Original Article

Characterisation of novel strains of multiply antibiotic-resistant *Salmonella* recovered from poultry in Southern Senegal

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Abstract

Introduction: Non-typhoidal *Salmonella* (NTS) contamination in poultry and poultry products is a major cause of food-borne disease in humans. This study presents the molecular epidemiology of NTS isolated from poultry in Senegal.

Methodology: A total of 261 NTS recovered from broiler farms, chicken carcasses and street vendors were characterized using random amplification of polymorphic DNA (RAPD) and multilocus sequence typing (MLST) techniques.

Results: We observed 20 distinct RAPD profiles corresponding to 18 different serotypes. Strains from each of these 20 groups were further analysed using MLST. Consequently, 12 new MLST alleles and 17 new sequence types were discovered. Three sequence types (*S.* Kentucky ST198, *S.* Agona ST13 and *S.* Istanbul ST33) have previously been described in Senegal and other countries, suggesting that these clones are geographically widely distributed and are circulating in a wide range of hosts. Nine clones showed multi-resistance to the most commonly used antibiotics in both humans and animals. However, a novel multi-resistant clone of *S.* Kentucky ST832 was found.

Conclusion: This study gives new insights into the genetic diversity of NTS in Senegal. Molecular tools remain essential to improve our understanding of the epidemiology of NTS by tracking the sources of infection and/or contamination.

Key words: *Salmonella;* clones; antibiotic-resistant; Senegal

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Introduction

Enteric diseases caused by *Salmonella* in chickens are of great concern because they are an important cause of mortality and morbidity [1]. Poultry and its products, in particular chicken meat and eggs, are a major source of *Salmonella* infection [2]. In Senegal, several studies have reported the presence of *Salmonella* along the poultry production supply chain and its public health impact [3-7], withserotyping being used to characterise the *Salmonella* [8]. Due to the ubiquitous nature of *Salmonella*, a typing scheme capable of a more detailed strain identification is essential for epidemiological studies [9], because the ability to distinguish isolates of *Salmonella* is very important to trace the source of infection and outbreaks. Several methods have been used for deciphering the relatedness among isolates but some have low discriminating power, whereas others demand a considerable amount of expertise, time and equipment [10]**.** Multilocus sequence typing (MLST) was found to have a low discriminative power, but is easier to interpret and to compare between laboratories and provides the best phylogenetic-relationship inferences [11]. Our study reports for the first time in Senegal the characterization of the most prevalent NTS strains isolated from poultry and poultry products by using the random amplification of polymorphic DNA (RAPD) and MLST techniques.

Methodology

Bacterial isolates

From October 2007 to July 2008, a total of 261 strains of *Salmonella* were collected during a cross-sectional study in 57 broiler farms, 285 chicken carcasses from farms/sale points, and 42 dishes of chicken meat from street restaurants in southern Senegal [7]. *Salmonella* was cultured according to the standard technique ISO 6579 (1998, International Organization for Standardization, Geneva, Switzerland). Serotyping was done by slide agglutination using *Salmonella* polyvalent and monovalent O and H antisera (Diagnostic Pasteur, Paris, France) according to the Kauffmann-White classification scheme [8]. The data of the

antimicrobial resistance tests were extracted from a previous study [7].

RAPD-PCR reaction conditions

The RAPD reaction was performed on all 261 isolates using P1254 primer as previously described [12]. The reaction was as follows: 3 µl of primer (20 mM) was added to 10 µl of DNA free water (Invitrogen, Paisley, UK), 5 µl of 5x Buffer (Qiagen, Crawley, UK), 2 µl of dNTPS (2 mM), and 3.5 µl of MgCl2 (25 mM), 0.5 GoTaq polymerase (Promega, Madisson, UK) and 1 µl of template (cells) to get the final volume of 25 µl. Amplification was performed in a PCR machine (Techne, Hatboro, USA) as follows: one cycle at 94ºC (2 minutes); a series of 3 cycles at 94ºC (2 minutes), 35ºC (1 minute) and 72ºC (2 minutes); a second series of 34 cycles at 94ºC (10 seconds), 40ºC (20 seconds) and 72ºC (2 minutes); with final extension at 72ºC for 5 minutes. The amplified product was stored at 4ºC until required. The PCR product (10 µl each) was loaded on a 1.5% agarose gel containing 0.5ul/ml ethidium bromide at 25 V overnight in 1X (SIGMA, Steinheim, Germany). Two lanes of 50 bp and 100 bp of ladder were included in each gel for reference. The RAPD electrophoresis bands were photographed using UV illumination with a gel documentation system (gel Doc 2000, Bio-Rad, Hertfordshire, UK). Gel pictures were analysed with Bionumerics software (version 4.0; Applied Maths, Saint-Martens-Latem, Belgium) and finally similar profiles resolving into similar clones were selected.

Multi locus sequence typing

MLST was performed on 20 randomly selected *Salmonella* serotypes with unique RAPD profiles. The protocol was followed as previously described [13] . Briefly, the seven genes were targeted were *aroC*, *dnaN*, *hemD, hisD*, *purE*, *sucE and thrA*. Amplification of all genes was performed with a 25 µl reaction mixture of the following: 10x Buffer (Applied Biosystems, Foster City, USA) with 1.5mM $MgCl_2$ (2.5 µl); 2 mM dNTP (0.5 µl); 12.5 mM forward primer (1 µl); 12.5 mM reverse primer (1 µl); 5U/µl Qiagen Hotstart *Taq* Polymerase (Qiagen, Crawley, UK) (0.25µl); template (cell lysate) (2 µl) and 17.75 µl sterile DNA free water. PCR cycling conditions were 10 minutes at 94°C, followed by 32 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. From each PCR, 2 µl aliquots were separated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining and UV illumination, and using a gel documentation system (gel Doc 2000, Bio-Rad, Hertfordshire, UK). PCR products were purified using Qiagen kit (Qiagen, Crawley, UK). Sequencing was done on both strands with BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Warrington, UK). The labelled fragments were separated by size using a 3130xl Genetic Analyser (Applied Biosystems, UK). Sequences were edited and complementary sense antisense fragments were aligned using the Laser Gene DNA star 7.1 software. Finally, the sequences were submitted to the MLST database website [14] and assigned to existing or novel allele type numbers defined by the database. New sequence types were arbitrarily assigned a number for the purpose of the analysis.

Cluster analysis and mapping

To perform the cluster analysis of the serotypes, MLST data were analysed with Bionumerics software (version 6.5; Applied Maths, Sint-Martens-Latem, Belgium). Analysis using a hierarchic unweighted pair group method (UPGMA) with averaging was used to generate a dendrogram describing the relationship among *Salmonella* serotypes. A minimum spanning tree was used to compare Senegalese clones to Gambian and other African clones. The mapping of the locations of the cases was performed using Arc Gis 9.3 software.

Results

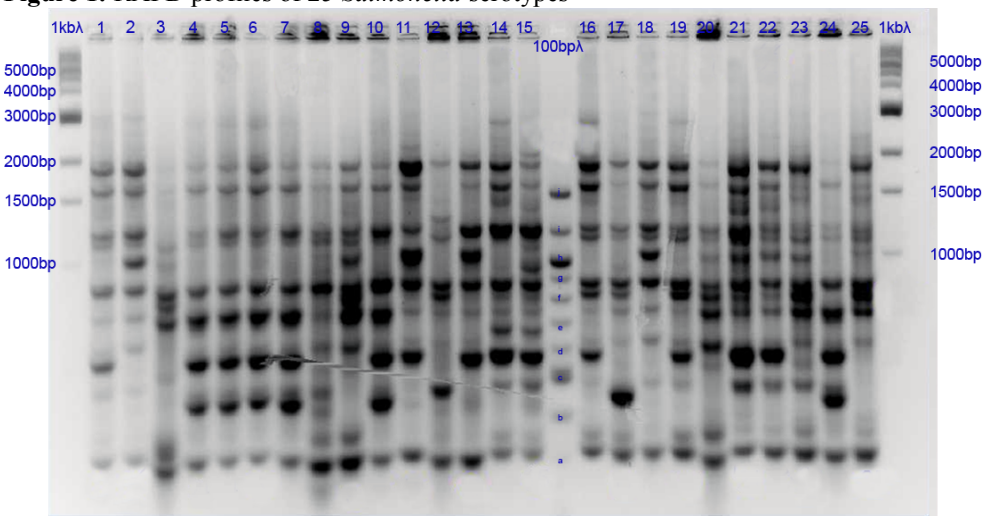
In this study, we analyzed the genomic relatedness between 261 NTS belonging to 18 different serovars, initially using RAPD profiling. All RAPD fragments amplified were between 200 bp and 3000 bp. Twenty different RAPD fingerprints were observed among the 261 NTS (Figure 1). Each serovar generated a unique RAPD fingerprints, with the exception of two serovars, *S*. Javiana (Figure 1, lanes 14 and line 15) and *S*. Kentucky (Figure 1, lanes 16 and line 17) which each showed two different fingerprints. To further characterise these NTS, MLST was performed on one strain from each unique RAPD type. Twelve new alleles were discovered [*aroC* (203), *aroC* (204), *aroC* (205), *dnaN* (195), *dnaN* (196), *hisD* (238), *hisD* (239), *hisD* (241), *purE* (199), *sucA* (195), *thrA* (185), and *thrA* (186)] as well as sixteen new sequence types (ST923, ST824, ST825, ST826, ST827, ST828, ST829, ST830, ST831, ST832, ST833, ST834,

Table 1. Multilocus Sequence Typing of *Salmonella* serotypes and antibiotic resistance patterns

		Allele number									
Strain ID	**Serotype**	***aroC***	***dnaN***	***hemD***	***hisD***	***purE***	***sucA***	***thrA***	**ST**	**Source (chicken)**	**Antibiotic resistance**
A1001	Agona	3	3	7	4	3	3	7	13	skin	SPT
A1002	Bandia	84	65	8	241	64	9	168	823	stool	STX, TMP, CIP, SSS
A1003	Bessi	204	11	8	241	161	195	186	824	skin	None
A1004	Brancaster	205	81	10	36	88	108	36	825	skin	STX, TE, TMP, S, SSS
A1005	Brunei	19	81	8	20	5	9	185	826	stool	AMX, TIC, STX, TE, TMP, SSS
A1006	Sp.	203	195	17	164	12	35	4	827	skin	SPT
A1007	Hadar	17	5	78	7	5	7	12	828	muscle	STX, TE, TMP, S, SSS
A1008	Hull	114	5	3	238	199	10	12	829	skin	None
A1009	Istanbul	2	5	6	7	5	7	12	33	restaurant	CF, STX, TE, GM, TMP, SPT, S, SSS
A1010	Javiana	11	196	78	74	40	13	4	830	skin	None
A232	Javiana	11	196	78	74	40	13	4	830	muscle	None
A1011	Kentucky	76	14	3	77	64	64	67	198	skin	None
A112	Kentucky	115	65	8	115	2	116	110	832	stool	STX, TMP, SPT, S, SSS
A1012	Magherafelt	84	65	3	241	64	9	110	833	skin	None
A1013	Sp.	76	14	3	77	64	132	67	835	skin	STX, TE, TMP, SPT, S, SSS
A1014	Sp.	203	14	17	164	12	35	4	836	skin	None
A1015	Poona	13	127	92	157	40	35	137	838	skin	AMX, TIC, STX, TE, TMP, SPT, S, SSS
A1016	Rubislaw	42	46	48	239	12	35	4	837	stool	None
A1017	Tamale	205	46	8	42	88	108	36	834	stool	STX, TE, TMP, SPT, S, SSS
A1018	Zanzibar	84	11	8	42	40	71	4	831	muscle	SPT, S

ST: sequence type, The underlined numbers are the new alleles and STs discovered in this study.
AMX, amoxicillin; TIC, ticarcillin; CF, ciprofloxacin cephalotin; STX, trimethoprim plus sulfamethoxazole; TE, tetracycline; C, chloramphenicol; GM, gentamicin; TMP, trimethoprim; CIP, ciprofloxacin; SPT, spectinomycin; S, streptomycin; SSS, sulfonamides

Figure 1. RAPD profiles of 25 *Salmonella* serotypes



Lanes: *S.* Agona (1), *S.* Bandia (2), *S.* Bessi(3), *S.* Brancaster (4, 5, 6, 7), *S.* Brunei (8), Sp (9, 10), *S.* Hadar (11), *S.* Hull (12), *S.* Istanbul (13), *S.* Javiana (14, 15), *S.* Kentucky (16, 17), *S.* Magherafelt (18), *S.* Molade (19), *S.* Oxford (20),) *S.* Poona (21, 22), *S.* Rubislaw (23), *S.* Tamale (24), *S.* Zanzibar (25)
100 bp ladder: a (300 bp), b (400 bp), c (500 bp), d (600 bp), e (700 bp), f (800 bp), g (900 bp), h (1000 bp), i (1200 bp), j (1517 bp)

ST835, ST836, ST837, ST838), giving a total of 19 different STs in the 20 strains (Table 1). The strains characterised belong to three clusters showing a DNA identity of 98.8 to 100% at MLST loci. Most of the antibiotic sensitive strains belonged to a different cluster from that containing predominantly resistant strains (Figure 2). The Senegalese and Gambian serovars cluster together and these are derived from the ST19, ST626 and ST937 clonal complexes (Figure 3).

Interestingly, both *S.* Javiana RAPD types belonged to the same novel sequence type (ST830) while both *S.* Kentucky RAPD types had different ST (ST198 and ST832). Ten of the strains tested were resistant to multiple antibiotics, with two resistant just to spectinomycin and eight strains were fully susceptible to all tested antibiotics (Table 1).

Discussion

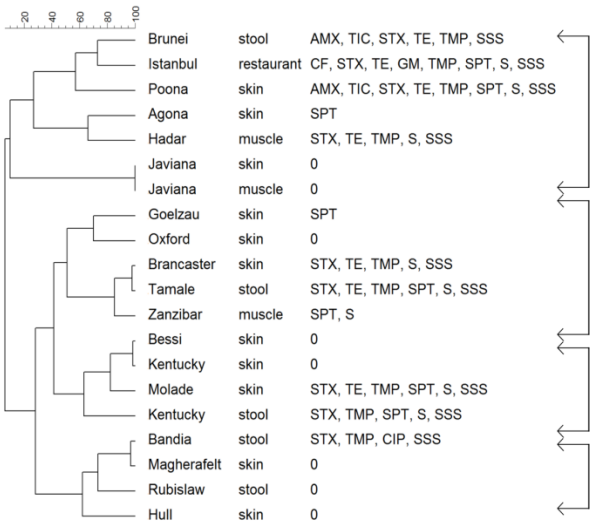
Salmonella is frequently isolated from poultry products in Senegal as shown by several studies [3-4,6] and confirmed in our previous study [7], with serotypes *S.* Brancaster, *S.* Hadar, *S.* Kentucky, and *S.* Agona found most commonly in poultry and poultry products.

RAPD profiling has been widely used to assess the genetic relatedness between *Salmonella* isolates indicating the reliable discriminatory power of this technique in subdividing strains within serotypes [1,10,12,15-16]. MLST has also been considered a method of choice for detailed characterization of a number of pathogens [14,17-18]. Although some authors have reported that MLST is not suitable to distinguish between closely related strains within the same serotype [19-20],in this study we showed that MLST is able to distinguish strains belonging to the same serotype, as in the case with the two *S.* Kentucky serotypes. Furthermore, both MLST and RAPD provided subtypes for *S.* Kentucky, meaning that MLST and RAPD techniques are more distinctive than serotyping for *S.* Kentucky; this result concurs with those of other studies that showed that MLST was more sensitive than serotyping [21-22]. The *S.* Kentucky ST198 and *S.* Kentucky ST832 did not share any alleles and while the former was fully susceptible to all antibiotics, the latter was multi-resistant to four antibiotics.

The high genetic similarity among serovars is not surprising as the clones were sampled from poultry farms in the same geographic urban area in Southern Senegal. The antibiotic sensitive strains cluster together and have been shown to belong to a new set of strains which is not frequently isolated in poultry in Senegal [7], Their high rate of sensitivity could be explained by the fact that they have not yet been under stress (antibiotic treatment) and thus have not adapted themselves to the environment by acquiring resistance determinants [24].

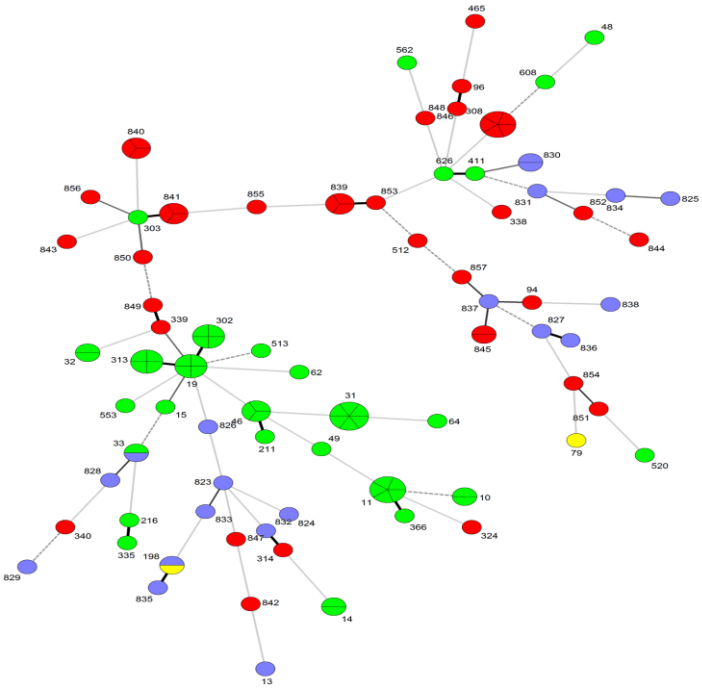
The currently accepted "gold standard" for the characterization of NTS is PFGE, and this, along with other methods such as plasmid profiling, could also be used to determine the diversity of isolates within this study. *S.* Kentucky ST198 has been

Figure 2. UPGMA dendrogram based on the similarity matrix showing the relationship between the concatenated seven housekeeping gene fragments from 20 *Salmonella*



Pairwise (OG: 100%,UG:0%) (Fast: 2.10) Gapcost: 0%. The scale bar represents percentage (%) of nucleotide identity.

Figure 3: Clustering of STs using minimum spanning tree



Each circle represents an ST. The area of each circle corresponds to the number of isolates. The length of the lines represents the number of locus variants. Thick, short, solid lines connect single locus variants; thick longer solid lines connect double-locus variants; thin long solid connect to 3 locus variants; dashed connect to 4 locus variants and dotted connect to 5 locus variants. Violet portions represent the Senegalese STs described in this study; Yellow portions represent Senegalese STs described earlier to this study; red portions represent Gambian STs and green portions represent STs found in the rest of the world.

previously isolated from humans in Senegal and from food and cattle in the United States (USA). Our study revealed its presence in chicken skin in southern Senegal. *S.* Agona ST13 found in chicken skin in our study was also previously described in Denmark from both human and veterinary sources, from milk in Ireland, from humans in Scotland, and from undetermined hosts in Australia, Germany, the USA, Canada and Spain [23], while *S.* Hadar ST33 was isolated from various sources in several European and American countries and South Africa [23]. However, the ST33 found in southern Senegal in our study belonged to serovar *S.* Istanbul. Collectively, ST13, ST33 and ST198 clones are widely distributed throughout the world and all three clones have been simultaneously identified in both human and animal sources, suggesting that the same clones are circulating in both hosts. *S.* Istanbul ST33 was multi-resistant to most commonly used antibiotics in humans and animals in southern Senegal, contrary to *S.* Kentucky ST198 and *S.* Agona ST13, which were fully sensitive to all antibiotics tested. However, a novel multi-resistant clone of *S.* Kentucky ST832 was found in poultry.

Conclusion

Our study showed that a variety of serovars and clones is circulating in southern Senegal; furthermore, 17 out of the 20 clones characterised in this study have never been reported elsewhere in the world.

This is the first report describing the use of MLST to characterise the most common NTS in poultry and poultry products in Senegal. The discovery of new alleles and sequence types is very useful for a better understanding of the epidemiology of NTS in Senegal.

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