

First report of the presence of enterotoxin gene in coagulase-negative staphylococci recovered from meat of snails (*Achatina achatina*)

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Abstract

Introduction: It has been suggested that coagulase-negative staphylococci can serve as reservoirs of virulence genes for other bacteria. This study assessed the presence of such genes in selected isolates recovered from meat of the giant African snail (*Achatina achatina*). **Material and Methods:** Virulence genes were detected using a polymerase chain reaction targeting specific primers. Two representative isolates were identified using 16S rRNA gene sequencing. **Results:** The results showed that the staphylococcal enterotoxin A gene (*sea*) was present in five out of the eight isolates studied. The isolates expressed resistance mainly to three antibiotics: chloramphenicol, norfloxacin and cloxacillin in descending order of incidence. Most importantly, the *Staphylococcus sciuri* isolate NEDU 181, in addition to being resistant to the three aforementioned antibiotics, also harboured the *sea* gene. **Conclusion:** Our findings demonstrate, for the first time, the presence of toxigenic and antibiotic-resistant coagulase-negative *Staphylococcus* spp. in commercially-available fresh snail meat. With staphylococcal enterotoxin A known to survive cooking temperature, this presents a food safety concern.

Keywords: fresh snail meat, *Staphylococcus sciuri*, 16S rRNA gene, *sea* gene, antibiotic resistance.

Introduction

Foodborne diseases have been recognised as a major challenge that strains the resources of several countries worldwide. Approximately 500 million cases of foodborne diseases are recorded *per annum* and the prevalence is heavily skewed towards developing countries (26, 37). Even though all categories of foods have been implicated in incidences of foodborne illness, foods of animal origin are most frequently their sources (26). Foods offer suitable growth environments for toxin-producing bacteria such as *Staphylococcus*, which is able to grow and exhibit virulence in a wide variety of foods (1). The presence of *Staphylococcus* in the intestinal tract of animals has been reported, and raw meat may contain *Staphylococcus* because of contamination with intestinal content during evisceration (8).

Staphylococcal food poisoning is often associated with the growth of *Staphylococcus* in protein-rich foods such as meat. The cooking temperature of food can eliminate *Staphylococcus* species, but enterotoxins, which are produced by some species, are heat stable (28). As little as 20 – 100 ng of staphylococcal enterotoxin can cause food poisoning (3). Staphylococcal enterotoxin A (SEA) is the most commonly reported enterotoxin in foods and it is the main cause of staphylococcal food poisoning. This might be due, not only to its heat-stability but also, to its extraordinarily high resistance to proteolytic enzymes (39). The predominance of SEA in most foodborne disease outbreaks in different countries is well documented. For instance, approximately 90% of food-poisoning isolates were reported to contain the *sea* gene in Korea (11). Since SEA is toxic even in low concentrations, detection of strains which harbour the

sea gene at any point along the food chain should raise food safety concerns.

Coagulase-negative staphylococci (CNS) are not among the classical food poisoning bacteria, yet there are reports of their association with intoxication (5, 36). The potential for CNS to serve as reservoirs of virulence genes for other bacteria has been considered (9). It has been proposed that some CNS such as the pathogenic strain of *S. sciuri* are primarily transferred by contact (14). The possible ease of transmission along the food chain, which this implies, is a risk that should not be ignored.

Snail meat is in high demand among consumers in Nigeria: the yearly demand has exceeded 7 million kg in this country alone (2, 30). However, commercially available edible snails consistently harbour high counts of viable bacterial pathogens (23, 32), probably because they are sourced from environments often associated with wastes. Snail meat is usually subjected to stages of culinary preparation which include shucking (extraction of the meat from the shell), evisceration, desliming and cooking. The preparation of snail meat in domestic kitchens might lead to the contamination of kitchen utensils, surfaces, sponges, food, and the dissemination of some pathogens such as *S. aureus*, *Bacillus cereus*, *Escherichia coli*, *Citrobacter* and *Aeromonas* species (23, 31, 32) within the home setting, thereby increasing the risk of infections. Edible snails also appear to be a neglected factor in foodborne disease epidemiology, which is evident in the lack of information about the virulent nature of coagulase-negative *Staphylococcus* in this foodstuff.

Therefore, the aim of this study was to determine the virulence potential of coagulase-negative *Staphylococcus* species isolated from edible snails. The investigation subjected this popular source of meat in Nigeria to phenotypic and genotypic methods.

Material and Methods

Source of bacterial strains. In this study, eight strains of coagulase-negative *Staphylococcus* predominant in raw snail meat (*A. achatina*) were used. The strains were isolated and phenotypically identified in our previous study on commercially available raw snail meat in Nigeria (31). The strains were subcultured on tryptone soya broth and incubated at 37°C.

Phenotypic tests for virulence factors. The strains were subjected to phenotypic tests for virulence factors

such as haemolysin, gelatinase and lecithinase according to published methods (13, 18, 22).

Determination of antibiotic resistance in isolates. The strains were tested for antibiotic resistance against cloxacillin, amoxycillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, levofloxacin, norfloxacin, rifampicin and streptomycin using the Kirby-Bauer disc diffusion method following the methodology in the Clinical and Laboratory Standards Institute M02-A12 Supplement (15).

Detection of two virulence genes in selected isolates. DNA extraction was achieved using a Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, CA, USA) in accordance with the manufacturer's instructions. A touchdown PCR was performed according to Don *et al.* (21) to detect the presence of the two virulence genes *sea* and *exhc* in eight selected isolates. The primer sequences were synthesised by Integrated DNA Technologies (Leuven, Belgium) (Table 1) (14, 19). The presence of amplicons was appropriately determined by electrophoresis of the reaction products and a 50 bp DNA ladder (New England Biolabs, Ipswich, MA, USA) as a molecular marker. Gels were stained with 5 µL of ethidium bromide solution at 10 mg/mL and documented using the Enduro GDS gel documentation system (Labnet International, Edison, NJ, USA) (19).

Identification of selected isolates using 16S rRNA gene sequencing. The DNA of the selected isolates was extracted as outlined above. The PCR assay was performed as described by Lane (27) using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1525R: 5'-AAGGAGGTGWTCARCCGCA-3' universal primers. Sequences of the DNA fragments were determined using a 3130xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) at the International Institute of Tropical Agriculture, Ibadan, Nigeria. The sequences of the isolates were automatically compared with reference sequences of related strains available in GenBank using BLAST (www.ncbi.nlm.nih.gov/Blast.cgi). The identity of each isolate was determined based on the highest percentage of sequence similarity to reference strains in the database.

Data analysis. Phylogenetic trees were constructed by the neighbour-joining method using a web version of a phylogeny/the ClustalW software package (<http://www.phylogeny.fr/index.cgi>). The 16S rRNA sequence data of the bacterial isolates sequenced in this study were deposited in GenBank and accession numbers were issued.

Table 1. Primers used for the detection of two virulent genes in coagulase-negative *Staphylococcus* recovered from edible snails

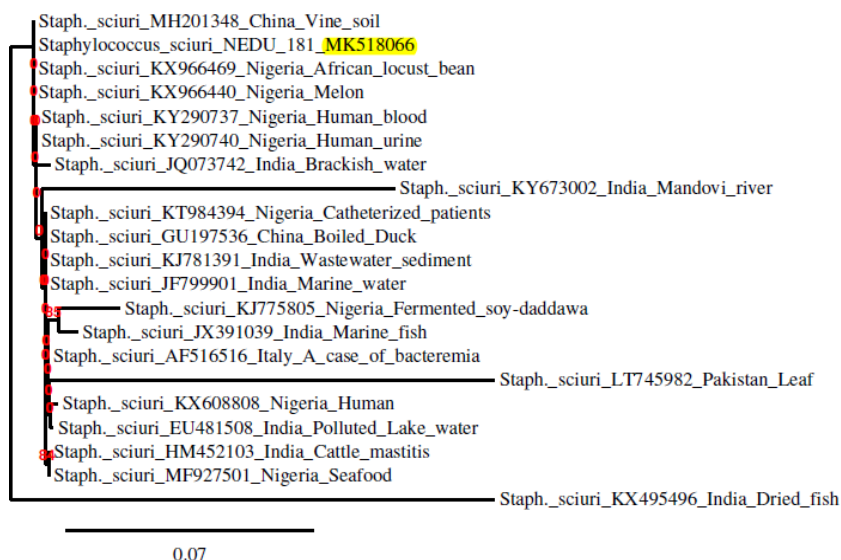
Target gene	Primer sequence (5'-3')	Amplicon size (Base pairs)	Reference
<i>Sea</i>	F: TAA GGA GGT GGT GCC TAT GG R: CAT CGA AAC CAG CCA AAG TT	180	(19)
<i>Exhc</i>	F: GAATAAATATTATGGAGTCTCTCTGATC R: CCATAGTATTTCAATCCAAAATCAGTAC	525	(14)

Table 2. Antibiotic resistance and some virulence factors in coagulase-negative *Staphylococcus* spp. isolated from edible snails

Isolate code	Cloxacillin	Chloramphenicol	Norfloxacin	Haemolysin	Gelatinase	Lecithinase	Sea gene	Exhc gene
0180 EN	S	R	R	+	+	+	–	–
0181 EN	R	R	R	+	+	+	+	–
0182 EN	R	S	S	+	+	+	+	–
0183 EN	S	R	S	+	+	+	+	–
0184 EN	S	S	R	+	+	+	–	–
0185 EN	S	S	S	+	+	+	+	–
0186 EN	S	S	S	+	+	+	–	–
0187 EN	S	R	S	+	+	+	+	–

R – Resistant;

S – Sensitive

**Fig. 1.** Phylogenetic tree showing the relationship of *Staphylococcus sciuri* NEDU 181 to reference strains based on 16S rRNA gene sequences in GenBank. Yellow highlighting indicates the GenBank accession number

Results

The eight CNS isolates tested positive for haemolysin, gelatinase, and lecithinase (Table 2). Generally, the isolates were sensitive to 70% of the antibiotics used in this study. The antibiotics to which isolates expressed resistance were chloramphenicol, norfloxacin and cloxacillin in descending order of incidence (Table 2). The results of our study showed that the most resistant isolate was *S. sciuri* NEDU 181 (0181EN), which expressed resistance to all three of these antibiotics. This isolate was found to be closely related to *S. sciuri* based on 16S rRNA gene sequencing (Fig. 1). The results of the PCR analysis for virulence genes in CNS isolates revealed that five of them harboured the *sea* gene but none had the *exhc* gene (Table 2). Two CNS isolates (0180EN and 0181EN) were selected for identification by 16S rRNA gene sequencing. Application of BLAST to the nucleotide sequences of the isolates on the US National Center for Biotechnology Information website revealed that isolates 0180EN and 0181EN were closest to *S. arlettae* and *S. sciuri*, respectively. The accession numbers assigned were MK518344 and MK518066. The phylogenetic tree showing the relationship between the 16S rRNA sequences of our strain, *S. sciuri* NEDU 181 and the sequences of other similar isolates in GenBank

is presented in Fig. 1. Our strain was revealed to be most phylogenetically related to other strains of *S. sciuri* isolated from soil and plants.

Discussion

Haemolysin, gelatinase and lecithinase, which were detected among the eight CNS isolates, are often reported among *Staphylococcus* species possessing virulence genes (7, 16). There was a low prevalence of antibiotic resistance among the isolates. Cloxacillin is one of the antibiotics that the isolates expressed resistance against and it is related to oxacillin, which is more sensitive for predicting methicillin resistance. Batista *et al.* (4) found that 100% of their isolates from clams containing the *mecA* gene were phenotypically resistant to oxacillin. In line with our findings, another study on *S. sciuri* isolates from poultry farms in Ghana found the isolates to be resistant to several antibiotics including chloramphenicol, ofloxacin and norfloxacin (10). A possible explanation for a connection between the snails in the present research and antibiotic-resistant isolates could be the herbivorous snails diet, primarily comprised of vascular plants (35). Their participation with other soil invertebrates in the decomposition of leaf litter could be another factor (25). Vascular plants may

have been contaminated with antimicrobial-resistant bacteria following sewage discharges or the use of irrigation water contaminated with the faeces of humans and animals reared with antibiotic abuse (6). Plant leaves, vegetables and kitchen wastes have come into common use by snail breeders as feed because formulated feeds for snails are not available on the Nigerian market (12). Furthermore, scientific studies have shown that soil is a major reservoir of antibiotic resistance genes (29).

Because five CNS isolates were found to harbour the *sea* gene, it is possible that the snails examined in this study were exposed to enterotoxigenic *S. sciuri* in their preharvest environment. This finding gives rise to food safety concerns because enterotoxin A is heat-stable and can survive cooking temperature. Although the occurrence of the *sea* gene in CNS is very rare, the need to screen for such a gene among CNS has been acknowledged in several articles since they were suggested as reservoirs of virulence genes associated with food borne disease (9). Dakić *et al.* (20) did not detect genes encoding staphylococcal enterotoxins in a panel of 48 CNS isolates recovered from clinical human samples and a hospital environment. Piechota *et al.* (33) detected *sec* genes in five *S. sciuri* isolates from cow's milk in Poland but reported the absence of the *sea* gene in these isolates. Furthermore, Pyzik *et al.* (34) screened for enterotoxin genes in CNS from broiler chickens and turkeys in Poland, but none of the isolates was reported to possess the genes for enterotoxin A, C or D. Since staphylococcal enterotoxin genes are often carried on mobile genetic elements such as plasmids (24), it is possible that the CNS strains isolated in this study may have gained the *sea* gene through genetic transfer, which could precipitate the transfer of this gene to other species capable of expressing it. It is important to note that the ability of *S. sciuri* to transfer genes of resistance to other bacteria pathogenic to man has been established (38). Efuntoye *et al.* (23) used phenotypic tests to demonstrate the enterotoxigenic potential of some coagulase-positive *Staphylococcus* isolates in varieties of snails obtained from south-west Nigeria.

As expected, *S. sciuri* NEDU 181 is genetically closest to other strains isolated from soil and plants. *Staphylococcus sciuri* is considered one of the most ancient and dispersed staphylococcal species, with a wide range of habitats such as the skin of several animals, and several environmental reservoirs, such as soil (17).

Our findings demonstrate for the first time the presence of toxigenic and antibiotic-resistant coagulase-negative *Staphylococcus* in fresh snail meat, which suggests a food safety concern. There is need for further investigation to clarify this novel hazard for the following reasons: commercially-available snails do not undergo any form of safety certification before being purchased by consumers, especially in developing countries; the stages of culinary preparation of snail meat (shucking, evisceration and removal of slime) in

domestic kitchens potentially disseminate pathogens within the home and the staphylococcal enterotoxin is known to survive cooking temperature.

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