment's used for each operation performed until the final product is eaten (Antwi-Agyei and Maalekuu 2014).

The genus Staphylococcus comprises several species of which *S. aureus* is the major bacterial agent causing food borne disease in human worldwide (EFSA 2010; Le Loir, Baron, and Gautier 2003). The *Staphylococcus* is gram –positive cocci with their primary habitat in the skin, glands and mucous membranes of warm-blooded animals including human. Infected sores and scratches are often harborage sites for *S. aureus.* The bacteria survive for weeks in the environment and may also be isolated from a range of sources that come into contact with man and animals. *S. aureus* can cause severe food poisoning out breaks and is probably responsible for even more cases in individuals and family groups than the record show (Tallent et al. 2001).

It is responsible for a wide range of human diseases including endocarditis, food poisoning, toxic shocks syndrome, septicemia, skin and soft tissues infections and borne infections as well as bovine mastitis (Costa et al. 2013). The incidence of *S. aureus* differs in different species of fish (Bujjamma and Padmavathi 2015).

Apart from food, these bacteria are ubiquitously found in human body as well as soil, water and air (Pinchuk, Beswick, and Reyes 2010). Furthermore, *S. aureus* enters in food chain as a result of poor hygiene conditions during processing and storage of food stuff (Afroz et al. 2008). The microbial association with fish compromises safety and the quality for human consumption, particularly critical when the microorganisms are opportunistic and /or pathogenic in nature (Mhango, Mpuchane, and Gashe 2010). The risks of contracting food borne diseases by fish users may be high, but also fish industry is one of the most important protein and economic sources for the rapid growing coastal population of Tanzania (URT 2013). The hygiene situation during fishing, handling processing and preservation techniques is still questionable in Tanzania fish industry. Therefore, the present study aimed at assessing microbiological quality of fish along the coast of Zanzibar in respect of *S. aureus*.

MATERIALS AND METHODS

Study Area

The study was conducted in Unguja Island, Zanzibar, Tanzania located between latitudes 5º40' and 6º 30' South and longitude 39º East. Samples were collected at famous landing sites of Malindi, Mkokotoni and Mangapwani.

Study Design and sample collection

Cross–sectional study design was used in this study and the sample size was computed by the formula earlier described (Thrusfield 1997). A total of 400 fresh Indian Mackerel fish (*Rastrelliger kanagurta*), 13cm to 25cm in size were collected from three fish

landing sites in Unguja Island. In Mkokotoni landing site 150 fish were collected, while 100 and 150 fish were collected from Malindi and Mangapwani respectively. From each fish one skin and one muscle sample was collected making a total of 800 samples.

Isolation of *Staphylococcus aureus*

Fish skin swabs were put in Stuart Transport Medium (HIMEDIA, Hi Media Laboratories PVT. Ltd), before further processing. A loopful of skin swab sample from transport media was inoculation onto Mannitol salt agar and incubated at 37ºC for 24hrs. On the other hand, one gram of fish muscle cut using sterile scalpel blade then ground and homogenized into peptone water before inoculated into Mannitol salt agar and incubated at 37ºC for 24hrs. For both skin and muscle sample cultures, medium sized yellow colonies suspected to be staphylococcus colonies were sub-cultured and purified on nutrient agar. The blood agar was then used for further characterization of suspected bacteria isolates such that suspected as S. aureus were medium sized, yellowish colonies with a zone of clear beta hemolysis. These colonies were subjected Gram staining and purple cocci in grape like shape was an indication of gram-positive bacteria. Catalase and coagulase slide and tube tests were performed, whereby staphylococcus isolates were expected to positive in both tests.

PCR amplification staphylococcus isolates

DNA extraction was by boiling as previously described (Sila, Sauer, and Kolar 2009). PCR was performed to target the *nuc*, *mec*A, and 16S rRNA genes, specific for S. aureus identification. Moreover, presence of *spa*, *pvl* and enterotoxin genes was assessed to determine virulence of the isolates. Table 1 shows primer pairs which were used to characterize the isolates.

Table 1: Oligonucleotide primer sequences used for typing of *Staphylococcus aureus*

Target gene	**Primer sequence (5-3)**	**Size (bp)**	**Reference**
16Sr RNA	GTACCAGCAGCCGCGGTAA	200	
	AGACCCGGGAACGTATTCAC		
nuc	GCGATTGATGGTGATACGGTT	270	(Chikkala et al. 2012)
	AGCCAAGCCTTGACGAACTAAAGC		
mecA	ATCATTAGGTAAAATGTCTGGACATGATCCA	293	
	GCATCAAGTGTATTGGATAGCAAAAGC		
PVL	GTAGAAATGACTGAACGTCCGATAA	443	(Sila et al. 2009)
	CCAATTCCACATTGTTTCGGTCTA		
Spa	ATGTGGTGGCGTAACACCTG	450-1500	(Shakeri et al. 2010)
	CGCTGCACCTAACGCTAATG		
SEA	GGTTATCAATGTGCGGGTGG	102	(Mehrotra, Wang, and Johnson 2000)
	CGGCACTTTTTTCTCTTCGG		
SEB	GTATGGTGGTGTAACTGAGC	164	
	CCAAATAGTGACGAGTTAGG		
SEC	AGATGAAGTAGTTGATGTGTATGG		
	CACACTTTTAGAATCAACCG		

The *S. aureus nuc* genes were determined as previously described by (Chikkala et al. 2012).

In summary, the PCR was performed in total volume of 25µl containing 2xready mix PCR of 12.5µl PCR primer for forward and reversed 1µl for each 6.5 µl of RNase free water 4µl of DNA template. The DNA thermocycler was programmed for initial denaturation at 94ºC for 5min, 30 cycles for

amplification denaturation at 94ºC for 30sec, annealing at 45ºC for 1 min and extension at 72ºC for 45sec and final extension was performed at 72ºC for 10min.

The *S. aureus* 16S rRNA and pathogenic genes of *mea* were amplified as described earlier (Chikkala et al. 2012). The PCR was performed in total volume of 25µl containing 2 x ready mix PCR of 12.5ul of RNase free water, 1 µl of each primer and 4µl of DNA template. The DNA thermocycler (Gradient PCR. TAKARA, Japan) was programmed for initial denaturation at 94ºC for 5min, 40 cycles for amplification denaturation at 94ºC for 30s, annealing at 55ºC for 40s, was performed at 72ºC for 50s and final extension was performed at 72ºC for 10 min.

Staphylococcus aureus spa gene was carried out as described by Shakeri and colleagues (Shakeri et al. 2010). The PCR was performed in total volume of 25µl containing 2x ready mix PCR of 12.5µl, PCR primer for forwarded and reverse 1µl for each, 6.5 µl of RNase free water ad 4µl of DNA template. The DNA thermocycler was programmed for initial denaturation at 94ºC for 4min, annealing at 56ºC for 1 min and extension at 72ºC for 3 min and final extension was perfumed at 72ºC for 5min.

The *S. aureus* enterotoxins genes of SEA, SEB and SEC were tested as earlier described (Mehrotra et al. 2000). The PCR was performed in total volume of 25µl containing 2 x ready mix PCR of 12.5 µl, PCR free water, 1µl of each primer and 4µl of DNA template. The DNA thermocycler was programmed for initial denaturation at 94ºC for 2min, annealing at 57ºC for 2min and extension at 72ºC 1 min annealing at 57ºC for 2 min and extension at 72ºC for 1 min and final extension was performed at 72ºC for 7min.

S. aureus pathogenic *pvl* genes were assessed as described by Sila and colleagues (Sila et al. 2009). PCR was modified in 16Sr RNA gene by using pvl gene condition. The PCR was performed in total volume of 25containing 2x ready mix PCR of 12.5µl, PCR free water and 4µl of DNA template. The DNA thermocycler was programmed for initial denaturation at 94^0C for 10sec, annealing at 56^0C for 20sec and extension at 72^0C 40sec and final extension. In all procedures, PCR products were analyzed using 1.5% agarose gel at 110 voltages and visualized under ultra violet light.

Data Analysis

Descriptive statistics such as frequencies and proportions were computed in MS Excel, whereas associations between categorical variables were determined by Chi-square test at significance level of 5% using SPSS software.

RESULTS

Bacterial contamination of fish samples

A total of 359 (89.75%) fish skin samples had bacterial growth while 102 (25.5%) fish muscle sample had bacterial growth. The fish skin swab samples from Mkokotoni landing site showed 88% (n=150) bacteria growth, whereas, 80% (n=100) and 98% (n=150) of fish skin swabs had bacteria growth in Malindi and Mangapwani respectively. Fish muscle samples from Mkokotoni showed 20% (n=150) bacterial contamination, 18% (n=100) for samples from Malindi and 36% (n=150) for Mangapwani muscle samples.

Contamination of fish samples with S.aureus

Out of all samples with growth of bacteria only 27 samples had yellowish medium sized colonies on mannitol salt agar, purple cocci on gram staining and showed beta hemolysis on blood agar. These 27 isolates were coagulase and catalase positive and hence confirmed *S*taphylococcus isolates.

Seven out of 27 staphylococcus positive samples were confirmed *S. aureus* by

showing bands for *nuc* genes, 16S rRNA and *mea* genes (Fig. 1 and 2).

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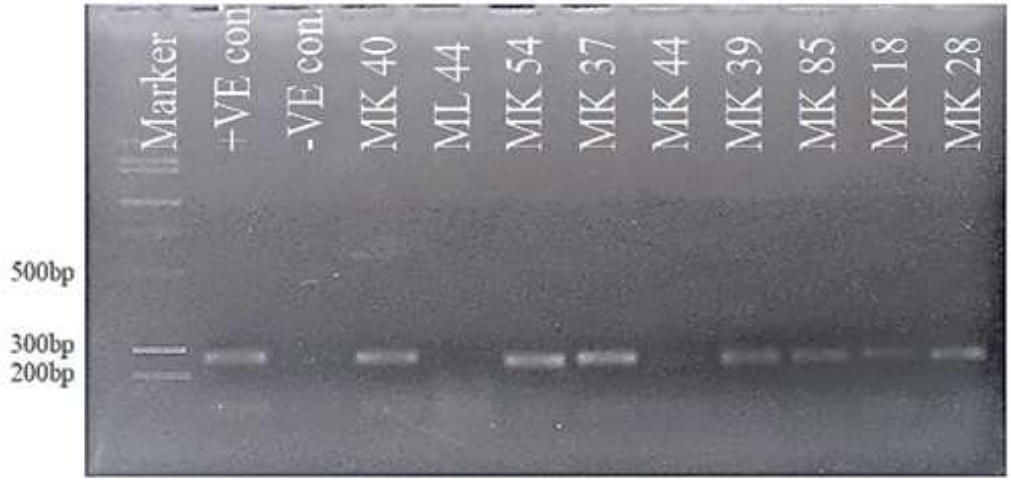

Figure 1: PCR gel picture for *nuc* gene detection in S. *aureus* from fish in Unguja island: MK 40, MK 54, MK 37, MK 39, MK 85, MK 18 and MK 28 are positive isolates; MK 44 is negative isolate, -VE con is negative control, +VE con is a positive control

All *S. aureus* were obtained from skin swabs from Mkokotoni landing site. Hence, the prevalence of S. aureus in Indian Mackerel Fish was 1.75% (95% CI: 0.0007 – 0.036).

Virulence genes in S. aureus

Out of seven samples two samples (MK40 and MK 54) had *mea* genes (Fig 2) and one sample (MK 39) was positive for enterotoxins B (SEB) and enterotoxins C (SEC) (Fig. 3). One *S. aureus* isolates has enterotoxins, hence posing a threat to public health

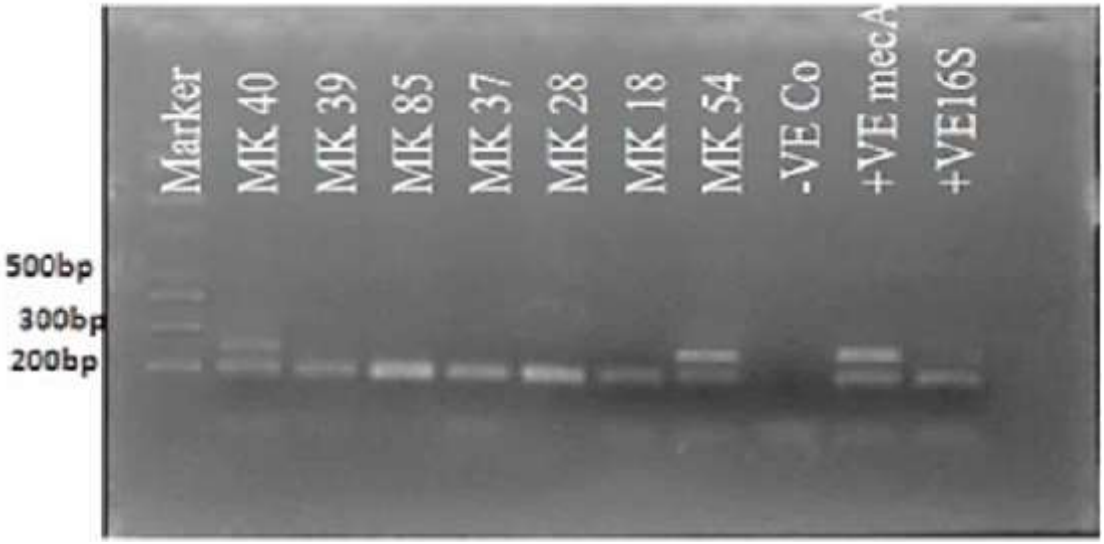

Figure 2: Multiplex PCR gel picture for *mea* and 16S rRNA genes detection in isolates from fresh Indian Mackerel Fish in Unguja island. MK 40 and MK 54 are *mea* and 16S rRNA positive isolates, MK 39, MK 85, MK 37, MK 28 and MK 18 are 16S rRNA positive, -VE Co is negative control, +VE *mea* is positive control for *mea* gene and +VE16S is a positive for 16S rRNA gene

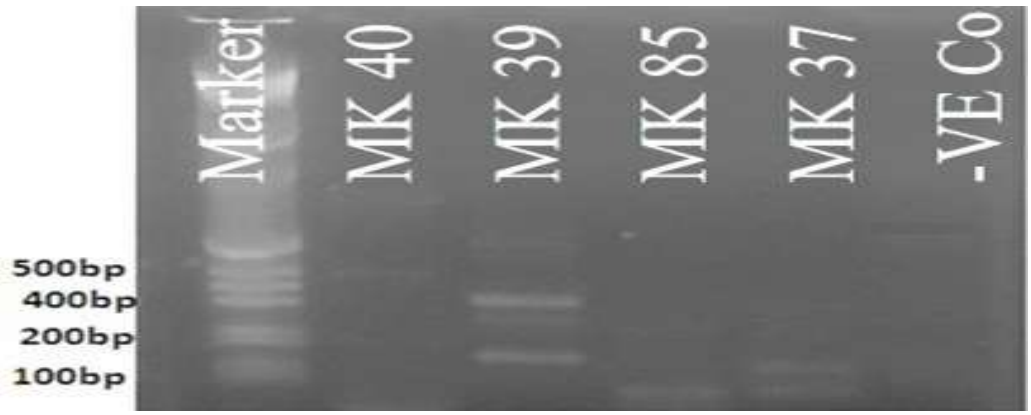

Figure 3: Multiplex PCR gel picture for enterotoxin detection in *S. aureus* isolates. MK 39 is positive for SEB and SEC, MK 40, MK 85 and MK 37 are negative isolates

DISCUSSION

Findings from the primary culture demonstrated a high growth of bacteria in Indian mackerel fish in all three studied landing sites. Staphylococcus aureus has also been isolated from skin swabs. These findings indicate contamination of the fish. Various factors pose risks to sea food safety and they range from contamination within the environment of its origin up to the point of consuming (Mohamed Hatha, Maqbool, and Suresh Kumar 2003; Rhee and Woo 2010). According to Reij and his colleague, potentially pathogenic bacterial present in foods can reach high number without necessarily producing noticeable alterations in features, odor or taste (Reij and Den Aantrekker 2004), hence jeopardizing public health.

Despite of very high contamination with other bacteria in this study, the prevalence of *S. aureus* is low compared with other studies that had prevalence from 17% - 61.67% in India and Japan (Rhee and Woo 2010; Shimizu et al. 2000; Simon and Sanjeev 2007). This difference could be due to difference in fishing and fish handling processes. Long distance from trapping sites to the shore, improper storage during transportation, inappropriate accumulation and packaging of frozen fish are some reported factors related to high contamination of frozen fish compared with fresh fish (Reij and Den Aantrekker 2004). The latter type was assessed in the present study. Contamination of Indian mackerel fish in this study might be due to direct contact of fish with contaminated environment, handling tools (knives, plastic bags, plastic buckets and fishing nets) used to unpack the fish from the boats and container used in freezing systems. This is because S. aureus was recovered from skin swabs and not from muscles. In the present study, through personal observation, contamination of fish with *S. aureus* could also be attributed to raw sewage that is discharged directly to the sea. This situation was observed in all three landing sites. Similar contamination of fish in relation to direct sewage into the sea has been reported in India (Sujatha et al. 2011)

The presence of *mea* gene in *S. aureus* strains was tested. In this study *mea* amplicons were identified in some strains. The presence of *mea* in species of *Staphylococcus* other than *S. aureus* was previously demonstrated (Carneiro, Queiroz, and Merquior 2004). Recent studies pointed out that the *mea* gene is present in mobile staphylococcal chromosomal cassette *mea* (Jansen et al. 2006; Katayama et al. 2003), and some of enterotoxin gene, along with other virulence factors are part of mobile pathogenicity Islands (Novick 2003; Novick and Subedi 2007). A recent study indicated that high mobility of the *mea* gene may be more prevalent than the movement of enterotoxins genes (WHO 2010).

In the study, the selected isolates were found negative for *pvl* genes. This could indicate that *pvl* is not normally found in the isolates of landing site environment. However, these isolates are not necessarily representative of landing sites environment in general. Absence of *pvl* in *S. aureus* from landing site environment may indicate limited role of antileucocytic activity outside the host. The absence of *pvl* in this study could also be contributed by the sample of *S. aureus* isolates being 7 out of 400 samples examined. Reports from various countries showed that there is increasing prevalence of *pvl* genes among isolates (Eckhardt et al. 2003; Linde et al. 2005). Prevalence of 64% *pvl* positive isolates in *S. aureus* was reported by Souza and colleagues in India (D'Souza, Rodrigues, and Mehta 2010). A lower prevalence of *pvl* has been reported from other parts of the world (5% in France, 4.9% in U.K, 8.1% in Saudi Arabia and 14.3% in Bangladesh) (Afroz et al. 2008; Holmes et al. 2005; Lina et al. 1999).

This study has revealed the presence of potentially pathogenic bacteria *S. aureus* in Indian mackerel fish in Unguja island. The presence of *S. aureus* in fresh fish is the source of health risk for fish handlers and consumers. Therefore, the public should be made aware and necessary preventive measure instituted so as to protect public health.

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