

PFGE *XbaI*[®] Indistinguishable properties of *Salmonella* Kastrup and *Salmonella* Larochelle isolates at beef processing and distribution continuum

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Abstract

Salmonellosis is a major cause of foodborne illnesses in humans throughout the world. Pulsed field gel electrophoresis (PFGE) is a commonly used tool for epidemiological survey and source tracking of *Salmonella* strains isolated from various sources. A total of 237 samples from various sources were collected at abattoir and butcheries in Ethiopia from December 2011 to April 2012. Isolates of *Salmonella* Kastrup (n = 3) and *Salmonella* Larochelle (n = 11) were characterized for antimicrobial resistance using disc diffusion and for genotypic profiles using PFGE *XbaI*[®]. *Salmonella* Kastrup and *S. Larochelle* were isolated from 1.3% and 4.6% of total samples (n = 237) examined. Except for water samples (n = 12) and truck swabs (n=11), all other sample sources were positive for one or more of *S. Kastrup* and *S. Larochelle*. *Salmonella* Kastrup was isolated in Ethiopia for the first time. *Salmonella* isolates from both Kastrup and Larochelle serotypes showed similar antimicrobial resistance profiles. Two pulsotypes consisting of the same three *S. Kastrup* and 10 *S. Larochelle* cluster and second cluster consisting of one *S. Larochelle* were identified at 1.5% and 1% band positions tolerance by unweighted-pair group method (UPGM) and showed $\geq 91\%$ similarity. *S. Kastrup* and *S. Larochelle* isolates showed similar antimicrobial resistance profiles with indistinguishable PFGE *XbaI*[®] patterns indicating possible clonal spread of the isolates along beef processing and distribution continuum.

Key words: *Salmonella*; PFGE; Antimicrobial resistance; Beef; Ethiopia

Introduction

Widespread distribution of *Salmonella* in the environment, their increase in prevalence in the global food chain, and their virulence and adaptability properties cause easy transmission resulting in enormous medical, public health and economic impact worldwide (Molbak *et al.*, 2006). Serology based on surface antigens (Grimont and Weill, 2007), phage typing based on bacteriophage host profile (Jay *et al.*, 2005), antimicrobial susceptibility; and biotyping, which uses biochemical tests to reflect metabolic activities of *Salmonella* strain (WHO, 2010), are used for phenotypic characterization. Pulsed field gel electrophoresis (PFGE) and whole genome sequencing are the most commonly applied genotyping techniques for epidemiological investigations of food borne gram-negative pathogens including *Salmonella* (Foley *et al.*, 2009; Miller, 2013; PulseNet, 2013). PFGE is based on the whole chromosomal DNA digestion with the use of one or more specific restriction endonuclease enzyme at selected genomic restriction sites. The restriction digestion results in 8- 25 high molecular weight DNA fragments of 10-800 kilobase (kb) in size (Wiedmann, 2002; Jay *et al.*, 2005). Some of the restriction enzymes used for *Salmonella* PFGE are *XbaI*, *BlnI*; *SpeI*, *SfiI*, *PacI* and *NotI*. Zheng *et al.* (2007) showed the differences in discriminatory power of each of these enzymes independently and/or in different combinations. Resulting DNA fragments are then separated by PFGE in agarose gels. The fingerprints derived from the process are analyzed by BioNumerics® software (BioNumerics, 2011) programmes to determine clonal diversity and relationship between the isolates (Olive and Bean, 1999).

In Ethiopia, different *Salmonella* serovars were isolated from animals and humans (Alemayehu *et al.*, 2003; Tibaijuka *et al.*; 2003; Ejeta *et al.*, 2004; Molla *et al.*, 2004; Aragaw *et al.*, 2007; Zewdu and Cornelius, 2009; Sibhat *et al.*, 2011; Beyene *et al.*, 2011; Hiko *et al.*, 2016; Kiflu *et al.*, 2017; Hiko *et al.*, 2018). However, PFGE analysis of the isolates was not conducted; thus establishing genetic relationships for epidemiological purposes is impossible. Using antigens, *S. Kastrup* (6, 7:e,n,z₁₅:1,6) and *S. Larochelle* (6, 7:e, h:1,2) shows closes while serotyping, (Grimont and Weill, 2007). But, the genotypic *XbaI* PFGE and phynotypic drug resistance profiles of *Salmonella* Kastrup and *Salmonella* Larochelle were not yet assessed in Ethiopia. The objective of the current study was to characterize *Salmonella* Kastrup and *Salmonella* Larochelle isolates obtained from various samples collected from beef processing and distri-

bution continuum in Ethiopia, for their genotypic profiles and antimicrobial resistance profile.

Materials and Method

Ethical Consideration

Samples were also collected from personnel's hands. Taking into consideration that the sampling was less invasive and also the samples were from adults working in abattoirs, individuals involved on the sample were informed regarding the details the research. Hence, informed consent was obtained from the study participants.

Description of beef processing and distribution continuum

The *Salmonella* strains we characterized in the current report were isolated from different sample types as part of a study conducted to investigate *Salmonella* at food processing plants and distribution. Addis Ababa Abattoir Enterprise (AAAE) and butchery which distribute raw beef in Addis Ababa city as well as selected beef processing plant and supermarkets in Addis Ababa city which distribute the processed beef (Mortadella) were investigated for sample collection. Ethical clearance for the study was obtained Ethiopian Engineering Capacity Building in collaboration with Health sector.

Sources of the Salmonella isolates

Swab samples from personnel hands, aprons, knives, hooks, rooms, refrigerators and beef transporting trucks were collected with sterile gauze moistened with normal saline. About 10 ml of tap-water samples were collected from the pipe line. Cattle feces were collected from the rectum; and mesenteric lymph nodes were collected after evisceration. Beef cut samples were collected at abattoir after meat inspection. Similarly, following the unique carcass identification number, raw beef of the respective cattle was sampled at retail butchers' shops (public meat supply station) in Ethiopia (Table 1). Samples were collected from December 2011 to April 2012. Bacterial isolation was conducted at Microbiology Laboratory, Akililu Lemma Institute of Pathobiology, Addis Ababa University, Ethiopia following standard protocols (Grimont and Weill, 2007).

Pre-enrichment was done using one portion of sample by volume or gram which was homogenized in 10 portions of buffered peptone water (BPW) (Merck, Germany) at 1:10 proportion. From the pre-enriched samples, 0.1 ml and 1 ml was transferred to 10 ml of Rappaport-Vassiliadis (RV) medium (Oxoid Hampshire, England) and 10 ml of Muller Kaufmann tetrathionate with novobiocin (MKTTn) (Merck) broths respectively for selective enrichment. RV and MKTTn broth cultures were then incubated at 43°C and 37°C respectively for 18-24 hrs. A loopful was plated on Brilliant phenol lactose sucrose agar (BPLS) (Merck) and Xylose lactose Tergitol™ 4 (XLT4) (Merck) in parallel and incubated at 37°C for 24 hrs and 48 hrs, respectively. Presumptive colonies based on their characteristic morphological appearances on the selective agar plates were sub-cultured onto standard-I nutrient agar (Merck) and biochemically using lysine test, TSI test, catalase test, indole production test and citrate utilization were confirmed for serotyping.

Serotyping

The isolates were stereotyped at Microbiology Laboratory, Institute of Meat Hygiene and Technology, Panel Veterinary Public Health, FAO Reference Center for Veterinary Public Health, Freie Universität Berlin, Germany. Serotyping was done using *Salmonella* antisera (Sifin, Berlin, Germany) with O-antigen and H-antigen agglutination test (Grimont and Weill, 2007).

Antimicrobial susceptibility testing

All of the isolates were tested for their phenotypic antimicrobial resistance by agar disc diffusion method with antimicrobial impregnated discs (Oxoid, Hampshire, England). The drugs were selected based on its used in health sectors in Ethiopia and availability on the market. The test was done against polymyxin-B (POL-B; 300U), trimethoprim-sulfamethoxazole (STX; 1.25/23.75 µg), chloramphenicol (CHL; 50µg), gentamycin (GEN; 10µg), trimethoprim (TRM; 5µg), neomycin (NEO; 10µg) and oxytetracycline (OxyTET; 30µg). Antimicrobial susceptibility tests were done on Mueller-Hinton agar (Oxoid) according to Bauer Kirby agar disc diffusion (Bauer *et al.*, 1966) following Clinical Laboratory Standards Institute's protocol (CLSI, 2007). The isolates were sub-cultured onto standard-I nutrient agar (Merck) and incubated at 37°C for 24 hrs. They were then inoculated into 3 ml of brain heart infusion broth (BHI) (Merck) and again incubated for 1 hr at 37°C. The inoculum density was standardized to 0.5 McFarland standards; from which 0.1 ml was spread onto Mueller-Hinton agar (Oxoid). After the plates were allowed to absorb the moisture;

antimicrobial impregnated discs were applied; and the plates were incubated at $35 \pm 2^\circ\text{C}$ for 16-18 hr. Based on the diameter of zone inhibition for *Enterobacteriaceae*, results were recorded as susceptible, intermediate or resistant (CLSI, 2007).

Pulsed field gel electrophoresis (PFGE)

The PFGE was conducted following PulseNet protocol (PulseNet, 2013) at Molecular Biology Laboratory, Institute of Meat Hygiene and Technology, Panel Veterinary Public Health, FAO Reference Center for Veterinary Public Health, Freie Universität Berlin, Germany. Agarose-embedded whole genomic DNA of the isolates was digested with *XbaI*[®] enzymatic restriction using 60U *XbaI*[®] (Roche Diagnostics GmbH, Germany). DNA fragments were separated by PFGE in agarose containing gels. *S. Braenderup* STSAL82 (Merck, Germany) was used as a reference strain. A 50-1000kb Pulse marker[™] (Sigma-Aldrich Co, USA), test strains and reference strain were loaded into 1.2% Pulsed Field Certified Agarose[®] gel. The gel running condition was set according to Pulse Net (2013) and followed by stained with 1mg/l ethidium bromide solution. Using Digital Imaging and Analysis System II (DIAS-II), the gel image was taken as “.jpg” processed into “.tif” file. The PFGE files were transferred into computer and processed using BioNumerics[®] software (Applied Maths BVBA, Kortrijk, Belgium).

Data analysis

Results of isolation and antimicrobial susceptibility testing were expressed as percentages. Isolates were compared using genomic cluster analysis method in BioNumerics[®]. The banding patterns were analyzed by Bionumerics software to determine clonal diversity and relation between the isolates. The cluster analysis was based on a variety of algorithms that have the common feature of hierarchical relatedness between and among the isolates using dendrogram or tree. During analysis, an optimization of 1.0 with three different band position tolerances of 1.5%, 1% and 0.8% by the unweighted-pair group method (UPGM) were used in BioNumerics[®] software program to assess genomic properties of these isolates.

Results

Distribution of isolates in samples

Out of 237 samples tested, 3 (1.27%) *S. Kastrup* and 11 (4.64%) *S. Larochelle* isolates were isolated (Table 1). *S. Kastrup* was isolated from Ethiopia for the first time. The three *S. Kastrup* isolates were isolated one each from personnel hand swab, animal feces and mesenteric lymph nodes. *S. Larochelle* was isolated from swab samples taken from aprons (1), knife (1), hook (1), refrigerator (1), room floor (2); raw beef (3) and cattle feces (2).

Table 1: Distribution of *S. Kastrup* and *S. Larochelle* isolates from various samples obtained from cattle slaughter plant and retail shops

Sample category	Sample source	Sample type	No. samples tested	No. Isolates	Serotypes detected (no.)
Abattoir environment	Personnel's hands	Swab	13	1	<i>S. Kastrup</i> (1)
	Aprons	Swab	14	1	<i>S. Larochelle</i> (1)
	Knives	Swab	13	1	<i>S. Larochelle</i> (1)
	Water	Water sample	12	0	-
	Hooks	Swab	11	1	<i>S. Larochelle</i> (1)
	Rooms	Surface swab	17	2	<i>S. Larochelle</i> (2)
	Refrigerators	Swab	10	1	<i>S. Larochelle</i> (1)
	Trucks	Swab	11	0	-
	Sub total			101	7
Cattle at abattoir	Feces	Fecal samples	34	3	<i>S. Kastrup</i> (1); <i>S. Larochelle</i> (2)
	Mesenteric lymph nodes	Lymph nodes	34	1	<i>S. Kastrup</i> (1)
	Beef	Carcass samples	34	1	<i>S. Larochelle</i> (1)
	Sub total			102	5
Retail shop	Beef at butcheries	Beef samples	34	2	<i>S. Larochelle</i> (2)
	Grand total (no; %)		237	14	<i>S. Kastrup</i> (3; 1.27); <i>S. Larochelle</i> (11; 4.64)

Antimicrobial resistance

Of the 14 isolates, 7.1%, 35.7% and 21.4% of them were resistant to gentamicin, neomycin and oxytetracycline respectively. As shown in Table 2, only two of the three (66.7%) *S. Kastrup* isolates were resistant to neomycin alone and one (9.1%) of *S. Larochelle* isolates was resistant to gentamycin. Three (27.3%) of *S. Larochelle* isolates each were resistant to neomycin and oxytetracycline. Of these three, one isolate was resistant to both drugs. No multiple drug resistance was observed.

Table 2: Antimicrobial susceptibilities of *S. Kastrup* and *S. Larochelle* isolates obtained from various samples at beef processing plant and retail shops

Serotypes	a Resistance category	b Antimicrobial resistance profile, n (%)						
		POL-B	GEN	CHL	TRM	SXT	NEO	OxyTET
<i>S. Kastrup</i> (n = 3)	S	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	1 (33.3)	3 (100)
	I	0	0	0	0	0	0	0
	R	0	0	0	0	0	2 (66.7)	0
<i>S. Larochelle</i> (n = 11) ^c	S	11 (100)	10 (90.9)	11 (100)	11 (100)	11 (100)	3 (27.3)	8 (72.7)
	I	0	0	0	0	0	5 (45.4)	0
	R	0	1 (9.1)	0	0	0	3 (27.3)	3 (27.3)
Total (n = 14)	S	14 (100)	13 (92.9)	14 (100)	14 (100)	14 (100)	4 (28.6)	11 (78.6)
	I	0	0	0	0	0	5 (35.7)	0
	R	0	1 (7.1)	0	0	0	5 (35.7)	3 (21.4)

^a S = susceptible; I = intermediate; R = resistant

^b POL-B = polymyxin-B; GEN = gentamicin; CHL= chloramphenicol; TRM =trimethoprim; SXT = trimethoprim-sulfamethoxazole; NEO= neomycin; OxyTET = oxytetracycline

^c one isolate was resistant to neomycin and oxytetracycline

Pulsed field gel electrophoresis finding

Two pulsotypes with 96.8% similarities at 1.5% band position tolerance (Fig. 1) and with 91.7% similarity at 1% band position tolerance (Fig. 2), both consisting of the same thirteen isolates (three *S. Kastrup* and 10 *S. Larochelle*) of 100% similarity and one *S. Larochelle* were obtained. Using 0.8% band posi-

tion tolerance, four pulsotypes consisting 2, 1 and 1 isolates of *S. Larochelle*, and 100% similar 10 isolates (3 *S. Kastrup* and 7 *S. Larochelle*) were observed (Fig. 3).

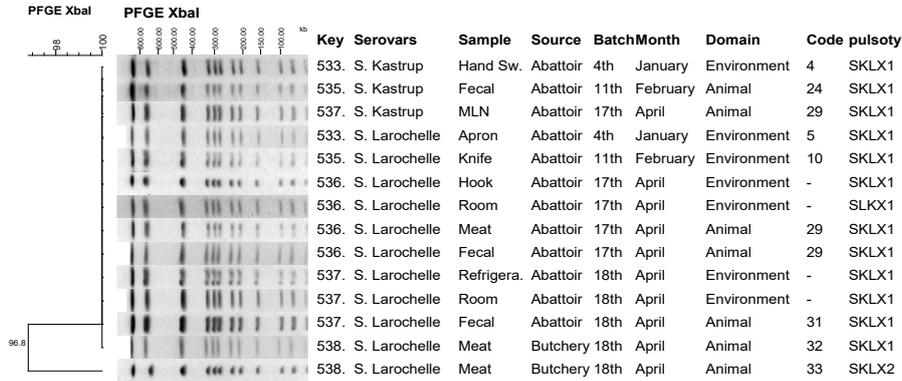


Fig. 1: *Salmonella* Kastrup and *Salmonella* Larochelle using PFGE *XbaI*[®] endonuclease at 1.5% band position tolerance by the UPGM. Hand Sw = Hand swab; Refrigerera = Refrigerator swabs; MLN = mesenteric lymph node; SKLX = *Salmonella*-Kastrup-Larochelle-*XbaI*[®] pulsotype; numbers following SKLX are grouping of pulsotype(s).

Discussion

Except for water and beef transporting truck swab samples, all sample sources were positive for one or more *Salmonella* Kastrup and *S. Larochelle* isolates. In current study, *S. Kastrup* was detected from 1.27% of samples collected from cattle feces, swabs from personnel’s hands and mesenteric lymph nodes. This is the first report of *S. Kastrup* from Ethiopia. Menghistu et al. (2011) reported overall prevalence of 2.7% *Salmonella* including *S. Kastrup* from poultry in India, showing epidemiological diversity of this serotype in food animals. The predominant occurrences of *S. Larochelle* serotype (4.64%) in the food chain including in the production environment, cattle feces and raw beef at different stages of the abattoir line poses risk to the public. Beyene (2008) reported one isolate of *S. Larochelle* from hospital cases in Ethiopia.

The 7.1% gentamicin resistance observed in the current study is similar to 3.6% reported by Reda et al. (2011), but lower than the 74.3% reported by Beyen et al. (2011) in *Salmonella* isolated from human cases in Ethiopia. The low

gentamicin resistance observed here could be due to infrequent use of this drug in food animal in Ethiopia. The 35.7% neomycin resistance we observed was higher than the 6.3% report of Oliveira et al. (2006) in *S. Enteritidis* isolated from foods involved in foodborne outbreaks in Brazil. The 21.4% oxytetracycline resistance observed in the present study was lower than 41.2% (Molla et al., 2003), 94.5% (Asrat, 2008), 71.4% (Reda et al., 2011), 39.8% (Beyene et al., 2011), 31.8% (Aragaw et al., 2007) reported from *Salmonella* isolates from humans and animals in Ethiopia. These studies show the widespread occurrence of tetracycline resistance in *Salmonella* isolates from Ethiopia. The Drug Administration and Control Authority of Ethiopia report (DACA, 2009) shows that tetracycline is a commonly used antibiotic in Ethiopia. *S. Kastrup* isolates showed resistance only to neomycin as opposed to *S. Larochelle* isolates which showed resistance to gentamicin and oxytetracycline in addition to neomycin indicating some degree of phenotypic difference.

Although *S. Kastrup* (6,7:e,n,z₁₅:1,6) and *S. Larochelle* (6,7:e, h:1,2) (Grimont and Weill, 2007) are antigenically differentiated by serotyping, our results indicate that the two serotypes demonstrate genetically indistinguishable PFGE *XbaI*[®] profiles. Using PFGE *XbaI*[®] as epidemiological tool for tracing possible sources of food borne infections, our observation of 100% genetic similarity among *S. Kastrup* isolates from personnel's hands, mesenteric lymph nodes and cattle feces shows their possible sources for carcass contamination at the studied abattoir. Similarly, the 100% similarity of *S. Larochelle* isolated at abattoir line shows the possible cross-contamination and cross-transmission to the level of meat supply (butchers'). On the other hand, the difference of pulsotype 2 from others and observing from the butchers' (meat supply station) might indicate contamination of carcass during handling and supply.

In conclusion, the finding of indistinguishable PFGE *XbaI*[®] patterns of *S. Kastrup* and *S. Larochelle* from various samples collected along beef production, processing and retail shops indicates the possibility of transfer of these serotypes along the processing and handling. The prevalence of antimicrobial resistance of these *Salmonella* serotypes was low with similar susceptibility patterns to the drugs tested. *Salmonella* *Kastrup* was isolated from Ethiopia for the first time with indistinguishable PFGE *XbaI*[®] profiles from *S. Larochelle*.

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