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Campylobacter and *Salmonella* in Scavenging Indigenous Chickens in Rural Central Tanzania: Prevalence, Antimicrobial Resistance, and Genomic Features

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Abstract: Introduction: *Salmonella* and *Campylobacter* spp. are commonly reported bacterial foodborne pathogens causing morbidity and mortality worldwide. In rural areas, where there is a high occurrence rate of human–animal interactions and poor hygiene practices, shedding animals present a high risk to humans in acquiring animal-associated infections. **Materials and methods**: Seasonal prevalence of *Campylobacter jejuni*, *Campylobacter coli*, and *Salmonella* spp. in scavenging indigenous chicken faeces was determined by polymerase chain reaction (PCR). Antimicrobial resistance was studied in *Salmonella* isolates by disc diffusion method, and whole-genome sequenced isolates were used to determine *Salmonella* serovars, antimicrobial resistance genes, virulence genes, and plasmid profile. **Results**: The overall prevalence of *Campylobacter* in chickens was 7.2% in the dry season and 8.0% in the rainy season ($p = 0.39$), and that of *Salmonella* was 11.1% in the dry season and 16.2% in the rainy season ($p = 0.29$). *Salmonella* serovars detected were II 35:g,m,s,t:-, Ball, Typhimurium, Haardt/Blockley, Braenderup, and Enteritidis/Gallinarum. One *S.* II 35:g,m,s,t:- isolate was resistant to ampicillin and the rest were either intermediate resistant or pansusceptible to the tested antimicrobials. The resistance genes observed were *Cat*A, *tet*J, and *fos*A7, most common in Ball than in other serovars. Seven plasmids were identified, more common in serovar Ball and less common in II 35:g,m,s,t:-. Serovar II 35:g,m,s,t:- isolates were missing some of the virulence genes important for *Salmonella* pathogenicity found in other serovars isolated. **Conclusions**: PCR detection of *Campylobacter* spp. and *Salmonella* spp. in chickens necessitate the improvement of hygiene at the household level and reducing human–chicken interaction as a strategy of preventing humans from acquiring chicken-associated bacteria, which would enter the human food chain. Infrequent use of antimicrobials in this type of poultry is most likely the reason for the low rates of antimicrobial resistance observed in this study.

Keywords: *Campylobacter*; *Salmonella*; antibiotic resistance; scavenging chickens; whole-genome sequencing

1. Introduction

Foodborne diseases are a major public health concern all over the world, in high-, middle-, and low-income countries. Current estimates indicate that 35 foodborne hazards cause 601 million illnesses, 476,000 deaths, and 42 million disability-adjusted life years annually worldwide [1,2]. *Campylobacter* spp. and non-typhoidal *Salmonella* spp. are important foodborne pathogens causing human morbidity and mortality in low-, middle-, and high-income countries. According to the report by the European Food Safety Authority and the European Centre for Disease Prevention and Control, *Camypylobater* and *Salomonella* accounted for more than 68% and 25%, respectively, of the infectious foodborne diseases cases reported in Europe in 2018 [3]. Broiler meat, turkey meat, pork, and eggs and egg products for *Salmonella,* and pork and milk and milk products for *Campylobacter* are frequently reported as the principal sources of infections in humans, with consumption of contaminated poultry products accounting for the majority of cases [3,4]. Birds infected with *Campylobacter* spp. and non-typhoidal *Salmonella* spp. remain carriers; shed organisms through their faeces; and, in the case of *Salmonella* Enteritidis, transmit the infection to their chicks transovarially [5,6]. Faecal shedding of bacteria by the colonised animals contaminates environments, which serves as a significant source of human infection, especially in poor hygiene conditions.

There is an extensive body of literature on poultry *Campylobacter* spp. and non-typhoidal *Salmonella* spp. infections in intensive or commercial production systems, unlike in scavenging indigenous chickens, where the data are limited. The available literature in Tanzania indicates a significantly higher prevalence of *Campylobacter* spp. infection in rural, extensively raised, local chickens (76%) than in urban broilers (60%), and a higher prevalence in local, rural chickens than in those raised in urban areas [7]. A recent study of *Salmonella* spp. prevalence conducted on indigenous chickens in central Tanzania reported a flock prevalence of 29% based on overnight floor dropping samples [8]. In high-income countries, *Campylobacter* spp. and non-typhoidal *Salmonella* spp. infections from poultry are mostly acquired through the handling and consumption of raw or undercooked intensively produced poultry meat and eggs [9–11]. In low-income countries, the infections are reported to be commonly acquired through poor hygiene and sanitation and animal faecal contamination as a result of living in close proximity to animals [12,13]. In the current study areas, poultry products are not frequently consumed, as chickens are retained for sale in times of need or for income generation, and eggs are used for the hatching of chicks [14]. Therefore, human *Campylobacter* spp. and non-typhoidal *Salmonella* spp. infections of poultry origin in these areas are thought to be more likely from the environmental contamination of food and water with chicken faeces than from handling and consuming raw or undercooked poultry products. There are few studies on the prevalence of human salmonellosis and campylobacteriosis conducted in some localities of Tanzania. A study conducted on children under five years of age in Dar es Salaam city reported a prevalence of 10.6% and 3.0% in diarrhoeic and non-diarrhoeic children, respectively [15]. Invasive *Salmonella* infections account for 17.4% of the total community-acquired and 8.6% of the nosocomial bacterial infections in the same locality [16]. In rural areas of Tanzania, non-typhoid infections account for 29% of the total bacteriaemia cases in individuals between the ages of two months and 14 years [17]. The health facilities-based study conducted in Morogoro Region in the Eastern Zone of Tanzania on human campylobacteriosis five years ago reported a prevalence of 16.7% in individuals less than 15 years of age and 10% in individuals older than 15 years of age [18]. The occurrence of *Salmonella* and *Campylobacter* gastroenteritis in Tanzania may be higher than what is stated in the available data because not all cases are reported, and sometimes antibiotics are prescribed before laboratory confirmation.

Health care services in low- and middle-income countries are facing significant challenges due to antimicrobial multi-drug resistance, which is increasing rapidly as a result of poor sanitation, increased global human mobility, and irrational use of antibiotics in both the health and livestock sectors [19]. Human infections with multi-antimicrobial drug

resistant bacterial strains, including *Salmonella*, can result in prolonged hospitalisation and increased mortality, especially in children with invasive infections [16]. The spread of antimicrobial resistance in *Salmonella* can be through the clonal expansion of resistant strains, as observed in the multi-national spread of serotype Typhimurium DT104 [20], and through horizontal resistance genes transferred via mobile genetic elements, including plasmids and class I integrons [21,22]. Modern techniques of whole-genome sequencing have enabled easy screening for antimicrobial resistance genes in bacterial genomes and the determination of the presence of mobile genetic elements carrying antibiotic resistance genes. These factors strengthen antimicrobial resistance surveillance [23].

Chickens kept in the study area are mostly managed under the small extensive scavenging production system [24], with human houses being mostly rustic, functioning as both children's playgrounds and chickens' scavenging and housing locations. The lack or poor quality of chicken houses, which cannot guarantee the safety and physical security of chickens, contribute to most households securing chickens inside their houses overnight. This system predisposes the environment, the house, and the kitchen utensils to contamination with chicken faeces, which can increase the risk of acquiring chicken-related microbial infections. This study determines the seasonal prevalence of *Campylobacter* spp. and *Salmonella* spp. and antimicrobial resistance and genomic analysis of *Salmonella* isolates in local scavenging chickens in three wards of rural central Tanzania as a step towards identifying public health risks associated with chicken–human interactions in the study areas.

2. Materials and Methods

2.1. Participating Households and Sample Size Determination

The study was conducted in households randomly selected from the households participating in the main project titled "Strengthening food and nutrition security through family poultry and crop integration in Tanzania and Zambia." funded by Australian Centre for International Agricultural Research (ACIAR) [25]. The project involved three wards and four communities (villages) from each ward, with a total of 12 communities (villages). A sampling frame was generated from the household census with at least one child under two years of age and keeping chickens or intending to keep chickens. A lottery draw using household identification numbers was done to enrol 240, 280, and 300 households from Sanza, Majiri, and Iwondo Wards, respectively. The number of the participating households dropped with time due to different reasons and at the time of implementation of the current study, the number of the households was 711 from 820. The current study used the same remaining households (711) to prepare the sampling frame, the inclusion criteria being having at least 5 chickens and being willing to participate in this study, followed by running a rotary to select the required number of households to participate in this study.

The chicken sample size was calculated using Statulator, the sample size calculation tool available at http://statulator.com/SampleSize/ss1P.html (accessed on 20 September 2016). The sample size estimation for determining the prevalence of *Campylobacter* and *Salmonella* was based on the previously reported prevalence of 38% [26] and 28% [8], respectively, in indigenous chicken populations in Sanza Ward with an intra-class coefficient of 0.05; a cluster size of 5; and at 5%, absolute precision and 95% confidence. These calculations gave a sample size of 363 and 310 chickens for *Campylobacter* and *Salmonella* prevalence estimation, respectively. In order to include equal representation from all 12 communities from the three wards (Sanza, Majiri and Iwondo Wards) that participated in the study, five chickens were sampled from seven households in each community (village) to give a total of 420 chickens from 84 households randomly selected from the prepared sampling frame of households owning at least five chickens.

2.2. Sample Collection

A dry swab (FLOQSwabs™, Copan Diagnostics, Murrieta, CA, USA) and Cary Blair media swab (Transwab® Cary Blair, Medical Wire & Equipment, Corsham, UK) were used to collect faecal samples from each of the chicken cloaca. A total of 420 and 390 chicken faecal samples were collected in the mid-dry (September 2017) and mid-rainy (February 2018) seasons, respectively, from the same households but not necessarily from the same chickens. Among the 84 participating households, six were no longer keeping chickens during follow-up sampling in the rainy season. The samples were kept on ice and transferred to the vehicle refrigerator before being preserved at −20 °C for dry swabs and refrigerator temperature (2–8 °C) for swabs in transport media at the Tanzania Veterinary Laboratory Agency (TVLA) central zone branch in Dodoma. The samples were then transported in a mobile refrigerator to Dar es Salaam and kept at the same temperatures as above at TVLA, Central Veterinary Laboratory, Department of Bacteriology until processing. The samples collected for the screening of both bacteria by polymerase chain reaction (PCR) and *Salmonella* isolation were collected as dry swabs and in transport medium, respectively.

2.3. Data Analysis

Data was first entered into an Excel 2007 spreadsheet and then transferred to STATA® software (College Station, TX, USA) version 14.2 for analysis [27]. Data analysis involved the calculation of the prevalence of *Salmonella* spp. and *Campylobacter* spp. infection in chickens, the difference in prevalence of both bacteria in chickens across all three wards in the dry and rainy seasons using STATA® software. To evaluate statistical differences in the prevalence among wards in the dry and rainy seasons for both bacteria, χ^2statistics were used, whilst difference in prevalence between *C. jejuni* and *C. coli* among wards in the dry and rainy seasons was determined using Fisher's exact test. The differences were determined among the wards in each season based on ward-specific samples and between seasons using overall samples for the dry and rainy seasons.

2.4. Molecular Methods Used to Determine Prevalence of Campylobacter and Salmonella

The distance between the study areas and the sample processing laboratory is more than 600 km, hence, daily sample processing was not possible. This resulted in sample processing being delayed for two to three weeks, as processing was not commenced until all sampling exercises were completed. Additionally, poor infrastructure contributed to further delay of the sampling exercise, as moving within and between the wards, especially during the rainfall, was not easy. Due to this delay, estimation of the prevalence through classical bacterial isolation methods was considered unreliable, therefore, the prevalence estimation of *Campylobacter* and *Salmonella* was based on PCR screening. Additionally, *Campylobacter* isolation was not attempted, considering the fastidious nature of these bacteria, as successful recovery was unlikely due to long storage, despite maintaining an appropriate storage temperature.

2.4.1. DNA Extraction

DNA extraction and amplification were carried out at TVLA, Centre for Infectious Diseases and Biotechnology, in Dar es Salaam. DNA was extracted using Quick-DNA™ Faecal/Soil Microbe Miniprep Kit (Zymo Research) (D6010). The DNA extraction process followed the kit manufacturer's protocol except that a lower quantity of faecal material (5–10 g) than recommended (150 g) was used as a starter, due to the difficulty of obtaining sufficient faecal sample quantities via cloacal swab sampling. Some samples were excluded because of low DNA concentrations measured by NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000, Waltham, MA, USA). The total number of chicken faecal samples used for *Campylobacter* and *Salmonella* PCR analysis was 780 corrected in dry and rainy seasons.

2.4.2. PCR Based Detection of Campylobacter

The PCR performed for the detection of *C. jejuni* and *C. coli* simultaneously was based on 16S rRNA gene sequences, and for *C. jejuni* based on the hippuricase (*hipO*) gene as described by Linton and Lawson [28]. The 16S rRNA PCR reaction mixture was prepared using 12.5 µL of OneTaq Quick-Load 2X Master Mix with standard buffer (Biolabs, New England), 0.5 µL of each of the forward and reverse primers (CCCJ609F and CCCJ1442R) (Table 1) (10 pmole), and 5 µL (~10 ng/µLL) of gDNA, then water was added to yield a final volume of 25 µL. Nuclease-free water was used as a negative control and in-house isolated and PCR confirmed *C. jejuni* and *C. coli*, obtained from Kenya Medical Research Institute laboratory, were used as the positive controls. The *hipO* PCR assay was performed using the same reagents and concentrations as the 16s rRNA PCR with the exception of replacing the 16S rRNA primers with the HIP400F and HIP1134R primers (Table 1) for the identification of *C. jejuni* in all samples that were positive for the 16s rRNA PCR reaction. The 16S rRNA PCR-positive samples and hippuricase PCR-negative samples were *C. coli*, and those positive for both PCRs were *C. jejuni*. Both assays were run for 30 amplification cycles under the conditions shown in Table 1 using a programmable thermal cycler (MJ Research, Watertown, MA, USA). The PCR products from both reactions were analysed by electrophoresis run at 100 V for 40 min in 1% agarose gel stained with GelRed (Biotium Inc., Fremont, CA, USA). A Quick-Load 100 bp DNA ladder (Biolabs, New England) was used to determine the amplicon size.

Table 1. Amplification conditions and the primers used for the co-amplification of *Campylobacter jejuni* and *C. coli*, and the amplification of *C. jejuni* and *Salmonella* spp. in faecal samples collected from chickens.

Assay	Amplification Conditions			Primers (5′-3′)	Expected Product Size
	Step	Temperature	Time		
C. coli/*C. jejuni* 16S rRNA gene-based PCR	Initial denaturation	94 °C	30 s	CCCJ609F (AATCTAATG-GCTTAACCATTA) CCCJ1442R (GTAAC-TAGTTTAGTATTCCGG)	854 bp
	Denaturation	94 °C	1 min		
	Annealing	58 °C	1 min		
	Extension	72 °C	1 min		
	Final extension	72 °C	7 min		
C. jejuni hippuricase gene-based PCR	Initial denaturation	94 °C	30 s	HIP400F (GAA-GAGGGTTTGGGTG) HIP1134R (AGCTAGCTTCG-CATAACTTG)	735 bp
	Denaturation	94 °C	1 min		
	Annealing	66 °C	1 min		
	Extension	72 °C	1 min		
	Final extension	72 °C	7 min		
Salmonella spp. *invA* gene-based PCR	Initial denaturation	94 °C	30 s	*invA*-1 (TTGTTACGGC-TATTTTGACCA) *invA*-2 (CTGACTGCTAC-CTTGCTGATG)	521 bp
	Denaturation	94 °C	1 min		
	Annealing	55 °C	1 min		
	Extension	72 °C	1 min		
	Final extension	72 °C	7 min		

2.4.3. PCR Based Detection of Salmonella

The PCR was performed based on the amplification of the invasive (*invA*) gene using *invA*-1 and *invA*-2 primers [29] (Table 1). The PCR reaction mix was composed of 12.5 µL of OneTaq Quick-Load 2× Master Mix with standard buffer (Biolabs, New England), 0.5 µL of each of the forward and reverse primers (10 pmole), and 5 µL (~10 ng/µL) of gDNA, then water was added to yield a final volume of 25 µL. Nuclease-free water was used as a negative control, and DNA extracted from *Salmonella* Typhimurium ATCC® 13311™ (KWIK-STIK™, Minnesota, USA) was used as a positive control. The assay was run for 35 amplification cycles under the conditions shown in Table 1. PCR products were analysed as in Section 2.4.2.

2.5. Salmonella Antimicrobial Susceptibility Test and Sequencing

2.5.1. Salmonella Isolation and PCR Identification

The isolation procedure was performed following the method described in the Global Foodborne Infections Network laboratory protocol for the isolation of *Salmonella* spp. from food and animal faeces [30]. The protocol was followed closely except that instead of using the recommended sample amount of 25 g, 1 mL of media mixed with the sample was used. One mL of Cary Blair transport media inoculated with the faecal sample was mixed with 9 mL of buffered peptone water (BPW) (CM0509B, Oxoid) and incubated at 36 °C for 24 h. One mL of the pre-enrichment broth was transferred to 10 mL tetrathionate broth (Müller-Kaufmann) (CM0343B, Oxoid) and 0.1 mL of pre-enrichment broth was transferred to 10 mL of Rappaport–Vassiliadis soy peptone (RVS) broth (CM0866B, Oxoid). The inoculated tetrathionate (Müller-Kaufmann) and Rappaport–Vassiliadis soy peptone (RVS) broths were incubated at 36 °C and 41.5 °C, respectively. A loopful each of the inoculated and incubated tetrathionate and RVS broth was spread on xylose lysine deoxycholate (XLD) (CM049B, Oxoid) and Brilliant Green Agar (BGA) (CM0263B, Oxoid) plates and incubated at 36 °C for a further 24 h. On day 4, two colonies were slightly transparent with a red halo with a black centre (showing the production of hydrogen sulphide gas—H_2S), and some of them were surrounded by pink-red zone on the XLD agar plates, and red and impart red/pink colour colonies on the BGA agar plates were sub-cultured on nutrient agar (CM0003B, Oxoid) for biochemical tests.

Colonies that were Gram stain-negative, short rods that were negative for indole production from tryptophan (Indole test) and urease negative (urea agar base, CM0053B, Oxoid) tests, but positive on triple sugar iron agar (red slant, yellow butt, black butt—H_2S gas production) (CM0277B, Oxoid) were subcultured onto agar slants for *Salmonella* confirmation by PCR. The DNA extraction from suspected *Salmonella* colonies was performed by the heat lysis method [31]. Briefly, two to three colonies of bacterial cells were re-suspended in 200 µL of nuclease-free water, then kept at −80 °C for 10 min followed by boiling in a water bath at 100 °C for 5 min. The procedure was repeated twice followed by cooling on ice for 5 min. The suspension was centrifuged at 16,000× *g* for 5 min, and the supernatants were stored at −20 °C until PCR was performed. DNA was quantified by nanodrop (Thermo Scientific™ NanoDrop 2000), and all samples were diluted to a concentration of ~10 ng/µL using nuclease-free water. The PCR was performed based on *invA* gene PCR following the same procedure as described in Section 2.4.3.

2.5.2. Antimicrobial Susceptibility Testing

An antimicrobial susceptibility test of PCR positive *Salmonella* spp. isolates was performed using the disc diffusion method (i.e., Kirby–Bauer method) according to the guidelines of the Clinical and Laboratory Standards Institute (accessed on 10 November 2016) [32]. The antimicrobial groups tested were aminoglycosides (amikacin—30 µg, streptomycin—10 µg, gentamycin—10 µg, kanamycin—30 µg), penicillins (amoxycillin—20 µg, ampicillin—10 µg), cephalosporin (ceftriaxazone—30 µg), fluoroquinolones (ciprofloxacin—5 µg, norfloxacin—10 µg), tetracycline (tetracycline—30 µg), and sulphonamides and folic acid inhibitors (trimethoprim/sulphamethoxazole in combination of 1.25 µg/23.75 µg).

2.5.3. DNA Extraction from Salmonella PCR Positive Isolates

DNA extraction from the colonies testing positive for *invA* gene PCR amplification was performed using a commercial extraction kit (Presto™ Mini gDNA Bacteria Kit Protocol—GBB100/101, Geneaid Biotech Ltd., New Taipei City, Taiwan) following the manufacturer's instructions with some modifications. A single overnight colony was picked and immersed in 10 mL of buffered peptone water (BPW) (CM0509B, Oxoid), mixed well, and incubated overnight at 36 °C. Three hundred microlitres of the BPW broth culture was dispensed into Eppendorf tubes and centrifuged for one minute at 14–16,000× *g* followed by the removal of as much of the supernatant as possible with a pipette. DNA was extracted from the resulting bacterial pellet using the abovementioned extraction

kit following the manufacturer's instructions. The DNA quality was checked for whole-genome sequencing by NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000). One ng of DNA (5 µL of 0.2 ng/µL) sample with absorbance ratios of OD A260/A280 at 1.0–2.0 and A260/A230 at 1.2–2.3 was used for the library preparation, quantified by PicoGreen method using a Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Scoresby, Australia).

2.5.4. Whole-Genome Sequencing of the Salmonella Isolates

Whole-genome sequencing (WGS) was performed on the NextSeq 500 platform (Illumina) (Illumina, San Diego, CA, USA) at the Centre for Infectious Disease and Microbiology—Public Health Laboratory Services, Westmead Hospital, Institute of Clinical Pathology Medical Research in Western Sydney, Australia. Sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina). The quality and quantity of the libraries were checked through a bioanalyser (4200 TapeStation, Agilent Technologies, Inc., Waldbronn, Germany) and qPCR-based quantification (KAPA library quantification Kits, Roche, Basel, Switzerland) procedures. A total of 18 raw sequences were submitted to the National Center for Biotechnology Information GenBank database under BioProject accession number PRJNA604270, titled Human-chicken interaction.

2.5.5. Primary and Secondary Analysis of Sequencing Data

The quality of raw sequence reads was checked using FastQC and QUAST software. Nullarbor pipeline v.2.0 (https://github.com/tseemann/nullarbor (accessed on 13 December 2018) was used to identify plasmids, virulence genes, antimicrobial resistance gene profiles, and multilocus sequence types. *Salmonella* serovar identification was determined by *Salmonella* In Silico Typing Resource (http://www.denglab.info/SeqSero (accessed on 13 December 2018) (SeqSero2 v1.0.2) by uploading the assemblies. The somatic (O) antigen group was ascertained through analysing *wzx* and *wzy* genes and the *rfb* cluster, and flagellar (H) antigen was determined by analysing the *fliC* and *fljB* genes.

Ethical considerations: This study was approved by Animal Research Ethics Committee of the University of Sydney.

3. Results

3.1. Prevalence of Campylobacter and Salmonella

The overall prevalence of *Campylobacter* in chickens was 7.2% in the dry season and 8.0% in the rainy season with no significant difference between the seasons (p = 0.39), and that of *Salmonella* was 11.1% in the dry season and 16.2% in the rainy season with no significant difference between the seasons (p = 0.29). The combined dry and rainy season data did not show any significant differences among the wards between *Salmonella* (p = 0.74) and *Campylobacter* (p = 0.13) prevalence in chickens. Prevalence of both bacteria in chickens was higher in the rainy season compared to that seen in the dry season in all wards, except in the case of *Campylobacter* prevalence in Majiri Ward (Table 2). The highest prevalence of *Campylobacter* and *Salmonella* in chickens was recorded in Majiri during the dry season (11.8%) and in Iwondo during the rainy season (17.4%), respectively; however, the difference between the wards and the seasons was not significant (Table 3). The highest proportion of chickens with mixed infections (both *Campylobacter* and *Salmonella*) was recorded in Majiri during the dry season (7.3%), and there was no significant difference in the prevalence of mixed infections in chickens among the wards across both seasons. *C. jejuni* prevalence in chickens was highest in Majiri in both the dry (5.5%) and rainy seasons (6.0%) compared to the other wards studied (Table 3). *C. coli* prevalence in chickens was highest in Majiri during the dry season (6.4%), and the difference among wards in this specific season was significant (p = 0.042) (Table 3).

Table 2. Prevalence of *Campylobacter* and *Salmonella* in chickens, by ward and by season.

	Seasonal Prevalence (%)							
	Campylobacter				***Salmonella***			
Wards	**Dry Season**		**Rainy Season**		**Dry Season**		**Rainy Season**	
Sanza	5.7	(*n* = 172)	7.1	(*n* = 126)	10.5	(*n* = 172)	16.7	(*n* = 126)
Majiri	11.8	(*n* = 110)	9.0	(*n* = 100)	10.9	(*n* = 110)	14.0	(*n* = 100)
Iwondo	6.7	(*n* = 134)	8.0	(*n* = 138)	11.9	(*n* = 134)	17.4	(*n* = 138)

Table 3. Comparison of the prevalence of *Campylobacter*, *Salmonella,* and mixed infections, by ward and by season.

	Prevalence (%), by Ward			**Wards Variations**	**Seasonal Variations**
Variable	**Sanza**	**Majiri**	**Iwondo**	***p*-Value**	***p*-Value**
Campylobacter					
Dry season	5.7	11.8	6.7	0.073	0.629
Rainy season	7.1	9.0	8.0	0.877	
Salmonella					
Dry season	10.5	10.9	11.9	0.919	0.483
Rainy season	16.7	14.0	17.4	0.771	
Mixed infections (*Campylobacter* and *Salmonella*)					0.635
Dry season	3.5	7.3	3.7	0.315	
Rainy season	2.4	6.0	5.1	0.363	
Campylobacter jejuni					
Dry season	3.5	5.5	2.2	0.425	0.747
Rainy season	2.4	6.0	4.4	0.380	
Campylobacter coli					
Dry season	1.2	6.4	4.5	0.042	0.576
Rainy season	4.8	3.0	3.6	0.836	

3.2. Salmonella Genome Sequence Quality

The average coverage depth was 89.8×, ranging between 54× and 120×. The contig number ranged between 26 and 55 with a mean of 34.6, while <500 contigs indicates good quality. The mean size of the contig was 4,717,392 bp, with a range of 4,599,633 bp to 4,878,892 bp. The mean of the N_{50} was 349,599 bp, with a range of 177,385 bp to 454,996 bp, whereby a minimum of size of 30,000 bp is considered to be of good quality.

3.3. Salmonella Serovars

The most common serovar was *S*. II 35:g,m,s,t:-, which constituted 10 out of the 18 sequenced isolates. Four isolates were identified as *S*. Ball, followed by one each of *S*. Enteritidis/Gallinurum, *S.* Typhimurium, *S.* Haardt/Blockley, and *S.* Braenderup. Multilocus sequence type (MLST) was inferred from the assemblies of *S*. Enteritidis/Gallinurum, *S.* Typhimurium, and *S.* Braenderup as sequence type (ST) 78, 19, and 22, and were assigned as such, respectively (Table 4).

Table 4. *Salmonella* serovars isolated from chicken cloacal swabs, identified by whole-genome sequencing indicating the laboratory and household identification numbers, the ward and village of isolation, the sequence types *, antimicrobial resistance genes, and plasmid profiles.

Laboratory ID	Household ID Number	Ward	Village	Serovars	Resistance Profile	Sequence Type *	Resistance Gene	Plasmids
1	537	Sanza	Ntope	*S*. II 35:g,m,s,t:-	STR (I)	New 1	None	None
2	173	Majiri	Mpandagani	*S*. II 35:g,m,s,t:-	Pansusceptible	New 2	None	IncI1_1_Alpha
3	678	Majiri	Kinangali	*S*. II 35:g,m,s,t:-	STR (I)	New 3	None	None
4	678	Majiri	Kinangali	*S*. II 35:g,m,s,t:-	KAN (I)	New 4	None	None
5	678	Majiri	Kinangali	*S*. II 35:g,m,s,t:-	Pansusceptible	New 5	None	None
14	438	Sanza	Ikasi	*S*. II 35:g,m,s,t:-	Pansusceptible	New 6	None	None
8	33	Sanza	Ntope	*S*. II 35:g,m,s,t:-	Pansusceptible	New 7	None	None
16	286	Iwondo	Iwondo I	*S*. II 35:g,m,s,t:-	Pansusceptible	New 8	None	None
17	286	Iwondo	Iwondo I	*S*. II 35:g,m,s,t:-	AMP (R), STR (I)	New 9	None	None
18	633	Sanza	Sanza	*S*. II 35:g,m,s,t:-	KAN (I), STR (I)	New 10	None	IncI1_1_Alpha
9	516	Majiri	Majiri	*S*. Ball	Pansusceptible	New 11	*Fos*A7	IncFIB(pB171)_1_pB171, IncFIB(pCTU3)_1_pCTU3, IncFII(pECLA)_1_pECLA, IncFII(S)_1
10	516	Majiri	Majiri	*S*. Ball	Pansusceptible	New 12	*Fos*A7	IncFIB(pB171)_1_pB171, IncFIB(pCTU3)_1_pCTU3, IncFII(pECLA)_1_pECLA, IncFII(S)_1
11	611	Sanza	Ikasi	*S*. Ball	Pansusceptible	New 13	*Fos*A7	IncFIB(pB171)_1_pB171, IncFII(pECLA)_1_pECLA, IncFIB(pCTU3)_1_pCTU3, IncFII(S)_1
24	285	Iwondo	Igoji II	*S*. Ball	STR (I)	New 14	None	IncFIB(pB171)_1_pB171, IncFIB(pCTU3)_1_pCTU3, IncFII(pECLA)_1_pECLA, IncFII(S)_1
13	117	Iwondo	Chamanda	*S*.Typhimurium	Pansusceptible	19	None	IncFIB(S)_1, IncFII(S)_1
15	577	Sanza	Ikasi	*S*. Haardt/Blockley	Pansusceptible	New 15	None	ColRNAI_1
6	466	Sanza	Ikasi	*S*. Braenderup	KAN (I), STR (I)	22	None	None
7	33	Sanza	Ntope	*S*. Enteritidis/ Gallinarum	Pansusceptible	78	None	IncFII(S)_1, IncFII(pECLA)_1_pECLA, ColRNAI_1

STR = streptomycin, KAN = kanamycin, I = intermediate resistance, R = resistant. * Sequence type based on MLST derived from genome sequence assembly.

3.4. Antimicrobial Susceptibility and Antimicrobial Resistance Genes

One isolate of *S*. II 35:g,m,s,t:- was resistant to ampicillin and was the only resistant isolate observed in this study. Two and four strains of *S*. II 35:g,m,s,t:- showed intermediate resistance to kanamycin and streptomycin, respectively. Intermediate resistance was also observed in *S*. Braenderup and one *S*. Ball isolate for streptomycin and kanamycin, and streptomycin only, respectively. Antimicrobial resistance genes were more common in *S*. Ball than in other serovars isolated. Four out of five *S*. Ball carried the *fos*A7 gene conveying resistance to fosfomycin, and one *S*. Ball serovar, in addition to *fos*A7, had *Cat*A and *tet*J genes conveying resistance to chloramphenicol and tetracycline, respectively. One of the 11 *S*. II 35:g,m,s,t:- isolates was *Cat*A and *tet*J gene positive (Table 4). Gene *tet*J was the only antimicrobial resistance gene detected corresponding to the antimicrobials tested (tetracycline); however, all isolates were susceptible to this antimicrobial, regardless of their *tet*J gene carriage status.

3.5. Virulence Genes

S. Typhimurium was the serovar with the highest number of virulence genes (152), while *S*. II 35:g,m,s,t:- serovar isolates had the lowest, ranging between 127 and 128. Among others, *S*. II 35:g,m,s,t:- serovar isolates were missing *Ste*A and *Avr*A genes coding for proteins important for pathogenesis, which were present in the rest of the serovars. *S*. Ball was the serovar with the second lowest number of virulence genes, ranging between 130 and 131. *S*. Typhimurium serovar had all three *Salmonella* plasmid virulence genes (spv) B, C, and R, and *S*. Enteritidis/Gallinurum had B and C, while the rest of the serovars were missing these genes.

3.6. Plasmid Analysis

Plasmid replicon types ColRNAI_1, IncFIB(S)_1, IncFIB(pB171)_1_pB171, IncFIB(pCTU3)_1_pCTU3, IncFII(S)_1, IncFII(pECLA)_1_pECLA, and IncI1_1_Alpha were identified in half of the isolates sequenced. Plasmid IncFII(S)_1 was the most common, found in six isolates, followed by IncFIB(pB171)_1_pB171, IncFIB(pCTU3)_1_pCTU3, and IncFII(pECLA)_1_pECLA, each detected in four isolates. Plasmids were rarely observed in *S*. II 35:g,m,s,t:–, with only 2 out of 10 isolates carrying plasmids. Plasmid IncFII(S)_1 was found in *S*. Ball, Typhimurium and Enteritidis/Gallinurum serovars and was the only plasmid shared among different serovars. The details of the serovars carrying plasmids are presented in Table 4.

4. Discussion

The overall prevalence of *Campylobacter* carriage or infection in chickens in Sanza Ward in the dry (7.2%) and rainy (8.0%) seasons was far lower than that previously reported from the same area and type of flock in 2014 from overnight droppings (38%) [26], in cloacal swabs of the same type of flock in the eastern part of the country in 2006 (76%) [7], and from the caecal contents of broilers in the eastern part of the country in 2011 (78%) [33]. *Salmonella* prevalence in the dry (11.1%) and rainy (16.2%) seasons in Sanza Ward was also lower than that previously reported in 2017 from on-floor overnight droppings of scavenging chickens (28%) [8]. The higher prevalence of *Campylobacter* and *Salmonella* reported in the two previous studies conducted in Sanza Ward may be attributed to sampling floor droppings, which may increase the possibility of contamination from either other animals or human sources [8,26].

This study identified II 35:g,m,s,t:-, Ball, Typhimurium, Haardt/Blockley, Braenderup, and Enteritidis/Gallinarum as the *Salmonella* serovars circulating in the study area. *S*. Braenderup is of particular public health importance. It has been associated with several severe gastroenteritis outbreaks as the result of consumption of contaminated plant and animal-source foods [34,35]. There is limited information on the occurrence of *S*. Ball, but a recent notifiable diseases report indicates the occurrence of five human cases from 2016 to 2018 in the state of Victoria in Australia [36]. The *Salmonella* serovars Haardt and

Blockley were identified as different serovars in the White–Kauffmann–Le Minor scheme for *Salmonella* serotyping, principally based on the presence or absence of the O:6 antigen. Pulse field gel electrophoresis and the current whole-genome sequencing-based study shows Haardt and Blockley as the same serovars, an indication of a variable expression between O:6^{-} and O:6^{+} in the same serovar [37]. The first report of *S.* Blockley cases in humans in South Africa (suggested as the same serovar, Haardt) was accompanied by diarrhoea, stomach cramps, and headache [38]. Seafood and poultry have been the suspected vehicles of *S*. Blockley for human infection [39,40]. *S.* Typhimurium DT104, which belongs to ST19, has been implicated in foodborne gastroenteritis outbreaks and has been isolated in different domestic animal species worldwide [41]. In 2010 and 2014, *S.* Enteritidis caused gastroenteritis outbreaks in the USA and in multiple nations, respectively, through the consumption of contaminated eggs, and resulted in the recall of over 500 million eggs during these outbreaks [10,42]. Therefore, all *Salmonella* serovars isolated in this study are of public health importance except *S*. II 35:g,m,s,t:-, of which there has been no report of cases of human infection in the literature.

All *S*. II 35:g,m,s,t:- isolates lack most of the virulence genes found in many of the other serovars recovered in this study, including a*vr*A and *ste*A genes, coding for avrA and steA effector proteins, respectively, which are important for *Salmonella* pathogenesis. The *avr*A gene is a seen on the *Salmonella* pathogenicity island 1 (SPI-1), encoding for a multiple-function protein that plays a critical role in inhibiting the eukaryotic innate immune response and preventing cell apoptosis signalling. These are critical host mechanisms enabling the clearance of pathogens [43,44]. The ability of *Salmonella* to control the inflammation process of the host cell, normally through the reversion of the activation signalling pathway, is vital for the protection of bacteria after cell invasion. Serovar II 35:g,m,s,t:- may be less pathogenic to humans because it lacks these two virulence factors important for *Salmonella* host cell invasion and survival. Relatively high recovery rate of this serovar observed in the current study may be associated with a lack of virulence, rendering it easily tolerable by chickens and humans, hence, more dominant over more pathogenic strains. As observed in *S.* Typhimurium, the expression of different categories of genes is essential at different phases of *Salmonella* infection [45]. However, experimental infection of a mouse with *S*. Choleraesuis and *S*. Schwarzengrund, 9,12:l,v:- isolates, both lacking a*vr*A, elicited an acute systemic infection in the mouse. This resulted in death with the former and persistent infection accompanied by extended pathogen shedding in the latter [46].

Salmonella plasmids contribute to the fitness and survival of bacteria in the host. *Salmonella* serovars, mostly those associated with human and animal infections, carry different plasmids with specific virulence or antibiotic resistance genes [47]. The *Salmonella* plasmid virulence genes B, C, and R were observed in *S*. Typhimurium and *S*. Enteritidis/Gallinurum isolated in the current study. These genes are carried on IncF plasmids. Although the role of *Salmonella* plasmid virulence in the pathogenesis of *Salmonella* in different hosts remains uncertain, some evidence indicates that they may play an important role in cell invasion and host immune response inhibition during *Salmonella* infections [48]. As well as virulence genes, plasmids are known to carry antibiotic resistance genes. Analysis of the six IncA/C plasmids isolated from six serovars from poultry sources reported carrying 7 to 14 antibiotic resistance genes, with all genotypes correlating positively with phenotypic antimicrobial resistance [49]. Additionally, the presence of integron class 1 in *Salmonella* isolates significantly related to phenotypic antimicrobial resistance, which further indicates the role of integron in horizontally acquired antimicrobial resistance [50].

The prevalence of antimicrobial resistance was relatively low in the *Salmonella* serovars isolated from scavenging indigenous chickens in this study. Only one isolate belonging to serotype II 35:g,m,s,t:- was phenotypically resistant to ampicillin, and the remaining isolates were either intermediate resistant or pansusceptible to the tested antimicrobials. The number of antimicrobial resistance genes observed in this study was also lower than that reported in other studies that used a similar or lower number of isolates than that seen in

this study [50]. Analysis of antimicrobial resistance genes may provide vital information on phenotypic antimicrobial resistance of the pathogen, as other studies reported agreement between the presence of antimicrobial resistance genes and phenotypic resistance against several antimicrobial groups [23]. However, in the current study, the isolates carrying the *tet*J gene were phenotypically susceptible to tetracycline. Tetracycline resistance in bacteria is mediated by a group of *tet* genes through different mechanisms, including ribosomal protection and efflux pumps, of which, in the bacterial family *Enterobacteriaceae*, which includes the *Salmonella* genus, the role is mostly played by *tet*A to *tet*E genes [51]. On the other hand, *tet* genes have different modes of action, which, in some cases, necessitate the presence of more than one type of *tet* gene for the bacteria to fully express phenotypic antimicrobial resistance [52]. All of these may be contributing to the susceptibility to tetracycline of *Salmonella* isolated in this study, despite carrying the *tet*J gene. Lack of consistency in the relationship between the presence of resistance genes and phenotypic antimicrobial resistance of the isolates observed in the current study indicates the importance of accompanying the resistance gene analysis with phenotypic antimicrobial resistance testing. Scavenging indigenous chickens, especially in the area studied, are rarely treated with antibiotics, which may be a reason for the pansusceptibility of *Salmonella* isolates from these chickens. This contrasts sharply with many commercial poultry settings, where frequent use of antimicrobials and multiple antibiotic resistance are commonly encountered [53].

This study recovered a relatively small number of isolates per serovar. This was possibly due to the delay in sample processing, despite maintaining appropriate storage temperature, as the laboratory and study areas are 600 km apart and because of difficulties moving within and between wards due to poor infrastructure. Recovery of more than one isolate in different serovars may have given a clearer picture of the movement of *Salmonella* strains in chickens through phylogenetic analysis among the households and three wards studied. Although this study managed to isolate only a few *Salmonella* isolates in chicken faecal samples and did not attempt to isolate *Campylobacter*, PCR detection of both organisms in chickens indicates the presence of these pathogens, albeit at relatively lower prevalence compared to previous studies conducted in Tanzania. All *Salmonella* serovars isolated, except one, have been associated with human infections, necessitating the creation of awareness regarding appropriate animal husbandry and hygiene practices and proper food handling in these localities. More than half of the *Salmonella* isolates were S. II 35:g,m,s,t:-, but information on the pathogenicity of this serovar in humans is lacking. It will be crucial to conduct a pathogenicity study in different models to be able to establish the health risks associated with this serovar. This study further confirms the delayed development of *Salmonella* antimicrobial resistance in animals when antimicrobials are not excessively used. It is essential to identify localities without antimicrobial resistance problems through frequent surveillance, and create public awareness about its prevention, rather than waiting for the problem to occur.

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Informed Consent Statement: Informed consent was obtained from all owners of chickens involved in this study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available as they are still used for other research works.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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