Genome analysis of Salmonella strains isolated from imported frozen fish in Burkina Faso

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Abstract

Purpose

Fish is an excellent source of protein and vitamins for humans, but improperly handled fish can expose consumers to pathogenic bacteria. This study aimed to isolate and characterize the genomes of Salmonella strains isolated from imported fish sold in the open market in Ouagadougou.

Methods

One hundred and fifty-nine fish were collected from open markets. Antimicrobial susceptibility was determined by broth microdilution. Whole Genome Sequencing was done to further study antibiotic resistance genes, plasmid replicons, and MSLT types. Serotyping was done using SeqSero 2.

Result

Out of the 159 fish samples analyzed, 28 (17.61%) were found to be contaminated with Salmonella. Among the isolated Salmonella strains, 6 different serotypes, Nima, Liverpool, Kokomlemle, Gaminara, Derby, and Tennessee, were found using SeqSero2. S. Tennessee was the predominant serotype. All the isolates possessed at least one resistance gene. The aac6-Iaa and aac6-Iy conferring resistance to aminoglycosides was the most prevalent gene found in the strains. The gene fosA7 was detected in two. All the S. Nima isolates were of Multilocus Sequence Type (MLST) 2258, Gaminara was ST 5197; Liverpool was ST 1959; Derby was ST 3997; Kokomlemle was ST 2696. The serotype Tennessee isolates gave many different STs such as ST 3763; 3997; 3135.

Conclusion

The presented results highlight the prevalence of Salmonella on imported fish purchased from the open markets. More attention should be paid regarding fish selling conditions in the country to prevent the potential health risk for consumers.

Introduction

Burkina Faso is a landlocked tropical country located in Sub-Saharan Africa. This country is characterized by a dry season from October-May with hot temperature (35–45 Celsius) and a short rainy season (June-September). In recent years, fish consumption has increased exponentially in this country with more than 96% of commercially sold fish imported from another country (1). Fish is an important source of essential amino acids and good fatty acids for humans, but fish can be contaminated by pathogenic bacteria that pose a high risk for consumer's health (2; 3). These pathogenic bacteria can contaminate ready to eat fish product through cross-contamination during fish processing (4). *Salmonella* has been implicated in fish outbreaks worldwide (5; 6).

Nowadays, the use of antibiotics in aquaculture practices as growth promoters or for treatment and prevention of fish diseases is increasing the risk of development of antibiotic resistant bacteria among the microbiome of fish gut and/or fishing water (7). Many studies have shown widespread transmission of antibiotic resistant bacteria of the aquatic or fish to human trough environment and/or fish consumption (8, 9).

According to the Centers for Disease Control and Prevention (CDC), antibiotic resistant infection is responsible for 25,000 annual deaths in the European Union and 23,000 annual deaths in the U.S (10). The World Health Organization (WHO) report on the burden of food-borne disease clearly shows that this burden is similar to the burden of malaria, tuberculosis and even HIV AIDS (11). The report also shows that the burden of food-borne disease is disproportionately borne by the least developed countries and by children. Since imported fish is widely consumed in Burkina Faso, it is important to know the microbiological quality of these fish. Therefore, the present study aims to understand the epidemiology and antibiotic resistance of *Salmonella* strains isolated from fish using whole genome sequencing and phenotypic methods.

Materials And Methods

Sampling

Imported fish samples were purchased from different open markets. All fish samples during collection were placed in sterile polypropylene bag, placed in polystyrene box containing crushed ice and the temperatures was 4°C during transportation. The samples were transported to the laboratory and examined on the same day for the presence of *Salmonella* spp.

Bacteriological analysis

Salmonella strains were isolated from fish samples following the methodologies described in the International Organization for Standardization 6579-2017 (12). The fish samples were gently removed from coolers and processed in aseptic condition. The gills, intestines parts and skin parts were removed using sterile knifes. About 10 g of samples (fish gills, intestines parts and skin) were placed into a stomacher bag containing 90 mL of buffered peptone water (Liofilchem, Teramo, Italy) and homogenized using a stomacher (400 Circulator, Seward, London,UK) for 1 min and incubated for 24 h at 37 °C. From this non selective pre-enrichment, 0.1 mL were transferred into 10 mL of Rappaport-Vassiliadis broth (Oxoid, Basingstoke, England) and incubated for 24 h at 42°C. A loopful from the selective enrichment broth was streaked onto XLD (Oxoid, Basingstoke, England) agar and incubated for 24 h at 37 °C. Suspected colonies on selective agar plates were purified and bio-typed by using biochemical tests and API 20E strips (BioMerieux, Marcy l'Etoile, France).

Confirmed colonies were sent to the United States Department of Agriculture, Bacterial Epidemiology and Antimicrobial Resistance Research Unit for future analysis.

Antimicrobial susceptibility testing

The isolates were streaked onto Blood agar plates and incubated for 24 h at 37°C, and one colony from each plate was streaked onto new Blood agar plate for another 24h at 37°C. Minimum inhibitory concentrations (MIC, µg/mL) of all *Salmonella* isolates were determined by broth-microdilution using the Sensititre™ semi-automated antimicrobial susceptibility system (TREK Diagnostic Systems Inc., Cleveland, OH, USA) and the Sensititre™ Gram-Negative plate format, with plate code GN4F (Thermo, Fisher Scientific, USA), according to manufacturer's directions. MICs of the isolates for the 24 antimicrobials were determined using colonies from the last 24h Blood agar plates, and each isolate was classified as resistant, intermediate, or susceptible to the antimicrobials tested using the breakpoints set by Clinical and Laboratory Standards Institute (CLSI) [13]. Antimicrobials used breakpoints were as follows: Amikacin (≥ 64 µg mL-1); Piperacillin/tazobactam (≥ 128/4 µg mL-1); Tigecycline (≥ 1 µg mL-1); Ticarcillin/clavulanic acid (≥ 128/2 µg mL-1); Levofloxacin (≥ 2 µg mL-1); Nitrofurantoin (≥ 128 µg mL-1); Tetracycline (≥ 16 µg mL-1); Doripenem (≥ 4 µg mL-1); Minocycline (≥ 16 µg mL-1); Ertapenem (≥ 2 µg mL-1); Trimethoprim/sulfamethoxazole (≥ 4/76 µg mL-1); Imipenem (≥ 4 µg mL-1); Piperacillin (≥ 128 µg mL-1); Meropenem (≥ 4 µg mL-1); Gentamicin (≥ 16 µg mL-1); Cefazolin (≥ 8 µg mL-1); Tobramycin (≥ 16 µg mL-1); Ceftazidime (≥ 16 µg mL-1); Ampicillin/sulbactam (≥ 32/16 µg mL-1); Aztreonam (≥ 16 µg mL-1); Ampicillin (≥ 32 µg mL-1); Cefepime (≥ 32 µg mL-1); Ciprofloxacin (≥ 4 µg mL-1); Ceftriaxone (≥ 4 µg mL-1). For the analysis, isolates identified as intermediate were considered susceptible to the drug. *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213 were controls for determination of MIC.

For each isolate, a final inoculum of 5 x 10^5 CFU/ml was targeted. The panels were read after 18 h of incubation at 35°C.

Whole genome sequencing

Genomic DNA was isolated using the GenElute bacterial genomic DNAkit (Sigma-Aldrich, St. Louis, MO, USA) following instructions for Gram-negative bacteria, from 5 mL of overnight cultures grown in Luria-Bertani Broth, Miller (Difco™, Becton Dickinson and Company, Sparks, MD) at 37°C with shaking. The extracted DNA quality was read using NanoDrop 2000c spectrophotometer (Thermo, Fisher Scientific, USA). DNA was stored at -20°C prior to library preparation.

Extracted DNA was quantified using the Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit according to the manufacturer's instructions (Life Technologies, Inc., USA). The Illumina libraries were prepared using the Nextera XT DNA library preparation kit and Nextera XT index primers (Illumina, USA). The library fragment size distribution was checked using the Bioanalyzer 2100 with an Agilent HS DNA kit (Agilent Technologies, USA) and quantified using a Qubit DNA HS assay kit in a Qubit fluorometer (Thermo, Fisher Scientific, USA). The generated libraries were then sequenced using a MiSeq version 2 reagent kit with 500 and 300 cycles. The paired-end read length of 2 X 250 bp was used for 500 cycles and 2 X 150 bp for 300 cycles on the Illumina MiSeq platform. The quality metrics of the reads were

performed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The sequence data were assembled using the A5-miseq assembler (14), and the genome sequence was annotated via the NCBI Prokaryotic Genome Annotation Pipeline (15).

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession XXXXXX000000000. The version described in this paper is version XXXXXX010000000.

Identification of antibiotic resistance genes, chromosomal mutations, serotypes, MLST and plasmid from total genome sequence

Antibiotic resistance genes and chromosomal mutations were identified using ResFinder 3.2 (16). SeqSero 2 was used to determine the serotypes of salmonella strains from genome assembly data. MLST sequence type was identified using MLST database from the center of genomic epidemiology (17). The PlasmidFinder were used to detect plasmid from the strains (18).

Results

Out of the 159 fish samples analysed, 28 (17.61%) were found to be contaminated with *Salmonella*. Among the isolated *Salmonella* strains, 6 different serotypes such as Nima, Liverpool, Kokomlemle, Gaminara, Derby and Tennessee were found using SeqSero2 with some being N/A. *S*. Tennessee was the predominant serotype. All the isolates possessed at least one resistance gene. The non-functional *aac*6-Iaa and *aac*6-Iy conferring resistance to aminoglycosides was the most prevalent gene found in the strains. The gene fosA7 conferring resistance to Fosfomycin was detected in two strains. The isolates were susceptible to all drugs tested. Four *S*. Nima isolates were identified from the 28 isolates and were all MLST Sequence Type (ST) 2258. One *S*. Gaminara isolate was identified and was ST 5197; two *S*. Liverpool isolates were detected and were identified as MSLT ST 1959; one *S*. Derby isolate was found and was ST 3997; One *S*. Kokomlemle was detected and was MLST ST 2696. Thirteen *S*. Tennessee isolates were found with different sequence types including ST 3763, ST 3997, ST 3135, and ten were unknown. Two strains possessed plasmid replicons: one IncFII(S) and one IncFII(pCRY) (Table 1). The amino acid substitution Thr57Ser and Ser80Ile in ParC were detected in 25 isolates. Point mutations in the quinolone resistance-determining regions (QRDRs) were detected in 25 (89.28%) isolates at positions 57 (Thr57Ser) and 80 (Ser80Ile) for ParC (Table 1).

Table 1
Resistance genes, plasmid replicon, MSLT type of salmonella isolates from fish

Sample ID	Serotype	Resistances genes	Plasmid replicon	QRDR point mutation ParC	MLST sequence Type (ST)	Accession Numbers
87	Nima	aac(6')-Iaa	-	Thr57Ser	2258	SAMN18249072
88	Tennessee	aac(6')-Iaa; fosA7	-	Thr57Ser	3763	SAMN18249073
89	N/A	aac(6')-Iaa	IncFII(pCRY)	0	530	SAMN18249074
95	Gaminara	Aac6-Iy	-	Thr57Ser	5197	SAMN18249075
98	N/A	Aac6-Iy	-	Thr57Ser	565	SAMN18249076
100	N/A	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249077
101	N/A	Aac6-Iy	-	0	3531	SAMN18249078
103	Derby	aac(6')-Iaa	-	Thr57Ser	3997	SAMN18249079
108	Tennessee	Aac6-Iy	-	Thr57Ser	3997	SAMN18249080
113	Liverpool	aac(6')-Iaa	-	Thr57Ser	1959	SAMN18249081
116	Tennessee	fosA7; aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249082
117	Tennessee	Aac6-Iy	-	Thr57Ser	3135	SAMN18249083
119	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249084
122	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249085
127	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249086
128	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249087
129	Nima	aac(6')-Iaa	-	Thr57Ser	2258	SAMN18249088
130	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249089
131	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249090
134	Kokomlemle	aac(6')-Iaa	IncFII(S)	Thr57Ser	2696	SAMN18249091
135	N/A	aac(6')-Iaa	-	0	Unknown	SAMN18249092
136	Liverpool	aac(6')-Iaa	-	Thr57Ser	1959	SAMN18249093
137	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249094

QRDR: quinolone resistance-determining region; MLST: Multilocus Sequence Typing

Sample ID	Serotype	Resistances genes	Plasmid replicon	QRDR point mutation ParC	MLST sequence Type (ST)	Accession Numbers
138	Nima	aac(6')-Iaa	-	Thr57Ser	2258	SAMN18249095
139	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249096
141	Tennessee	aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249097
142	Nima	aac(6')-Iaa	-	Thr57Ser	2258	SAMN18249098
144	N/A	aac(6')-Iaa; aac(6')-Iaa	-	Thr57Ser	Unknown	SAMN18249099
QRDR: quinolone resistance-determining region; MLST: Multilocus Sequence Typing						

Accession numbers generated during the current study are assigned in Table 1.

Discussion

The present study was initiated to determine the microbiological quality of imported and local fish consumed in the city of Ouagadougou, Burkina Faso. The prevalence of *Salmonella* strains was 17.41% from imported fish. This result could be explained by the fact that imported fish are exposed to several stages of handling including packaging at the farm in the origin country, transport to Burkina Faso, reception at wholesalers, delivery to semi-wholesalers, and delivery to different retailers. All these steps undoubtedly favor the contamination by bacteria like *Salmonella*. However, the consumption of imported fish is very high in Burkina Faso because it is very accessible and inexpensive in all the localities of the country. In these localities, the imported fish is cut into small pieces by small traders and sold at a minimum price of 50 FCFA (about 1 cent of dollar). This necessitates permanent monitoring of the prevalence of germs that can affect the health of consumers as well as chemicals. The population of Burkina Faso is over 80% illiterate, which will undoubtedly lead to an increase in contamination of raw fish and the possibility of cross-contamination due to a lack of training and information on the causes and consequences of foodbornes diseases (19). The prevalence of *Salmonella* in fish in this study is higher than those reported by Broughton and Walker, (20) from fish in China (5%) and by Heinitz et al. (21) in U.S.-imported raw seafood from several Asian countries (10%). These variations in prevalence can be explained by differences in farming methods, and in the food safety regulations of each country. For example, in Burkina Faso, many researchers demonstrated that good hygienic practices are not respected yet by food sellers and domestic food safety regulation and / or training program still missing (22; 23; 24).

Salmonella Tennessee was the most prevalent serotypes among fish samples. This serotype of *Salmonella* was detected in different types of samples and in the stools of patients with diarrhea in other studies from Burkina Faso (25). *S.* Tennessee has also been implicated in outbreaks in the United States

due to contaminated peanut butter; powdered milk products and infant formula (26; 27). These facts show us that the Tennessee serotype is not necessarily linked to a specific food or environment. Depending on the handling of food, this serotype can contaminate humans through any contaminated food.

We also have the presence of *S*. Liverpool, which is a pathogenic serotype and has not been identified in our previous studies carried out in Burkina Faso in diarrheal patients, chickens, the environment, or animals (25; 28; 29). *S*. Derby identified was the most dominant in our previous studies in chickens and slaughter animals (Kagambèga *et al*., 2013). *S*. Nima and *S*. Kokomlemle also have been isolated in chicken and beef previously in Burkina Faso (30). *S*. Gaminara has been identified in a patient suffering from diarrhoea in Burkina Faso (28).

The presence of these serotypes in fish shows that chicken, slaughter animals, the environment and humans share the same pathogens that circulate in our country.

Twelve different MLST sequence type were find from the strains. A diversity of MSLT type was detected in our study with *S*. Tennessee. This may show that *S*. Tennessee has genetic diversity within its population. We can say that the other serotype with a unique MSLT type retained their genetic characteristic during their evolution while keeping the same type of MLST. On the other hand, *S*. Tenessese population structure has changed during evolution.

All the *Salmonella* strains found in this study possessed aminoglycoside resistance genes, encoding acetyltransferases (aac(6')-Iaa; aac(6')-Iy). While these genes were not functional in this study and are commonly non-functional in *Salmonella*, mutations in the promoter of the gene can lead to expression and phenotypic resistance (31). Rather *et al*. (32) demonstrated that aminoglycoside resistance in *Salmonella* strains is usually secondary to increased gene expression following regulatory mutations.

Point mutations in the quinolone resistance-determining regions (QRDRs) were detected in 25 (89.28%) isolates at positions 57 (Thr57Ser) and 80 (Ser80Ile) for ParC with known acquired antibiotic resistance as Nalidixic acid and Ciprofloxacin. However, all isolates were susceptible to both nalidixic acid and ciprofloxacin.

In this study, two *Salmonella* strains Kokomlemle and one unknown serotype possessed IncFII-type plasmids, which have been important in spreading resistance genes such as bla $_{NDM-1}$ and bla $_{CTX-M-15}$ (Xavier *et al*., 2016). Both strains did not harbour any beta-lactamase resistance genes. More investigation into these plasmid sequences are needed to determine any benefit they provide the strains.

Conclusion

This study has shown that widely consumed fish in Burkina Faso are contaminated with pathogenic bacteria of the genus *Salmonella*. The microbiological quality of fish sold in Burkina Faso must be improved to reduce the risks of contamination to consumers. Improved food safety will lead to reduced

losses, better access to markets and hence better incomes. The modern molecular biology technique used in this study as whole genome sequencing is a technique that is not yet available in the developing country. An urgent action is needed by decision-makers in Burkina Faso, other developing countries, and those around the world for collaboration in the regulation and monitoring of foodborne pathogens.

Abbreviations

WGS
Whole Genome Sequencing
NTS
Non-typhoidal *Salmonella*
CDC
Centers for Disease Control and Prevention
MLST
Multilocus sequence typing
MDR
Multidrug Resistant
LaBESTA
Laboratoire de Biologie Moléculaire, d'épidémiologie et de surveillance des bactéries et virus transmissible par les aliments
U.S
United States
USA
United States of America

Declarations

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Authors' contributions

AK, SB, SKD carried out the strain's isolation and characterization. LH, AK, SP, SKG, HR, EAM carried out the WGS analysis, AK drafted the manuscript. EAM, LH participated in manuscript writing. NB, CRJ, and JGF supervised the WGS and participated in writing the manuscript. All authors read, commented on, and approved of the final manuscript.

Availability of data and materials

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession XXXXXX000000000. The version described in this paper is version XXXXXX010000000.

Consent for publication

Not applicable.

Ethical considerations and consent to participate

Not applicable

Competing interests

The authors declare that they have no competing interests.

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