CHARACTERIZATION OF *SALMONELLA* ISOLATES OBTAINED FROM PIGS SLAUGHTERED AT WAMBIZZI ABATTOIR IN KAMPALA, UGANDA

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Abstract

Globally, non-typhoidal salmonellosis accounts for approximately 80.3 million cases of human infections annually. Estimates of salmonellosis due to consumption of pork or pork products is difficult to determine but, it ranges from < 1% to 25%. These invasive pathogens colonize intestinal mucosal surface but, they are self-limiting in health individuals due to a noble immunity. Utilization of antimicrobial agents in pig farming has been associated with the spread of resistant *Salmonella* species to man and the carrier status presents a major hazard to human health. This study examined 54 isolates for antimicrobial resistance, sequenced seven housekeeping genes, and performed Multi-Locus Sequence Type (MLST) analysis. We detected *β-lactamase* and *tetA(B)* genes in 100% and 80% of the isolates respectively. Data analysis using Codon-based Test of Neutrality analysis between sequences revealed *P*-value less than 0.05, an indication of strong forces of natural selection pressure acting at the sequence type level. Further data analysis using the Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution discovered frequencies of 0.177 (A), 0.244 (T/U), 0.263 (C), and 0.317 (G). The transition/transversion rate ratios were found to be k_1 = 2.698 (purines) and k_2 = 20.089 (pyrimidines) with an overall transition/transversion bias of R = 6.565, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$ further confirming that indeed the *Salmonella* isolates studied here were divergent. These results suggest that, *Salmonella* isolates of sequence types (STs) coexist in the intestine thereby providing for an efficient intestinal colonization and multiple adaptations. Our results offer general and rapid approaches for identifying genetic diversity of *Salmonella* serotypes in individual pig carcasses which can be adopted for molecular epidemiological surveys of important food contaminating bacterial pathogens.

Key words: Salmonellosis, antimicrobial resistance, isolates

1.0 Introduction

Salmonellosis is an infectious disease of humans and animals which is caused by two species of *Salmonella,*
namely *S. enteric* and *S. bongori* (Grimont and Weill, 2007). *Salmonella* organisms are etiological agents that cause diarrheal and systemic infections in humans, arising mostly from consumption of contaminated food (Yang *et al*., 2010). Animal source food, fruits and vegetables have been implicated to be the main sources of salmonellosis infections in man (Guentzel *et al*., 2008). Many animals, especially pigs and poultry, are colonized showing no clinical illness hence maintaining carrier status. In this condition, carrier animals continue shedding *Salmonella* and thereby acting as sources of human contamination and infections (Molla *et al*., 2003; Kalule *et al*., 2012).

The two serotypes of *S. enterica* species which are primarily responsible for human and non-human gastroenteritis are *S*. *Typhimurium and S. Enteritidis*. Similarly, *S. Typhi* and *S. Paratyphi* serotypes are associated with human enteric fever, while *S.* Choleraesuis is associated with bacteremia in pigs (Bywater *et al*., 2004). *Salmonella* Typhi is the etiological agent of typhoid fever, a serious invasive bacterial disease of humans with an annual global burden of approximately 16 million cases, leading to 600,000 fatalities Ivanhoff (1995). It is estimated that 80.3 million cases of foodborne (Non-typhoidal) salmonellosis occur annually in the world (Majowicz *et al*., 2010).

In the U.S, *Salmonella* is the second leading cause of foodborne illness, and the leading cause of hospitalization and death (Scallan *et al*., 2011). Although the precise number of human salmonellosis cases directly attributable to pork or pork products is difficult to determine, reported estimates range from < 1% to 25% (Hald *et al*., 2004). Salmonellosis is more prevalent where livestock are intensively confined (Pang *et al*., 1995). CDC (2005), reported an annual incidence of salmonellosis of 100 to 1000 cases per 100 000 population in which the estimated typhoid cases were 21.6 million in the United States of America alone. (Crump *et al*., 2003), noted a decline in the global mortality due to typhoid fever, which dropped from 600000 to 200000 per year however; this was based on extrapolated data which may be a misrepresentation of facts. The annual, cost of salmonellosis in the USA is estimated at $2.4billion, arising mainly from medical cost, loss of productivity, and premature death USDA-ERS (2005).

Published reports from Southern Asia on population-based studies, indicates that the incidence of salmonellosis was highest in children aged less than 5 years Bhutta (2006). The high rates of complications and hospitalization is attributed to early exposure due to relatively large infecting doses of the organisms in the study population Bhutta (2006). In industrialized countries, *Salmonella* surveillance and monitoring systems are implemented to identify specific serotypes involved and to

facilitate better management of disease outbreaks (Olsen *et al*., 2001). These data are limited in African developing countries largely due to inadequate funding for research, lack of proper testing laboratories, and absence of qualified personnel to isolate and characterize *Salmonella* (Scallan *et al*., 2011). Although developed countries of USA and Europe have seen tremendous gains in the management and control of salmonellosis, it still remains a major problem in developing countries in Africa, Asia and South America, and acts as a source of infection to foreigners returning from such countries (Santos *et al*., 2001).

Multidrug resistant salmonellosis is an increasing problem (Leekitcharoenphon *et al*., 2013) and is generally attributed to the unsupervised usage of antimicrobial agents for growth promotion and nutritional enhancement in livestock animals such as pigs (Rayamajhi *et al*., 2008). Drug resistance is a serious public health concern in human and veterinary medicine (Su *et al*., 2004). Antimicrobials such as sulphonamides, trimethoprim, fluoroquinolones, aminoglycosides, chloramphenicol and tetracycline are broadly used for both livestock and human treatment (Su *et al*., 2004).

However, the diversity, occurrence and spread of antimicrobial resistance and STs of non-typhoidal *Salmonella* in pigs slaughtered at the Wambizzi abattoir, the only farmers owned pig abattoir and which handles pigs from different parts of Uganda, has never been reported. Like in other pathogens, the diversity of within *Salmonella* isolates has major implications for treatment and resistance (Guo *et al*., 2011; Thong and Modarressi, 2011) for inferring transmission networks Lieberman (2011) and for understanding evolutionary processes Chung (2012). It was therefore important to undertake research to characterize existing ST serotypes ST lineages and explore opportunities to combat the spread of non-typhoidal *Salmonellosis* through the food chain to humans. Conventional culture and biochemical tests were used for preliminary identification of *Salmonella*. Similarly, antimicrobial disc diffusion tests and genetic analysis were performed to determine susceptibilities and identify the genes coding for resistance towards tetracycline, beta-lactam and sulfamethoxazole drugs. Multi-Locus Sequence Type (MLST) analysis (Achtman *et al*., 2012) was performed to determine the evolutionary relationships and clustering of the STs taxa. Codon-based Test of Neutrality analysis between sequences and Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution were performed to determine if the isolates were genetically divergent. Finally a generalized taxonomic tree of the isolates was constructed based on the seven housekeeping gene sequences.

2.0 Materials and Methods

2.1 Study Site and Sample Collection

A total of 100 gluteal muscle samples were taken using sterile knives and transferred into sterile plastic caps containing Stuart transportation medium which

was used to preserve the samples and maintain viability of bacteria. Similarly, 100 fecal samples were taken from the carcasses after opening the ileocecal junction of the intestines and drained into sterile plastic caps containing Stuart transport medium. The samples were transported to Makerere University, Department of Biosecurity, Ecosystems and Veterinary Public Health, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), where they were kept at -80^{0}C until further analysis.

2.2 Isolation of *salmonella* From Pork and Fecal Samples and Identification Using Biochemical Tests

Isolation of *Salmonella* was carried out according to the standard protocol described by the International Organization for Standardization 1993 -ISO 6579 and the White-Kauffmann-Le Minor scheme (ISO, 1993; Kang and Fung, 2000; Guibourdenche *et al*., 2010), with minor modifications. Twenty five grams of pork muscle and 25g pig fecal samples were weighed and transferred to sterile Stomacher bags (Seward, UK), containing 225ml of cold sterilized peptone water (Oxoid, UK). This was followed by homogenization using a pulsefying machine (Thermo Fisher scientific, UK). The homogenates were incubated at 37^{0}C for 24 hours for pre-enrichment. Broth was added to the 1 ml bug to make a 5 ml final volume in 5 ml of tetrathionate broth (Oxoid, UK) and incubated at 37°C for 24 hours. Xylose Lysine Desoxycholate (XLD) agar (Oxoid, UK), was used as selective medium to isolate *Salmonella* from the enriched samples. Plates were incubated at 37^{0}C overnight, observed for *Salmonella* colony forming units (CFU) and detected according to the protocol by Dixit and Shanker, (2009). Presumed *Salmonella* colonies were identified by using biochemical tests including indole test, triple sugar iron test, Simon citrate test and urease test.

2.3 Susceptibility Testing by Disc Diffusion

Susceptibility tests were performed according to CLSI guidelines (CLSI, 2010). The isolates were examined against tetracycline, ampicillin, penicillin and sulfamethoxazole. The disc concentrations in micrograms (µg) were 10, 10, 10, and 25 respectively (Oxoid, UK). The diffusion radii in millimeters (mm) were measured, recorded and compared with standard measurements in mm (CLSI, 2010) in order to identify resistant *Salmonella* isolates.

2.4 Data Management and Analysis

Salmonella isolates were examined and confirmed by biochemical tests including Indole, Tryple Sugar iron, Simon Citrate and Urease tests. Confirmed *Salmonella* isolates were subcultured in XLD and typical round colonies with black centers were found. Susceptibility examination revealed different minimum inhibition diameters in millimeters (mm). Both resistance and housekeeping gene analysis was performed by PCR and quality checked by gel electrophoresis. Sequence data were unzipped using CLC workbench, edited, gaps removed, and conflicts resolved

and assembled into contigs using predetermined statistical algorithms in the software (O'Neil *et al*., 2014; Perceptual Edge-Jonathan Koomey, 2006). The contigs assembled were converted into FASTA format and aligned at publicly accessible *www.ncbi.nlm.nih.gov* website to identify *Salmonella*. Further, FASTA sequences were converted into MEGA sequence format and aligned using ClustalW. Build-in algorithms in MEGA were used for sequence type analysis of six taxa, identifying changes due to selection between isolate sequences, nucleotide sequence substitutions by transitions and transversions and overall phylogenetic analysis.

2.5 Codon-based Test of Neutrality for Analysis between Sequences

This test was used to estimate the probability of rejecting the null hypothesis that there are no differences between *Salmonella* isolates investigated in this study by considering strict-neutrality ($d_N = d_S$) between sequences. Values of *P* less than 0.05 were considered significant at the 5% level. The test statistic ($d_N - d_S$) evaluated the d_S and d_N which are the numbers of synonymous and nonsynonymous substitutions per site, respectively. The variance of the difference between the variables was computed using the bootstrap method replicates in which analyses are conducted using the Nei-Gojobori method in MEGA4 (Nei and Gojobori, 1986; Tamura, *et al*., 2007). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

2.6 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

Finally, the Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (Tamura *et al*., 2007) was performed. The test is based on the probability of substitution from one base (row) to another base (column) instantaneously as defined by MEGA software algorithms. Only entries within a row are compared. The test yields the rates of different transitional substitutions which are shown in bold and those of transversional substitutions normally shown in italics Table 6. All positions containing gaps and missing data were eliminated from the dataset (Complete-deletion option). There were a total of 80 positions in the final dataset. All calculations were conducted in MEGA4 (Tamura *et al*., 2007).

2.7 Molecular analysis DNA Extraction, Polymerase Chain Reaction (PCR) and Antimicrobial Resistance Genes Analysis

Briefly, DNA was extracted using an Invitrogen PureLink Genomic DNA extraction kit (*Invitrogen*, USA) according to the manufacturer's instructions but, with minor modifications. DNA was eluted in 50µl nuclease free water and stored at -20° C until use. To detect genes coding for resistance towards tetracycline, beta-lactam and sulfamethoxazole, genomic DNA was PCR-amplified using specific primers. Table 1a in the appendix shows the specific primers used to perform the analysis. Briefly, PCR was performed using the Bioneer PCR, PreMix Kit (Bioneer, South Korea) and according to the manufacturer's instructions so as to identify the following targeted resistance genes (*tetA (A), tetA (B), tetC (A), Sul1, Sul2, Sul3 and* $bla_{CMY-2)}$. The 20 µl reaction master mix containing 0.5 µl - forward primer at 10 µM, 0.5 µl – reverse primer (for each gene) at 10 µM, 1 µl DNA template at 20ng/µl, 18 µl ddH_2O nuclease free and a pellet consisting of Top DNA polymerase 1U, each dNTP (dATP, dCTP, dGTP and dTTP) 250 µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, $MgCl_2$ 1.5mM, stabilizers and tracking dye was used. The master mix was loaded into the PCR Thermocycler (Applied Biosystems) with the following conditions; an initial denaturation at 95℃ for 2 min followed by 35 cycles of 95℃, 30s; 55℃, 30s; 72℃, 30s, and a single final extension of 10 min at 72℃. The amplicons were resolved by electrophoresis on 1.8 % agarose gel stained with gel red (Biotium, Hayward, CA) and visualized by UV trans-illumination.

2.8 PCR Amplification and Sequencing of Housekeeping Genes

Polymerase Chain Reaction (PCR) was performed targeting the following seven housekeeping genes, *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III cosynthase), *hisD* (histidinol dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase) and *thrA* (aspartokinase+homoserine dehydrogenase) using Bioneer Premix Kit (Bioneer, South Korea). Table 2a in the appendix shows the primers used to perform the primary PCR. 20 µl reaction mixture consisting of 0.5 µl - forward primer at 10 µM, 0.5 µl – reverse primer at 10 µM, 1 µl DNA template

at 20ng/µl, 18 µl ddH_2O nuclease free and a pellet consisting of Top DNA polymerase 1U, each dNTP (dATP, dCTP, dGTP and dTTP) 250 µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, $MgCl_2$ 1.5mM, stabilizers and tracking dye was used. The PCR mixture was loaded into PCR Thermocycler (Applied Biosystems, UK) with the following conditions: an initial denaturation at 95℃ for 2 min followed by 35 cycles of 95℃, 30s; 55℃, 30s; 72℃, 30s, and a single final extension of 10 min at 72℃. Sequences was performed using primers in Table 3a in the appendix and sequences were assembled using CLC Genomics Workbench downloaded from (http://www.clcbio.com) and BLASTn performed according to the protocol by (Korf *et al*., 2003), at the publicly available website at www.ncbi.nlm.nih.gov in order to identify the isolates.

2.9 Sequence Type (ST) Analysis at *Salmonella* MLST Website

Multi-Locus Sequence typing (MLST) was performed according to the protocol described by (Martinez-Murcia *et al*., 2011) with slight modifications. The sequences were concatenated and aligned using ClustalW (Thompson *et al*., 1994) algorithm in MEGA4.0 (Tamura *et al*., 2007). The complete nucleotide sequences of the seven housekeeping gene loci in the following order (*aroC, dnaN, hemD, hisD, purE, sucA and thrA*), were used to query the MLST website. Interrogation of the *Salmonella* MLST website automatically assigned existing or new allele type (sequence type (ST)) numbers. Composite sequence types (STs) were assigned based on the predefined MLST algorithms (Aanensen and Spratt, 2005). A reference ST was obtained by downloading *Salmonella enterica subsp. enterica* serotype Typhimiurium, strain VNP20009, accession number CP007804.2 from the publicly accessible database at www.ncbi.nlm.nih.gov, and comparatively aligned with the composite ST sequences using ClusalW (Thompson *et al*., 1994) in MEGA4.0 (Tamura *et al*., 2007). A sequence types taxonomic tree was constructed in MEGA 4.0 (Tamura *et al* 2007) using the neighbor- joining method algorithm (Saitou and Nei, 1987; Tamura *et al*., 2007) (Figure 1).

2.10 Overall Taxonomy of the Isolates Based on the Seven Housekeeping Gene Sequences

The seven housekeeping gene sequences were aligned using ClusalW (Thompson *et al*., 1994) in MEGA 4.0 (Tamura *et al*., 2007) relative to their reference sequences downloaded from the MLST website. The analysis was performed with the Dice coefficient using the unweighted pair group method with arithmetic averages (UPGMA dendrogram). An overall taxonomic tree of the isolates was constructed (Figure 2).

3.0 Results

3.1 Isolation and Iidentification of *Salmonella* by Plate Culture

Out of 200 samples (100 fecal and 100 muscle samples) collected and cultured 54 samples (33 fecal and 21 muscle samples) were found to be contaminated with *Salmonella* (Table 1).

Table 1. Summary of results upon conventional plate culture and isolation of Salmonella

Type of Sample	Sample Size	No. Positive samples	% of positive
Fecal	100	33	16.5
Muscle	100	21	10.5
	200	54	27

Phenotypes of resistance against tetracycline, ampicillin, penicillin and sulfamethoxazole were detected (Table 2).

Table 2. Isolate susceptibility counts

Category	**Tetracycline**	**Ampicillin**	**Penicillin**	**Sulfamethoxazole**
Susceptible	22	6	30	44
Intermediate	11	11	5	10
Resistant	21	37	19	0

3.2 Molecular analysis of the confirmed *Salmonella* positive isolates

Isolates carrying $^{bla}_{CTX\text{-}M2}$ were correctly detected by PCR as being equally distributed among all the isolates. None of the sulfonamide resistance gene markers including *sul1, sul2* and *sul3* were detected in the isolates whereas, only *tetB(B) gene* was detected in 80% of the isolates analyzed for tetracycline resistance *tet* genes. All the seven housekeeping genes; *aroC, dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA* evaluated using specific primers (Table 3 in the appendix) were correctly amplified and sequenced. Sequence identity analysis using BLASTn revealed an E-value of 0.0 and a percentage identity value of between 98%-99% with a query cover of 95%. Accession numbers of *Salmonella* serotypes with which the isolates studied here were found to be identical include, *S*. Paratyphi, *S*. Abony, *S*. Newport, *S*. Bovismorbificans, *S*. Bareilly, *S*. Thompson, *S*. Dublin and *S.* Typhimurium (Table 3).

Table 3. BLASTn results

Report	Description	Max score	Total score	Query cover%	Evalue	Ident%	Accession
1	*S*. Paratyphi	1077	1077	95	0.0	99	CP000886.1
2	*S*. Abony	1077	1077	95	0.0	99	CP007534.1
3	*S*. Newport	1072	1072	95	0.0	99	CP007216.1
4	*S*. Bovismorbificans	1072	1029	95	0.0	99	HF969015.2
5	*S*. Bareilly	1072	1072	95	0.0	99	CP006053.1
6	*S*. Thompson	1066	1066	95	0.0	99	CP006717.1
7	*S*. Dublin	1061	1061	95	0.0	99	LK931502.1
8	*S*. Dublin	1061	1061	95	0.0	99	CP001144.1
9	*S*. Typhimurium	1044	1044	95	0.0	99	CP007235.1
10	*S.* Typhimurium	1044	1044	95	0.0	98	CP009102.0

Table 3 serotypes obtained by BLASTn of the FASTA format sequence at www.ncbi.nlm.nih.gov based on the *sucA* housekeeping gene sequences.

3.3 Multi-Locus Sequence Typing Analysis

Sequence types (STs) related to ST5 lineage was found. The STs obtained belonged to the ST157 complex but, they were not identical to each other. Novel STs were assigned new allele numbers as follows; ST684, ST466, ST1676, ST1229, ST741, ST98, ST725, ST569, ST546, ST429, ST92, ST78, ST747, ST739, ST640, ST945,ST 861, ST86, ST787, ST772, ST694, ST599, ST582, ST534, ST533, ST1776, ST999, ST998, ST997 and ST996. A unique evolutionary tree, drawn to scale based on these sequence types (Table 4) was constructed (Figure 1).

Table 4 MLST sequence types

MLST allele Sequence Analysis and Assignment of the New Allele Sequence Types								
ST	*aroC*	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	ST Complex
Unknown ST	147	378	10	123	10	19	17	NC
ST684	147	13	15	123	15	19	17	157
ST466	147	13	15	123	140	7	17	157
ST1676	147	13	15	123	15	9	17	NC
ST1229	313	13	15	123	15	19	17	157
ST741	2	201	146	9	6	19	17	NC

These sequence types were automatically generated by the predefined MLST algorithms and allocated new (for unknown STs) or existing sequence types (for known STs). NC=no complex.

3.4 Analysis of Evolutionary Relationships Based on ST

The tree shows the evolutionary relationships of six (6) selected ST taxa. The genetic differences between *Salmonella* isolates STs and a reference ST isolate

were found. The isolates were clearly differentiated into two clusters 1 and 2. Cluster 1 consists of ST19, ST147 and ST17. Cluster 2 consists of ST123 and ST378. *Salmonella* Typhimurium (ST446) was used as a reference ST. It was revealed that, the genetic distances between the ST147 and ST17 in cluster 1 relative to the reference ST was negligible compared to ST19. However it can be inferred that, these STs are closely related to the reference isolate (ST466). This implies that these isolates were more similar to each other and to the reference ST. The genetic distances between isolates in cluster 2 were found to be negligible but protracted as compared to the reference ST466, an indication that these isolates were different from each other and from the reference ST (Figure 1).

Figure 1. Evolutionary relationships of 6 ST taxa

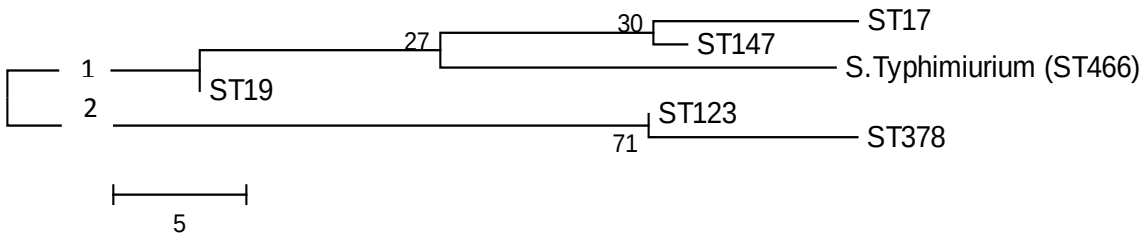

Figure 1. Dendogram showing two clusters 1 and 2. Cluster 1 consists of ST19, ST147 and ST17. Cluster 2 consists of ST123 and ST378. S. Typhimurium (ST466) is the reference.

3.5 Codon-based Test of Neutrality for Analysis Between Sequences

This test statistic yielded different probability levels between the sequences. Probability levels greater than 0.05 indicates the highest level of neutrality (similarity) between sequences. On the other hand, probability levels less than 0.05 indicates lack of neutrality. Similar to MLST analysis ST based evolutionary taxonomic tree (Figure 1), these results pointed to differences between isolate sequences and the reference sequence (Table 5).

Table 5 Codon-based Test of Neutrality for analysis between sequences

	1	2	3	4	5	6
[1] *S.* Typhimurium (ST466)		0.137	-0.877	-0.0618	-0.386	1.726
[2] ST123	0.891		0.762	2.114	0.878	0.910
[3] ST147	0.382	0.448		-0.233	-1.207	-0.0467
[4] ST17	0.951	0.0366	0.816		2.00813	1.569
[5] ST19	0.700	0.382	0.300	0.0469		-2.344

[6] ST378	0.0868	0.364	0.963	0.119	0.0207

Table 5 shows the test statistic (d_N - d_S) above the diagonal. d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively. *P* Values of less than 0.05 at the 5% level are highlighted.

3.6 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

The nucleotide frequencies of 0.177 (A), 0.244 (T/U), 0.263 (C), and 0.317 (G) were obtained. The transition/transversion rate ratios were found to be k_1 = 2.698 (purines) and k_2 = 20.089 (pyrimidines). The overall transition/transversion bias was R = 6.565, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. The results further showed that the sequences types obtained by MLST analysis were genetically diverse (Table 6)

Table 6 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	**T**	**C**	**G**
A	-	*1.81*	*1.94*	**6.33**
T	*1.31*	-	**39.06**	*2.35*
C	*1.31*	**36.27**	-	*2.35*
G	**3.54**	*1.81*	*1.94*	-

3.7 Assignment of Isolate to Serotypes Taxonomy

The small bootstrap values indicate that the genetic distances between the isolates with a given cluster are negligible. The large bootstrap values conversely indicate that the genetic distance between evaluated isolate sequences are protracted. These are important indicators of genetic divergence between closely related isolates. The isolates differentiated into two main clusters 1 and 2 (Figure 2).

Figure 2: Overall phylogenetic tree

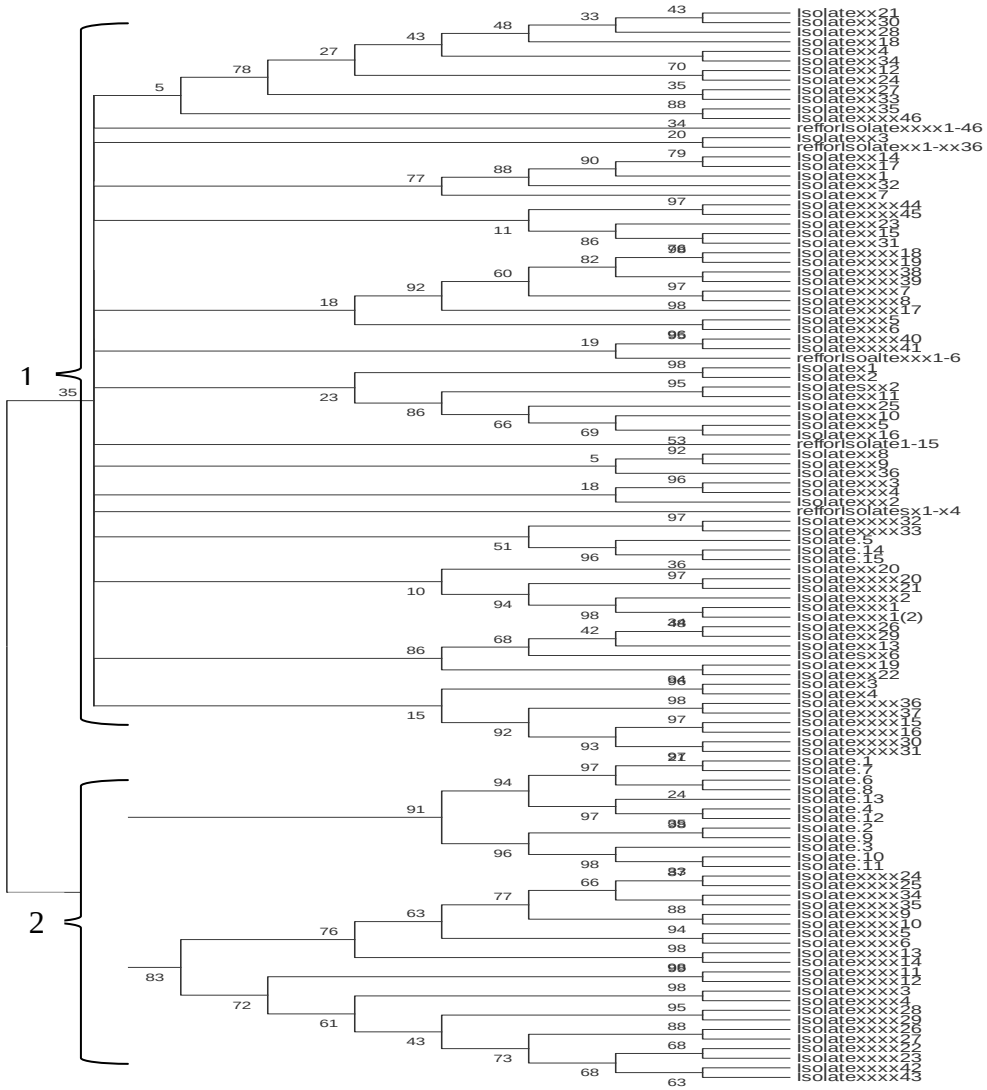

Figure 2: The dendogram showing two clusters 1 and 2. The tree was constructed using Unweighted Pair Group Method using arithmetic Averages (UPGMA) algorithm. The results further confirm that the isolates studied here were indeed divergent.

4.0 Discussion

Worldwide, salmonellosis is one of the most important public health zoonotic diseases of man and animals (Majowicz *et al*., 2010; Schneider, 2011). Salmonellosis is one of the pathogens which have been seen to represent an increasing emergence, whose origins are associated with consumer food handling, livestock production and food contamination (Gantois *et al*., 2009). Pathogenic bacterial gain or loses essential regions of their core genome leading to the accumulation of several genetic factors such as pathogenicity islands, sequence types (STs) and modified proteins consequently, making them resistant to antimicrobial drugs (Gantois *et al*., 2009).

Reports by Gantois *et al*., (2009) identified *Salmonella* harboring ST lineages belonging to ST11 complexes, which were capable of colonizing and persisting in the chicken oviducts and consequently lead to chicken salmonellosis pandemic of between 1980s and 1990s in Japan. In this study, we identified ST5 lineage which was found to belong to ST157 complex. Similar to the observation by Gantois *et al*., (2009), *Salmonella* isolates studied here are equally likely to colonize, persist and initiate a pandemic not only in Ugandan pigs but in other pig rearing systems worldwide and perhaps result to human infections.

In our study a great deal of inter-isolate diversity was displayed, given that the genetic distances between ST147 and ST17 which were found to cluster close to each other and to the reference *S*. Typhimiurium (ST466) were negligible. It was also revealed that ST19 deviated from ST147, ST17 and the reference *S*. Typhimurium (ST466) (Figure 2). While the genetic distance between ST123 and ST378 was found to be negligible but, highly protracted in relation to the reference *S*. Typhimiurium (ST466). This suggests that the ST lineages constitute genetic departures or genetic divergents from the reference *S*. Typhimiurium (ST466) and from each other (Figure 1).

Certainly, ST variations are thought to contribute to *Salmonella* serotypes and lineages which conceivably may be the major causes of diarrhea observed in pigs. The observed genomic diversity is a reflection of a multiple of coexisting lineages in the intestinal mucosal surface. Probability levels greater than 0.05 indicates the highest level of neutrality (similarity) between sequences. On the other hand, probability levels less than 0.05 including 0.0366 in column two, 0.046 in column four and 0.021 in column five indicates lack of neutrality. The overall transition/transversion bias *R* was found to be = 6.565, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$, an indication of genetic divergence. In addition to MLST analysis ST based evolutionary taxonomic tree figure 3, these results points to differences between isolate sequences and the reference sequence.

Our findings contributes to filling in the gap in knowledge on the sequence types which are thought to contribute to genetic divergence in *Salmonella enterica* sub-species *enterica*, an impediments in animal source food safety, animal health and associated human infection with super-resistant *Salmonella* serotypes such as *S*. Typhimurium. Indeed, we demonstrated the combined usefulness of conventional

culture methods; molecular biology based genomic DNA analysis, MLST analysis, natural selection analysis using the Codon-based Test of Neutrality for analysis between sequences and Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution.

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Appendix (Supplementary materials)

Table1a. Oligonucleotide primers used for PCR amplification of antimicrobial resistance genes

Gene target	Primer name	Primers sequence(5'-3')	Nucleotide position	size	TM (^{0}C)	Acc. No
tetA (A)	tetA (A)-F	GCTACATCCTGCTTGCCTTC	7562-7581	20	55	S5243
	tetA (A)-R	CATAGATCGCCGTGAAGAGG		20		7
tetA (B)	tetB (B)-F	TTGGTTAGGGGCAATTTTTG	2003-2022	723	55	J01830
	tetB (B)-R	GTAATGGGCCAATAACACCG	2661-2642	20		
tetC (A)	tetC (C)-F	TCAACCCAGTCAGCTCCTTC	675-694	20 400	55	J01740
	tetC (C)-R	GAGCACATGGAACGGGTT	1192-1174			
sul1	S1-F	GTGACGGTGTTCGGCATTCT	1738-1758	400 400	55	M7381 9
	S1-R	TTTACAGGAAGGCCAACGGT	2406-2387			
sul2	S2-F	TTTTCGGCATCGTCAACATA	7894-7914	400 400	55	M2882 9
	S2-R	CAATGCTCTGCAGCGAGTGT	8579-8568			
sul3	S3-F	AAGTGGGCGTTGTGGAAGA	300-320	400 400	55	AY494 779
	S3-R	CTGACTTTGCCAAGCCTGAA	353-333			
bla$_{TEM-1}$	bla-F	AACTTTATCCGCCTCCATCC	43490-43509	450 450	55	AJ8510 89
	bla-R	GCCTTCCTGTTTTTGCTCAC	44079-44060			

Table 2a. Oligonucleotide primers used for MLST analysis based on the seven housekeeping genes.

Gene	PCR Primer pair sequence	Product size	TM (^{0}C)
thrA	F 5'-GTCACGGTGATCGATCCGGT-3'	852 bp	55
	R 5'-CACGATATTGATATTAGCCCG-3'		
purE	F 5'-ATGTCTTCCCGCAATAATCC-3'	510 bp	55
	R 5'-TCATAGCGTCCCCCGCGGATC-3'		
sucA	F 5'-AGCACCGAAGAGAAACGCTG-3'	643 bp	55
	R5'-GGTTGTTGATAACGATACGTAC-3'		
hisD	F 5'-GAAACGTTCCATTCCGCGCAGAC-3'	894 bp	55

	R 5'-CTGAACGGTCATCCGTTTCTG-3'		
aroC	F 5'-CCTGGCACCTCGCGCTATAC-3'	826 bp	55
	R 5'-CCACACACGGATCGTGGCG-3'		
hemD	F 5'-ATGAGTATTCTGATCACCCG-3'	666 bp	55
	R 5'-ATCAGCGACCTTAATATCTTGCCA-3'		
dnaN	F 5'-ATGAAATTTACCGTTGAACGTGA-3'	833 bp	55
	R 5'-AATTTCTCATTCGAGAGGATTGC-3'		

MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica), which is hosted at the University College Cork and funded by the Science Foundation of Ireland (05/FE1/B882). Table 2 above provides the list of primers which were used for specific amplification of the seven housekeeping genes in Salmonella isolates.

Table 3a. Oligonucleotide primers used for Sequencing MLST amplicons.

Primer name	**Primer sequence (5', 3')**	**Tm (^{0}C)**
***thrA*: sF**	5'-ATCCCGGCCGATCACATGAT-3'	55
***thrA*: sR**	5'-CTCCAGCAGCCCCTCTTTCAG-3'	55
***purE*: sF**	5'-CGCATTATTCCGGCGCGTGT-3'	55
***purE*: sF1**	5'-CGCAATAATCCGGCGCGTGT-3'	55
***purE*: sR**	5'-CGCGGATCGGGATTTTCCAG-3'	55
***purE*: sR1**	5'-GAACGCAAACTTGCTTCAT-3'	55
***sucA*: sF**	5'-AGCACCGAAGAGAAACGCTG-3'	55
***sucA*: sR**	5'-GGTTGTTGATAACGATACGTAC-3'	55
***hisD*: sF**	5'-GTCGGTCTGTATATTCCCGG-3'	55
***hisD*: sR**	5'-GGTAATCGCATCCACCAAATC-3'	55
***aroC*: sF**	5'-GGCACCAGTATTGGCCTGCT-3'	55
***aroC*: sR**	5'-CATATGCGCCACAATGTGTTG-3'	55
***hemD*: sF**	5'-GTGGCCTGGAGTTTTCCACT-3'	55
***hemD*: sF1**	5'-ATTCTGATCACCCGCCCCTC-3'	55
***hemD*: sR**	5'-GACCAATAGCCGACAGCGTAG-3'	55
***dnaN*: sF**	5'-CCGATTCTCGGTAACCTGCT-3'	55
***dnaN*: sR**	5'-CCATCCACCAGCTTCGAGGT-3'	55

MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica), which is hosted at the University College Cork and funded by the Science Foundation of Ireland (05/FE1/B882). Table 3 above provides the list of primers which were used for sequencing the seven housekeeping genes PCR products.

