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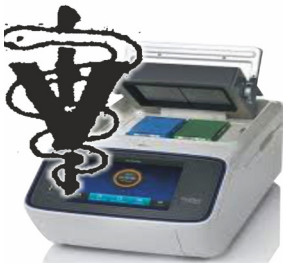

Occurrence and Antimicrobial Resistance of Salmonella enterica in Clinical and Food Samples in Northcentral Nigeria.

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Abstract

This study was conducted to determine the occurrence of typhoidal and non-typhoidal Salmonella (NTS) in ready- to-eat foods and human clinical cases. In northcentral Nigeria, NTS have not been well studied and the reservoirs, transmission and antimicrobial susceptibility patterns are poorly understood. A variety of commonly eaten foods including local fermented milk, grilled beef, pork and commercial chicken eggs as well as stool samples were examined for Salmonella. Samples were analyzed by pre-enrichment in lactose broth, selective enrichment with Selenite F and Rappaport–Vassiliadis (RV) broths and culture on Xylose Lysine Deoxycholate (XLD) agar. The bacteriological quality of foods was evaluated by bacterial counts and presence of Escherichia coli. Salmonella was identified by biochemical tests and confirmed by PCR with primers targeting invA gene. The antibiogram of the isolates was determined by disc diffusion assay. The occurrence of Salmonella in food and clinical fecal samples was 17% (12/72) and 55 % (11/20). Grilled pork, grilled beef, Egg and fermented milk had detection rates of 26.7 % (4/15), 25% (5/20), 13% (3/22) and 5% (1/20) respectively. Whilst all Salmonella isolates of fecal origin were typhoidal, 5 isolates from food were NTS. Antimicrobial resistance profile of the isolates indicated that 57.89% were resistant to multiple classes of antibacterial agents. However, all isolates were susceptible to ciprofloxacin and chloramphenicol. The results provide baseline data of typhoidal and NT Salmonella in Northcentral Nigeria, confirm under reportage of NTS, show gaps in food safety practices and the continued need for surveillance of Salmonellae in food and in clinical cases.

Keywords: Salmonella, Antimicrobial resistance, Occurrence, Northcentral Nigeria.

Introduction

Salmonella enterica is one of two species of the genus Salmonella and is further classified into six subspecies. Salmonella enterica subspecies enterica consist of ~ 2600 serovars and is a pathogen of warm-blooded mammals [1]. In terms of human health, Salmonella enterica subspecies enterica can be designated as typhoidal and non-typhoidal Salmonella (NTS) serovars [2]. Both typhoidal and non-typhoidal Salmonella enterica are genetically similar but infections result in different disease manifestations [3]. Together, Salmonella enterica infect over 100 million individuals annually and mortality is over 200 000 [4]. Whilst non-typhoidal Salmonella primarily cause gastroenteritis (salmonellosis) in many hosts including humans [5], typhoidal Salmonella is human-specific and causes enteric (typhoid and paratyphoid) fever. Typhoid (and paratyphoid) fever is a systemic disease and without treatment, mortality can reach up to 30% [6]. By contrast, NTS infections are associated with acute diarrhea, abdominal cramps, and fever that is usually self-limiting although treatment with antibiotics could be needed [7,8].

In Nigeria, typhoid fever is endemic with serious implications on health. Unlike typhoid fever that is transmitted via fecal-oral route, the transmission of NTS is more complicated. Many serovars are part of the normal microbiota of food animals including cattle, pigs and poultry [9]. Therefore, these animals may be involved in indirect or direct transmission to humans. For example, poultry, poultry products, meat contamination as a result of improper handling of the infected animal organs, such as the gut and liver are potential sources of contamination [10].

Due to the lower severity of NTS infections compared to typhoid fever, it has not been well studied in northcentral Nigeria [11,12]. However repeated cases of salmonellosis in children can result in malnutrition, poor cognitive and school performance and may compound these effects in populations already having high rates of malnutrition [13].

Antimicrobial resistance in developing countries including Nigeria has continued to rise [14] and this has also been reported in NTS [15]. Thus, continued surveillance data is required on the most effective drugs for current use whilst combating misuse of antibiotics and searching for new novel sources. This work sought to determine Salmonella prevalence and antimicrobial susceptibility/resistance trends to obtain baseline epidemiological data needed to plan an appropriate treatment and remedial measures on drug resistance.

Materials and Methods

A variety of commonly eaten foods in northcentral Nigeria that are of animal origin including local fermented milk, roasted beef and pork and commercial chicken eggs and stool samples were examined for Salmonella. Samples were analyzed by the Bacteriological Analytical Manual (BAM) protocol of pre-enrichment in lactose broth, selective enrichment with Selenite F and Rappaport–Vassiliadis (RV) broths and cultured on XLD agar [16]. Additionally, the bacteriological quality of foods was evaluated by bacterial counts and presence of Escherichia coli. Salmonella was identified by biochemical tests and confirmed by PCR. Antibiogram was also determined following a standard procedure from Clinical and Laboratory Standards Institute [18].

Stool samples were collected from patients presented with diarrhea at Primary Healthcare Centre, Kuje, FCT after ethical approval. Food samples including roasted pork and beef and commercial chicken eggs were purchased randomly from vendors within the FCT. Locally fermented milk was purchased from vendors within University of Abuja. All samples were immediately transported to Microbiology Laboratory, University of Abuja for analysis.

Determination of Total Bacterial Count

For solid samples, 1 gram of sample was crushed with sterile laboratory pestle and mortar (Fisher Scientific) and then added to 9 ml sterile saline water. Subsequent 10-fold serial dilutions were made and 0.1 ml were spread on sterile Plate Count Agar and incubated for 24 h at 37 °C. After incubation the plates were observed for bac-

terial growth and visible colonies were counted and colony forming unit per ml or g were determined respectively.

Detection of Escherichia coli

Three loopfuls of 1 g/ml of food material suspended in 1 ml of sterile saline water were streaked on Eosin Methylene Blue (EMB) agar and incubated at 37 °C for 24 h. Dark colonies with green metallic sheen indicated the presence of E. coli. Isolates were confirmed biochemically using the IMViC test (Indole, Methyl Red, Voges-Proskauer and Citrate utilization).

Isolation of Salmonella enterica

The isolation of Salmonella was carried according to the Bacteriological Analytical Manual (BAM) [16] protocol for Salmonella. One ml of locally fermented milk, commercial chicken egg and 1 g roasted beef, pork and stool samples of were each transferred into 9 ml of lactose broth for pre-enrichment. The broth were incubated overnight at 37 °C. Aseptically, 0.1 ml of aliquots were inoculated into 10 ml of Rappaport Vassiliadis (RV) broth (Soy peptone 4.5g/L, NaCl 8g/L, K_2HPO_4 0.4g/L, KH_2PO_4 0.6g/L, $MgCl_2$.6H2O 40g/L, Malachite green 0.036g/L) and incubated at 42 °C for 24 h. One milliliter of aliquots from lactose broth culture was also inoculated into 10 ml of sterile Selenite F (SF) broth and incubated at 37 °C for 24 h. A loopful of the samples in both RV and SF broth was streaked unto Xylose Lysine Deoxycholate (XLD) Agar and incubated at 37 °C for 24 h. Pink colonies with or without black center were presumed to be Salmonella. The presumptive colonies were sub-cultured to obtain a pure culture and stored in a refrigerator for further analysis. The isolates were confirmed using biochemical test including; IMViC, triple sugar iron (TSI) and Urease test. The isolates were confirmed using PCR.

Diagnostic PCR for S. enterica

Crude bacterial DNA was obtained from pure cultures on Nutrient agar plate by picking a single colony and suspending in 50 µl of dH_2O before heating to 100 °C for 5 min. The samples were centrifuged at 13000 g for 5 min and 15 µl of the supernatant was aspirated as template DNA and stored at -20 °C till use. The presence of invA gene in Salmonella was detected using 10 µl PCR volume that comprised 1µl template DNA, 1x Taq reaction buffer (New England Biolabs), dNTPs (200 µM; New England Biolabs), Taq DNA polymerase (0.625 units; New England Biolabs) as well as forward and reverse primers (0.2 µM). The following primers were used: forward 5'GTGAAATTATCGCCACGTTCGGGCAA 3' and reverse 5' TCATCGCACCGTCAAAGGAACC 3' with an expected 284 bp invA PCR product [17]. Thermocycling was performed using the cycling conditions: The cycling conditions were as follows: (1) 95°C for 30 sec; (2) 95°C for 30 sec; (3) 55°C for 1 min; (4) 68°C for 1 min; and (5) 68°C for 5 min. Steps 2 to 4 were repeated for 30 cycles. PCR products were mixed with 6X gel loading dye (New England Biolabs). One percent (1 % TAE) (40 mM Tris-acetate, 1 mM EDTA) agarose (MELFORD) gels plus fluorescent nucleic acid stain GelRed™ (Biotium; 1:25,000) were cast. Samples were electrophoresed with size markers (log2 DNA ladder, New England Bio-labs), in TAE buffer at 100 volts and DNA was visualized using a UV transilluminator (Bio-Rad).

Antibiotic Susceptibility Tests

The antimicrobial susceptibility profile of Salmonella enterica isolates was determined using the Kirby-Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute [18]. The bacterial isolates were standardized to 0.5 McFarland and the inoculum was carefully seeded onto Mueller Hinton agar plate. Antibiotic discs with the specified concentrations of [18] protocol were placed on seeded plates. The plates were incubated for 24 h at 37°C. Following incubation, the diameters of the clear zones produced by antimicrobial inhibition of bacterial growth were measured to the nearest millimeter for each disc using a transparent straight-line ruler and then classified as resistant or susceptible according to the CLSI. Six antibiotics belonging to different classes were selected. They include ampicillin 10µg, ciprofloxacin 5µg, chloramphenicol 30µg,

tetracycline 30µg, trimethoprim-sulfamethoxazole 1.25/23.75µg and gentamicin 10µg. Multi-drug resistance organism described as those resistant to at least one antibiotic in 3 or more classes of antibiotics was determined by observing the antibiotic-resistant patterns of the isolates.

Results

Bacteriological quality and incidence of E. coli in foods

All 77 samples showed no presence of Escherichia coli. Total bacteria count ranged between 2.8×10^3 to 4.07×10^6 and was dependent on type of food.

Table 1: Average mesophilic bacterial count in foods

Samples	Cfu/ml
Commercial Egg	2.8×10^4
Grilled Pork	3.3×10^4
Grilled Beef	2.7×10^3
Local Fermented Milk	4.07×10^6

Morphological and Biochemical analysis and PCR

Morphological identification of salmonellae

Morphology showed 19 isolates to be presumptively Salmonella e.g. 19 isolates appeared pink on XLD agar with 16 having dark centers. All stained isolates were Gram-negative rod-shaped motile bacteria.

Biochemical identification of salmonellae

The isolates tested were positive for catalase, methyl red, citrate utilization test and oxidase. Conversely, isolates were negative to indole, and Voges-Proskauer. On TSI slant, they showed typical black pigmentation indicating production of H_2S. S. enterica Typhi gave little black pigment in TSI, while S. enterica Paratyphi gave no black pigment (Plate 1)

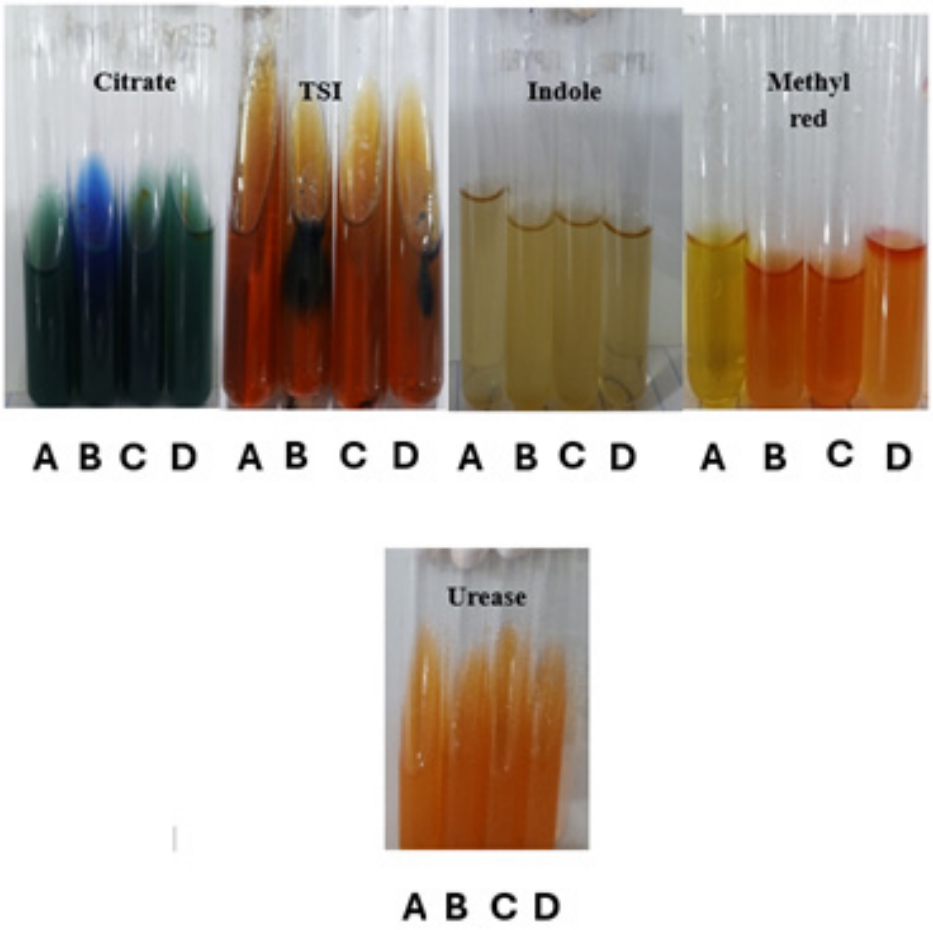


Plate 1: Biochemical analysis of Salmonella isolates. A=Control; B= S. enterica Enteritidis, C= S. enterica Paratyphi, D= S. enterica Typhi

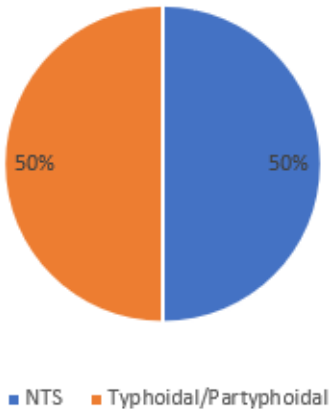
Distribution of Salmonella

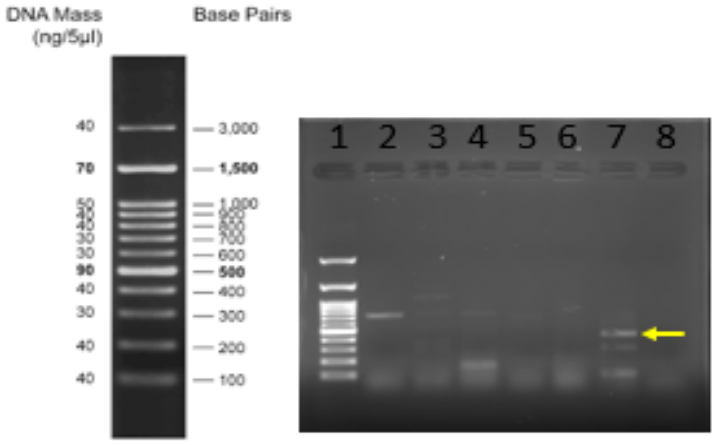
A total of 77 samples (22 commercial eggs, 20 locally fermented milk, 20 grilled beef, 15 grilled pork and 20 clinical samples) were screened for the presence of Salmonella sp. with an overall prevalence of 24.675%. The prevalence of Salmonella enterica in each sample is presented on Table 2. Fifty percent (50%) of isolates were Typhoid/paratyphoid while 50% were non-typhoidal (Figure 2).

Table 2: Prevalence of Salmonella

Samples	No. positive for *E. coli*	Number of *Salmonella* Isolates	Number of Samples	Prevalence (%)	Typhoid/paratyphoid	NTS
Stool	NA	11	20	55	9	2
Commercial egg	0	3	22	13	0	3
Grilled pork	0	4	15	26.7	0	4
Grilled beef	2	5	20	25	2	3
Locally fermented milk	1	1	20	5	1	0

Figure 2: Percentage NTS and Typhoid/Paratyphoid Salmonella recovered





Left to right, Lane 1: log2 DNA ladder; lane 2: *Shigella* sp; lane 3: *E. coli*; lane 4: *Proteus mirabilis*; lane 5: *Serratia marscence*; lane 6: *Klebsiella,* and lane 7: *S. enterica* Enteritidis. Yellow arrow indicates expected PCR product size.

PCR confirmation

Plate 2: PCR of Salmonella enterica isolates

Antibiotics Susceptibility Test

Antimicrobial resistance profile of the isolates indicated resistance to multiple antibiotics. The antibiotics resistance profile of Salmonellae is presented in Figure 3. A greater proportion (57.89%) of the isolates were multi-drug resistant (Figure 4).

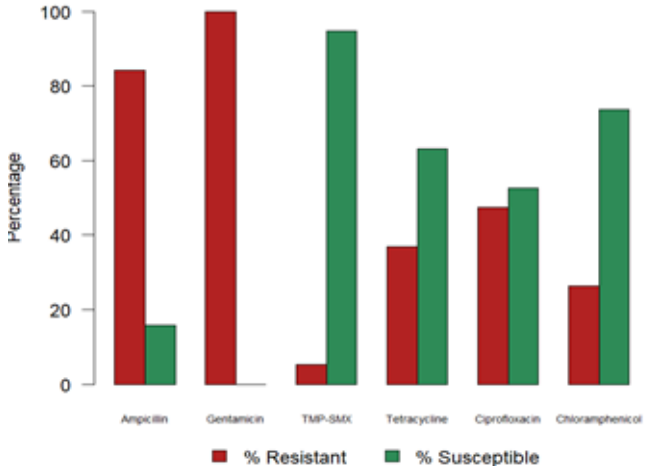


Figure 3: Antibiotic resistance of Salmonella sp.

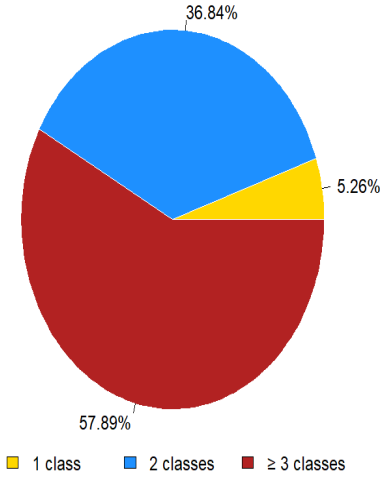


Figure 4: % MDR *Salmonella* Isolates

Discussion

The bacteriological qualities of ready to eat foods (RTE) are a source of concern in North central Nigeria [19,20]. This study further demonstrates the poor bacteriological quality of foods, most of which, undergo no additional processing prior to consumption.

The occurrence of Salmonella in food and clinical fecal samples was found to be 17% (12/72) and 55 % (11/20) respectively. Grilled pork, grilled beef, Egg and fermented milk also had detection rates of 26.7 % (4/15), 25% (5/20), 13% (3/22) and 0% (0/20) accordingly.

Studies on of Salmonella prevalence in grilled pork in Northcentral Nigeria is sparse, but in the South-south and South-east regions of Nigeria, grilled pork was associated with high bacterial counts and the presence of Salmonella enterica [21,22]. In pigs, horizontal and vertical transmission occurs and gastrointestinal tract (GIT) colonization with NTS is frequent and often results in asymptomatic chronic carriers that carry the bacteria in gut tissues, tonsils, and lymph nodes [23]. Therefore, the isolation and detection of NTS in this study is likely due to insufficient thermal processes employed.

For eggs, in Nigeria, studies in the Southeast region have consistently shown poor bacteriological quality with Salmonella prevalence of 10.3% [24]. Interestingly, the study of Okorie-Kanu et al. [24] detected E. coli in 54.4% of eggs examined with eggs from the farms having a higher prevalence than eggs from the retail outlets. By contrast, this study did not detect E. coli presumably because it focused on retailed eggs that had their shells cleaned prior to sale. In the present study, only the contents were analyzed and the differences in prevalence of Salmonella in eggs can be explained in the way it infects eggs. Salmonella infects eggs via vertical and horizontal routes. Vertical transmission also known as transovarian infection occurs during the formation of the egg and before egg contents are covered with a shell [25]. By contrast, horizontal transmission is by penetration through the egg shell [26]. Fecal contamination of the eggshell is significantly higher than the contamination of contents and there is a positive correlation between visible eggshell contamination and the Salmonella load in feces [26]hens were also taken for culture at the end of the laying period, and faecal and environmental samples were taken from the laying houses before and after cleaning and disinfection. Twenty-four batches of six egg shells from the 13 652 tested (0.18% [0.11 to 0.26 CI(95 . Other factors that may contribute to variable results include differences in poultry management, poultry personnel hygiene as well as storage conditions of the egg. Overall, our findings indicate eggs as potentially a major transmission link for salmonellosis, especially if undercooked or used in its raw form, for example in making ice creams.

In local fermented milks (‘nono’, ‘kindirmo’), the detection of Salmonella was zero. By contrast, other researchers have documented in its presence in street vended artisanal fermented milks. In Northcentral Nigeria, Oludairo et al. [27] reported 3.4% in Ilorin, Kwara state whereas Godwin et al. [28] reported 10% in the FCT. These differences probably reflect hygiene practices and access to potable water. Most of our samples originated from the University of Abuja campus. A key feature is the presence of potable pipe-borne water on campus and probably the exposure to the university community has had a positive influence on the artisanal producers and vendors. Although, Godwin et al. [28] reported 10% prevalence in the FCT, when the data is disaggregated by sampling location, all the 3 samples originating from University of Abuja campus (permanent site) were negative for Salmonella. Overall, these suggest that prevalence was impacted by personal hygiene and processing practices.

For stool samples, recent reports are sparce and it has been noted that inadequate published studies on stool culture and increasing multi-drug resistance has an impact on treating Salmonella infections in Nigeria [29]. Antibiotics resistance in Salmonella enterica has become a major healthcare concern with the growing resistance to commonly used antibiotics. Antibiotics resis-

tance profile of isolates recovered in this study revealed a 57.89 % multi-drug resistance. High percentage of MDR isolates may arise from inappropriate use of antibiotics as they are available over the counter, longtime use of antibiotics in animal production contributing to the misuse and abuse of antibiotics [30,31]. This could result in transfer of resistance genes and mutations [32]. By contrast, Okorie-Kanu et al. [24] reported a 100 % case of MDR. Resistance to ampicillin (84.21%), a beta-lactam antibiotics could be as a result of beta-lactamase production. This pattern is slightly lower than the 100% reported by Oludairo et al. [27]. These differences could be as a result of difference in the use of antibiotics in various locations. Resistance to different antibiotics could be linked to a number of resistance mechanisms in salmonellae including increased efflux pump activity, modification of drug target, reduced permeability and enzymatic degradation [33].

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