Research Article

iMedPub Journals
www.imedpub.com

Health Science Journal
ISSN 1791-809X

2021
Vol.15 No.9:4313

Isolation and Molecular Identification of Different Salmonella Serovars Distributed In Benue State, Nigeria

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Received date: May 26, 2020;**Accepted date:** August 19, 2021;**Published date:** August 28, 2021

Citation: (2021) Okpa Department of Microbiology, University of Agriculture, Makurdi. Health Sci J. Vol 15 No.8.

ABSTRACT

Isolation and molecular characterization of Salmonella serovars in Benue State was undertaken. Conventional cultural characterization and biochemical results showed that eighteen [18]positive samples of Salmonella species were obtained out of the four hundred and twenty [420] samples collected from Patients across the three [3] senatorial districts sampled. The organisms possessed characteristic pinkish colonies and black dots at the center on the XLD agar and whitish-green colonies on BGA. The API tests result showed the difference in the utilization of various substrates that distinguished between Salmonella species and other members of Enterobacteriaceae. Four [4] distinct serovars of S.enterica and one from S.bongori, totaling eight different groups of Salmonella were observed. S.enterica Typhimurium was the highest with 6 [33.33%] and prevalence of 1.43% followed by S.enterica Enteritidis with 4 [22.22%] and prevalence of 0.95%. Other serovars had <0.5% prevalence. S.entericaTyphi and S.enterica Heidelberg had 2 cases each while the remaining four [S.enterica Agona, S.enterica Paratyphi B, S.enterica Huaian and S.bongori] had a lone case each. Significant association was established between occurrence of Salmonella infection and causative serovars [$\chi 2$= 57.93, P<0.05]. Molecular characterization results obtained showed that the dendrogram formed 2 main clusters [numbered 36 and 81] with two divergent Salmonella strains. The first sub cluster of main cluster [numbered 57] has four strains isolated from different locations: S.entericaHeidelberg-MG663473.1 from Gboko; S.entericaTyphimurium-JQ228518.1 from Katsina-Ala; S.enterica Typhimurium-CP014981 from Makurdi and S.enterica Typhimurium-CP023166.1 from Kwande. Seventy five percent [75%] of strains in this group were Typhimurium serovars. In this group, S.entericaTyphimurium-CP023166.1 sourced from Kwande was a unique strain that showed wider genetic variability but closely related to the check strain [S.bongori strain KU060291.1]. The second sub cluster of a main [numbered 98] also consisted of 3 strains all of different serovars of S.enterica and one strain of another species. They are: S.entericaParatyphi B-JQ694526.1; S.enterica Heidelberg-CP019176.1; S.enterica Typhimurium-CP024619.1 and S.bongori-FR877557.1. The latter belongs to the same group with Paratyphi serovar and even closely related with Paratyphi B strain MF772492.1 used as a check. The second main cluster [numbered 81] comprised 8 strains belonging to 4 Enteritidis, 2 Typhi, 1 Huaian and 1 Typhimurium cutting across all locations except Gboko. Within this group, 4 strains showed wider relationships with other members and they were all of different serovars while Typhimurium strains were either closely clustered to themselves or to the Typhi strain. S.entericaTyphimurium-MH196335.1 is divergent from the source of the main clusters [88] bearing no close relationship with any existing check strain. The most unique of all the 18 strains identified was the S.enterica serovar Agona strain 392869-2 and is closely related with a known check Enteritidis strain.

KEYWORDS: Salmonella, Serovars, Paratyphi, Heidelberg

INTRODUCTION

The genus Salmonella consists of rod-shaped, Gram-negative, flagellated, facultative anaerobes that belong to the family Enterobacteriaceae that contains all important species of Salmonella [1]. S. enterica is an important food-borne pathogen that is currently divided into 2,587 serovars [2], while the typhoidal Salmonella serovars are host-restricted human pathogens and include S. Typhi and S. Paratyphi [3]. They are the main causes of enteric or typhoid fever that poses health threat in developing countries. The disease has been described as endemic in tropical and sub-tropical countries, with estimated annual incidences of 540 per 100,000 [4,5]. The world wide incidence was estimated to reach up to 17million cases [6] and about 600,000 deaths per annum [7].

Salmonellosisis a symptomatic bacterial infection caused by Salmonella species: S. bongori and S. enterica with many subspecies and serotypes. Infections occur when contaminated food or drink are taken in. A number of pets such as cats, dogs and reptiles can also carry or spread the infecton (8). S. enterica is an enteric bacterium causing typhoidal salmonellosis (Typhi and Paratyphi serotypes) and non typhoidal salmonellosis (Typhimurium and Enteritidis) [9]. Salmonellosis is one of the most common causes of diarrhea globally with high morbidy rate. Salmonella is carried by both humans and animals. Most serotypes of Salmonella cause non-typhoidal salmonellosis (9). Typhoidal type which causes typhoid fever is caused by S.Typhi and it is carried only by humans. WHO [6]expressed the need for more epidemiological data to estimate the incidence of Salmonella in Africa. Some workers estimated 123 persons per 100,000 in West Africa; 195 persons per 100,000 in South Africa and 465 persons per 100,000 in East Africa [10]. Incidence of 725 persons per 100,000 has also been reported in the sub-Saharan Africa [9]. Most human infections are caused by a

limited number of serovars, which may vary from country to country and over time.Very limited information is available on Salmonella isolation for African continent [11], particularly in Nigeria, due to drawbacks such as the limited number of samples, the lack of representativeness of the samples selected, lack of access to serotyping facilities or the restricted geographical coverage [11] The predominant serovars in some African countries however include S. Enteritidis, S. Typhimurium, S. Concordand S. Isangi [12,1, 13].

A study of Non-Typhoidal Salmonella in chicken farms in the six geopolitical zones of Nigeria reported the presence of S. Kentucky, S. Poona, S. Elizabethville, S. Iarochelle, S. Agama and S. Graz, with S. Kentucky as the most prevalent [11]. Several other studies in chicken farms reported the presence of Non-Typhoidal Salmonella in various parts of Nigeria including S. Kentucky and S. Hadarin Jos [14], S. Paratyphi A, S. Gallinarum and S. Typhinurium in Awka [15] and S. Hiduddify in Maiduguri, North East Nigeria [11].

In humans, Non-Typhoidal Salmonella including S. Apapa, S. Dublin, S.Enteritidis, S. Infantis, S. Jukestown, S. Monaschaui, S. Oritamerin and S. Typhimurium were reported in Ibadan with S. Enteritidis and S. Typhimurium as the most predominant [16]. In Lagos, Akinyemi et al. [17]reported only S. Enteritidis from stools of children under five years while in Abuja, Nigeria, three Salmonella serovars: S. Zanziba, S. Brancaster and S. Enteritidis were recovered from children with acute gastroenteritis. Similarly, some studies of Typhoial Salmonella have reported various prevalence rates including 45.0% of S.Typhi among other bacterial isolates in Zaria, Kaduna State [18], 27% for S.Paratyphi A, 25% for S.Paratyphi B, 13.7% for S.Paratyphi C and 20% for S. Typhi respectively in Biu, Borno state [19], while in Benue State, Umeh and Agbulu, [20]reported a prevalence rate of 57.6% for S.Typhi, 26.3% for S. Paratyphi and 15% for the mixture of both serovars in Okpokwu Local Government Area.

Non-typhoidal Salmonella serotypes such as Typhimurium and Enteritidis are generalist pathogen with a broad host specificity (pets, livestock and human) and global presence [9]. S. enterica serotypes such as Typhi and Paratyphi A, B and C are specialists as they are adapted to the human host as their exclusive reservoir [21, 9]. They are referred to as typhoidal Salmonella serotypes. They are the causative agents of typhoid fever (caused by serotype Typhi) or paratyphoid fever (caused by serotype Paratyphi). Enteric fever is an invasive, life threatening systemic disease with very high mortality rate [10, 21]. It is endemic in the developing world in regions that lack clean water and adequate sanitation thus facilitating the spread of the pathogens via faecal-oral route Njunda and Oyerinde, [21]. The average incubation period for typhoidal serotypes is 14 days with symptoms persisting for up to 3 weeks. Clinical manifestation of enteric fever include: sustained fever, chills, abdominal pain, hepatosplenomegaly, rash, nausea, anorexia, diarrhea, constipation, headache, bacteremia and related symptoms of salmonellosis [10].

S. enterica serotypes initially adhere to and invade the intestinal epithelium of the small intestine. After passing the intestinal mucosa, the bacteria gain access to underlying lymphoid tissues and multiply intracellularly within mononuclear phagocytes. Infection quickly becomes systemic with spreading of the pathogen from the intestine to the mesenteric lymph nodes, liver, spleen, bone marrow and gall bladder. Secondary infection to the small bowel occurs via secretion in the bile through enterohepatic cycle [9]. The high invasiveness of the typhoidal serotypes to the deeper tissues and their dissemination to the systemic sites are responsible for the higher morbidity rate than NTS serotypes. It was reported that 90,300 and 178,000 deaths occurred globally in 2015 from non typhoidal and typhoidal salmonellosis respectively [22]. Long term carriage of inactive, asymptomatic chronic cases is more predominant in the developing countries [10]. In most cases, the bacteria are gradually excreted in faeces. The long term persistence of typhoidal serotypes suggests an ability of these pathogens to invade the human immune system [9]. The most common symptoms are diarrhea, fever, abdominal cramps and vomiting. This occurs between 12-36 hours after exposure with symptoms lasting from 2-7 days. Severe cases may result in dehydration. The old, young and people with weak immune system are more susceptible to Salmonella infections [9].

Typhoid cases are stable with low numbers in developed countries, but non typhoidal salmonellosis has increased worldwide. Typhoid fever usually causes mortality in 5 to 30% of typhoid-infected individuals in the developing world. The World Health Organization (WHO) estimates 16 to 17 million cases occur annually, resulting in about 600,000 deaths. The mortality rates differ from region to region, but can be as high as 5 to 7% despite the use of appropriate antibiotic treatment [23]. In Nigeria, typhoid fever is among the major widespread diseases affecting both young children and young adults as a result of many interrelated factors such as inadequate facilities for processing human wastes and indiscriminate use of antibiotics. Morbidity associated with illness due to Salmonella continues to be on the increase, in some cases resulting in death [24, 25].

In 2011, Nigerian hen egg production totaled 636,000 metric tonnes ranking 19th in world hen egg production and the top producer in Africa [26]. Both large and small egg farms are scattered all over the country, although they are generally concentrated around the major urban centres [27]. Poultry meat and eggs are the major sources of animal protein in Nigeria, as in many developing countries, because of their affordability and acceptability [28]. This source is, however, being threatened by diseases such as salmonellosis and avian influenza [26]. Salmonellosis is a bacterial disease affecting both humans and animals worldwide and Nigeria is not an exception. Although most of the infections in humans cause mild gastroenteritis, life-threatening systemic infections are common especially among high risk categories [11]. Invasive nontyphoidal Salmonella commonly cause infection among infants, children, elderly and immunocompromised individuals worldwide and especially in African countries, where these diseases are driven in part by co-infection with malaria or human immunodeficiency virus (HIV) [2]. Sources and modes of transmission of nontyphoidal Salmonella are still poorly understood in Africa due to the lack of coordinated national epidemiological surveillance systems. In food-producing animals and especially in poultry, Salmonella is one of the leading causes of infection, and this has a direct impact on the global marketing of the respective food-producing

animals and animal-derived food products. Poultry salmonellosis related to host adapted serovars remains a major constraint on poultry production in all parts of Nigeria [11]. Farmers still experience great losses (due to mortality, morbidity and drop in egg production) caused by host adapted Salmonella serovars despite huge amounts spent on vaccination and medication. In early life, S. Pullorum causes extremely high mortality of both broilers and commercial laying birds. Similarly, older birds succumb heavily to other serovars of Salmonella and it is assumed that Salmonella infections of this category of birds are mainly due to S. Gallinarum [30]. In addition to these host adapted serovars causing systemic disease, poultry harbor in their gastrointestinal tracts zoonotic serovars with no apparent signs of illness.

Hence, these Salmonella serovars can be present in feaces excreted by healthy animals and may be transferred to raw foods of animal origin through contamination during slaughtering and processing [30]. Generally, Salmonella in food producing animals, including poultry, manifests as long periods of latent carriage with occasional faecal shedding, which is the leading source of contamination of feed, water and environment. Relatively few African countries report surveillance data on Salmonella and as such, very limited information is available on Salmonella isolation for this continent [31]. This is also the case in Nigeria, where the few studies conducted so far show different drawbacks, such as the limited number of samples considered, the lack of representativeness of the samples selected, lack of access to serotyping facilities, or the restricted geographical coverage. Raufu et al. [11], who collected samples from three poultry slaughterhouses and five intensively managed poultry farms in a circumscribed area of Nigeria, reported a Salmonella prevalence ranging from 2 to 16%. Moreover, a study conducted in three commercial hatcheries in the Jos area reported a prevalence of 9%, with S. Kentucky and S. Hadar as the most frequent serovars [32]. Finally, a study involving five farms in Awka reported a Salmonella prevalence of 38.3% in poultry droppings with S. Paratyphi A, S. Gallinarum and S. Typhimurium as the most common serovars [33].

In Nigeria, as for the rest of Africa, it is very difficult to have a clear picture about the real situation of human salmonellosis due to the non-availability of facilities to provide essential tests for the diagnosis of Salmonella infections. However, the limited amount of study concerning non-typhoidal Salmonella serovars responsible for human infections in Africa reported S. Enteritidis and S. Typhimurium as the most prevalent serovars [32]. Moreover, a recent study monitoring Salmonella from different sources, including humans, in the northeastern regions of Nigeria reported S. Eko, S. Enteritidis and S. Hadar as the most common serovars from humans [30].

Study Area

The study area is Benue State, North Central Nigeria. Benue state lies in the lower river Benue trough in the middle belt region of Nigeria (now North Central) and has 23 Local Government Areas (LGAs). It is inhabited predominantly by the Tiv, Idoma and Igede who speak Tiv, Idoma and Igede respectively. It is divided into three Senatorial zones, Benue North-East(A), Benue north-West (B) and Benue South (C). Its geographic coordinates are Longitude 7 47'' and 10 0'' East, Latitide 6 25" and 8 8" North, and it shares boundaries with five other states namely: Nasarawa state to the North, Taraba state to the East, Cross river state to the south, Ebonyi and Enugu states to the South-West and Kogi state to the West. The state also shares a common boundary with Cameroon on the South-East. Benue occupies a landmass of 34,059 square kilometers and has a population of about 4,253,641 in 2006 census with an annual increament of 85,073 persons based on 2% rise in population. The current population is expected to be 5,444,660.

Benue state has Guinea Savannah vegetation with flat to undulating topography and few hilly areas. It experiences two distinct seasons, the Wet and the dry seasons. The rainy season lasts from April to October with annual rainfall in the range of 100-200mm. The dry season begins in November and ends in March. Temperatures fluctuate between 21-37 degrees Celsius in the year. River Benue is the dominant geographical feature in the state. It separates Makurdi, the capital city into two segments, the North and South Banks.

Sample Collection and Sampling Distribution

Exactly 420 stool samples were collected aseptically as described by Cheersbrough [2006]from patients attending secondary health facilities in 6 Local Government Areas (LGAS), two [2] from each of the three Senatorial districts [A, B and C]. Table 1 gives the sampling distribution based on locations and sex of patients. Seventy [70] stool samples were collected from each of the six selected health facilities, one per each LGA. Exactly 226 samples were collected from male patients while 194 samples were collected female patients. Five gram [5g]of early morning stool sample was collected from each patient in a sterile plastic bottle and analyzed according to the method of Cheesbrough[34]. The samples were properly labeled and placed in ice box and transported to the Department of Microbiology, Federal University of Agriculture, Makurdi, laboratory for examination as described by Winn et al.[35].

Isolation and Identification of Salmonella from Faecal Samples

Isolation and identification of Salmonella was carried out according to the World Health Organization [WHO] global food borne infections network laboratory protocol, [2010]. Exactly 5grams of each specimen was suspended in 45ml of buffered peptone water, a pre-enrichment broth(Tm media, India) and incubated at 37 C for 24 hours. A100 ul of the suspension was transferred to 10 ml of Rappaport-vassiliadis enrichment broth [Tm media, India] and incubated at 37 C for 24 hours. A 1 ml of the suspension was also transferred to 10 ml of Selenite F broth, [Tm media, India] and incubsted at 42 C for 24 hours. A loop full of each of the enrichment broth was streaked into Xylose Lysine Deoxycholate [XLD] agar and Brilliant Green Agar[BGA] and incubated at 37 C for 24 hours. Cultural Identification of Salmonella was done using morphology, such as: appearance, color, size and shape. Bacterial morphology such as red-to pink

colonies with black centre on XLD and red to pink-white colonies on BGA respectively were suggestive of Salmonella species [36].

Biochemical Characterization of Isolated Salmonella Species

To further confirm the presumptive Salmonella isolates, the colonies were sub-cultured on Nutrient Agar (NA) to obtain pure and distinct colonies. The following conventional biochemical tests were carried out in the indentification of Salmonella species: Gram staining, Triple sugar iron (TSI), Citrate, Indole, Oxidase, Catalase, Urease, Methyl red and Voges Proskauer tests following different standard protocols. Motility test was carried out as described by Winn et al. [2005]. API20Ebiochemicalteststrip as a non conventional biochemical test was used to further detect and confirm Salmonella isolates.

Molecular Characterization and Identification of Samonella Serovars and Strains

Extraction of genomic DNA

The bacteria isolates confirmed by biochemical tests were sub-cultured on Luria-Bertani (LB) (Oxford UK) and incubated at 37 overnight from which broth cultures were prepared. The DNA was extracted from the bacterial cultures on broth using boiling method [37]. A 1.5ml of the sample broth was centrifuged at 10,000rpm for 5 minutes. The supernatant wasdecanted and the pellets washed twice with sterile deionized water. A 200 of sterile deionized water was added to the pellets, and vortexed to homogenize and boiled in a water bath at 100 for 10 minutes. This was followed by vortexing and centrifugation at 12,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to another test tube and stored at -20. The quantification and purity of the DNA so extracted was estimated using a NanoDrop spectrophotometer. The quantified DNA extract supernatant was prepared and used for PCR [37].

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was employed for rapid and definite identification of Salmonella serovars using Salmonella specific primers as described by Anejo-Okopi et al.[37]. Amplification was carried out in 50 reaction volumes containing; 25 Dream Taq Master Mix (Thermoscientific, USA), 15 of nuclease free water, 2.5 of each primer and 2.5 of extracted bacterial DNA. The Amplification was done in 35 cycles with an initial denaturation at 95for 5 min. followed by a denaturation step of 95 for 2 min, primer annealing at 55 and primer extension at 72 with the final extension at 72.The amplified DNA products from Salmonella specific-PCR was further analysed. Five microliter aliquots of reaction mixture were electrophoresed through 1.5 % agarose gels stained with ethidium bromide and visualized using ultraviolet illumination.

Sequencing

All PCR products were purified with Exo sap and sent to Epoch Life science (USA) for Sanger sequencing [38]. The corresponding sequences were identified using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi

Sequence Profiling and Identification of Salmonella Strains

The 16s rRNA sequences obtained were compared with known 16s rRNA sequence at National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLASTn) algorithm. Identification of the sequences at both genus; species and serovar level was defined as a 16s rRNA sequence similarity at between 95- 100% with that of the phenotype strain sequence in GenBank. The sequences together with reference sequences derived from the GeneBank were aligned using CLAUSTAL IV [38].

RESULTS

Cultural characterization gave 18 positive samples of Salmonella species out of the sample population of 420 patients. Table 1 presents the sample codes and locations of the positive samples. The organisms possessed characteristic pinkish colonies and black dots at the center on the XLD agar and whitish-green colonies on BGA.

Table 1: Sample Code and Locations of Salmonella Positive Samples in Cultural Characterization.

S/N	Sample Code	Location	Result
1	GBK 033	Gboko	Positive
2	GBK 034	Gboko	Positive
3	GBK 037	Gboko	Positive
4	GBK 039	Gboko	Positive
5	MKD 014	Makurdi	Positive
6	MKD 015	Makurdi	Positive
7	OJU 002	Oju	Positive
8	OJU 015	Oju	Positive
9	OJU 017	Oju	Positive

10	KWD 026	Kwande	Positive
11	KWD 023	Kwande	Positive
12	KWD 038	Kwande	Positive
13	KA 021	Katsina-Ala	Positive
14	KA 034	Katsina-Ala	Positive
15	KA 038	Katsina-Ala	Positive
16	KA 045	Katsina-Ala	Positive
17	OTK 005	Otukpo	Positive
18	OTK 014	Otukpo	Positive

Table 2 shows the results of biochemical tests using conventional methods and API 20E strips respectively. The API tests showed the difference in the utilization of various substrates that distinguished between Salmonella species and other members of Enterobacteriaceae. Results showed positive and negative reactions to different substrates utilization for Salmonella and control test organism (Citrobacter). The outcome of biochemical characterization confirmed the 18 positive cases from cultural method as Salmonella species.

Table 2: Biochemical Tests and Identification of Salmonella species using Conventional and API Test Kit.

Conventional Tests	**Gram staining**	**Triple sugar iron**	**Oxidase**	**Catalase**	**Citrate**	**Methyl red**	**Urease**		**Indole**	**Voges Proskauer**										**Identification**
1-18	-	+	-	+	+	+	-		-	+										**Salmonella spp**
API Biochemical Tests	O-nitrophenyl-D-galactopyranoside	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Citrate	Hydrogen sulphide	Urease	Tryptophan deaminase	Indole	Voges Proskauer	Gelatinase	Glucose	Mannose	Inositol	Sorbitol	Rhamnose	Sucrose	Melibiose	Amygdalin	Arabinose	**Identification**
1-18	-	+	+	+	+	+	-	-	-	+	-	+	+	-	+	+	-	+	-	+	**Salmonella spp**
19	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	**Citrobacter spp**

Samples number 1-18 of Salmonella isolates

Samples number 19 of Citrobacter

Table 3 gives the prevalence of Salmonella infection in Benue State and across the six Local Government Areas (LGAs) as sampled through each of its General Hospitals. Eighteen (18) positive samples out of 420 population size were found with

total prevalence of 4.29% in Benue State. At the LGA level, Gboko and Katsina-Ala LGAs recorded the highest number of positive cases out of 70 sub samples. Here, there were four (4) cases each representing 22.2% of the positive cases and prevalence of 5.71%. Oju and Kwande LGAs had 3 (16.7%) cases each and prevalence of 4.29%. Makurdi and Otukpo recorded the least number of cases (2 cases each representing 11.1% of the positive cases) and prevalence of 2.89%. In the study area, prevalence of Salmonella infection did not depend on locations of sampling since no significant association was established between the two variables ($\chi 2 = 1.89$, $P > 0.05$).

Table 3: Prevalence of Salmonella Infections in Different Locations of Benue State

General Hospital	Sample size	No and Percentage of positive sample	Prevalence (%)
Gboko	70	4 (22.2%)	5.71
Makurdi	70	2 (11.1%)	2.86
Oju	70	3(16.7%)	4.29
Adikpo	70	3(16.7%)	4.29
Katsina-Ala	70	4(22.2%)	5.71
Otukpo	70	2 (11.1%)	2.86
Total	420	18	4.29

$\chi 2$ (5)(location and prevalence) = 1.89, P= 0.863(P>0.05)

As given in table 4, occurrence of Salmonella infection was caused by two species: S.enterica and S.bongori. The latter had 5.6% occurrence with prevalence of 0.24% while the former was the dominant type with 94.4% occurrence and prevalence of 4.05%. Hence, occurrence of Salmonella infection was significantly tied to species type ($\chi 2 = 78.85$, $P < 0.05$)

Table 4: Prevalence of Salmonella Infections based on Species Type in Benue State.

Salmonella species	Frequency and Percentage Occurrence	Prevalence (%)
S.enterica	17 (94.4%)	4.05
S.bongori	1 (5.6%)	0.24
Total	18	4.29

There were 4 distinct serovars of S.enterica and one from S.bongori, totaling eight different groups of Salmonella pathogens (table 5). S.entericaTyphimurium was the highest with 6 cases (33.33%) and prevalence of 1.43% followed by S.enterica Enteritidis with 4 cases (22.22%) and prevalence of 0.95%. Other serovars had <0.5% prevalence. S.entericaTyphi and S.enterica Heidelberg had 2 cases each while the remaining four (S.enterica Agona, S.enterica Paratyphi B, S.enterica Huaian and S.bongori) had a lone case each. Significant association was established between occurrence of Salmonella infection and causative serovars ($\chi 2 = 57.93$, $P < 0.05$).

Table 5: Prevalence of Salmonella Infections

Salmonella serovars	Frequency and Percentage Occurrence	Prevalence %
S.enterica Agona	1 (5.56%)	0.24
S.enterica Paratyphi B	1 (5.56%)	0.24
S.enterica Heidelberg	2 (11.1%)	0.48
S.enterica Typhi	2 (11.1%)	0.48
S.enterica Typhimurium	6 (33.33%)	1.43
S.enterica Enteritidis	4 (22.22%)	0.95

S.enterica Huaian	1 (5.56%)	0.24
S.bongori	1 (5.56%)	0.24
Total	18 (100%)	4.29

Based on serovars relationships among 18 Salmonella strains identified using plasmid gene sequencing data. Dendrogram formed 2 main clusters (numbered 36 and 81) and 2 divergent strains. The first sub cluster of main cluster (numbered 57) comprised four strains isolated from different locations: S.entericaHeidelberg-MG663473.1 sourced from Gboko, S.entericaTyphimurium-JQ228518.1 sourced from Katsina-Ala, S.enterica Typhimurium-CP014981.1 sourced from Makurdi and S.enterica Typhimurium-CP023166.1 sourced from Kwande. Male and female patients were equally represented. All age groups were equally represented (18, 35, 45 and 50 years old patients respectively). However, 75% of strains in this group was of the Typhimurium serovars. In this group, S.entericaTyphimurium-CP023166.1 sourced from Kwande was a unique strain that showed wider genetic variability but closely related to the check strain (S.bongori strain KU060291.1). The second sub cluster of a main (numbered 98) also consisted of 3 strains all of different serovars of S.enterica and one strain of another species. They are: S.entericaParatyphi B-JQ694526.1; S.enterica Heidelberg-CP019176.1; S.enterica Typhimurium-CP024619.1 and S.bongori-FR877557.1. The latter belongs to the same group with Paratyphi serovar and even closely related with Paratyphi B strain MF772492.1 used as a check.

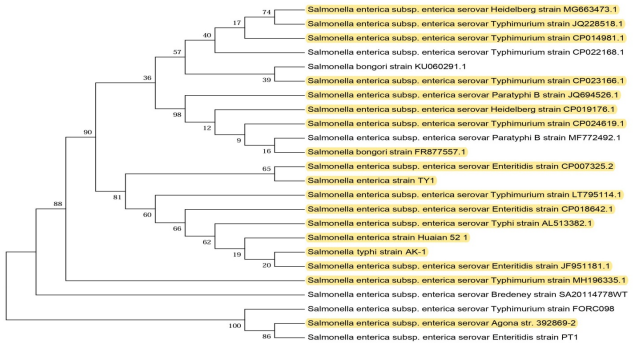

Figure 1: Phylogenetic Construction of Salmonella strains from Plasmid Gene Sequencing.

The second main cluster (numbered 81) comprised 8 strains belonging to 4 Enteritidis, 2 Typhi, 1 Huaian and 1 Typhimurium cutting across all locations except Gboko. Within this group, 4 strains showed wider relationships with other members and they were all of different serovars while Typhimurium strains were either closely clustered to themselves or to the Typhi strain regardless of the sex status of the infected patients. From the dendrogram, S.entericaTyphimurium-MH196335.1 was a divergent strain identified It diverged from the source of the main clusters (88) bearing no close relationship with any existing check strain. It was isolated from a 20 year old female patient in Adikpo General Hospital. The most unique of all the 18 strains identified was the S. enterica serovar Agona strain 392869-2 isolated from a 50 year old male patient in Gboko General Hospital. It bore a close relationship with a known check Enteritidis strain. The bands made up of 23,130 base pair (Figure 2).

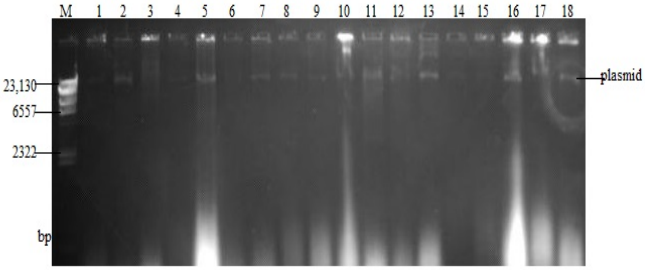

Figure 2: Amplified gel image of Plasmids from Salmonella strains (23,130bp)

- 1=S.entericaAgona-392869-2
- 2=S.entericaParatyphi B-JQ694526.1
- 3=S.entericaHeidelberg-MG663473.1
- 4=S.entericaHeidelberg-CP019176.1
- 5=S.entericaTyphi-AK-1
- 6=S.entericaTyphimurium-CP014981.1
- 7=S.entericaEnteritidis-CP007325.2
- 8=S.entericaTyphi-AL513382.1
- 9=S.entericaTyphimurium-CP024619.1
- 10=S.entericaTyphimurium-MH196335.1
- 11=S.entericaTyphimurium-CP023166.1
- 12=S.entericaEnteritidis-JF951181.1
- 13=S.entericaHuaian-H52.1
- 14=S.bongori-FR877557.1
- 15=S.entericaTyphimurium-LT795114.1
- 16= S.entericaTyphimurium-JQ228518.1
- 17=S.entericaEnteritidis-TY1
- 18= S.entericaEnteritidis-CP018642.1

DISCUSSION

Salmonella species, isolated from patients in selected health facilities of Benue State, have been successfully characterized using both conventional and molecular approaches. Prevalence and serovar distribution in relation to demographic parameters have been determined. Coventionally, both cultural and biochemical methods of characterization yielded accurate preliminary identification at the generic level within the limit of their diagnostic resolutions. For instance, all the 18 Salmonella positive cases were congruent in both cultural and biochemical methods. They were also confirmed true in the molecular analysis. However, the molecular approach was more specific as sequencing data led to precise Salmonella identification at the

sub species level including serovars and strains consummating in the determination of phylogenetic relationship among them. Similar view was previously upheld among epidemiologists (39). Information obtained from plasmid profiling has given insight into the antibiotic susceptibility or resistance pattern of the serovars and the nature of the genes carried on such plasmids.

Based on the result, thetotal prevalence of Salmonella cases in Benue State was 4.29%. This is lower than the 5% permissible limits of the WHO (40). Previous studies on Salmonella had higher seroprevalence rates than what was found in this study. For instance, 42.4% Salmonella incidence was reported among University of Ilorin Students (7). Also, 40% prevalence was reported in Biu Bornu State (41). Adeshina et al. (18) found 9.3% prevalence among Federal College of Education students and 16.5% prevalence among Ahmadu Bello University students in Zaria.

Salmonella infection is highly prevalent in tropical regions and in populations that lack access to safe water and adequate sanitation (42, 22). These are the prevailing factors in the six studied locations. Thus, it is expected that prevalence rate is higher than the present finding possibly because the subjects were hospital patients attending the selected health care facilities. Other infected persons in each locality who did not attend hospitals within the time frame of this study might not have been captured. It is possible that those uncaptured infected persons decided to use alternative methods of treatments such as herbal therapy. This view aligns with the findings of Kosek et al. (43) where 70% of infected persons in rural communities preferred herbal method treating typhoid fever as a more potent remedy than the orthodox medicine. Thus, studies that focus on achieving total seroprevalence rate must capture not only hospital patients but also populations of those not attending hospitals in the rural areas or students' populations.

In spite of this, Gboko and Katsina-Ala recorded the highest number of positive cases and prevalence of 5.71% higher than the WHO permissible limits. However, all the six LGAs have equal chances of Salmonella infection unlike in most studies in Nigeria where the infection is location dependent (42, 44). Thus, predisposing factors highlighted are equally present in the six locations of the study area. For instance, all locations are facing the challenges of unsafe drinkable water, poor food handling among farmers or vendors and poor sanitary conditions. Poultry and other poultry products thought to be the foremost means of transmission of Salmonella (45, 46) are commonly seen. All locations have similar market structures where fruits and vegetables are sold in the open.

Two Salmonella species (S.enterica and S.bongori) are present in all samples but the dominant one is S.enterica accounting for 94% of cases. This result is consistent with previous findings globally (47, 48). According to Brenner et al. (4), S. enterica subsp. enterica is the subspecies of most concern because the strains within these serogroups areknown to cause 99 % of Salmonella infections in humans.

Accordimg to Raufu et al. (11), most enteric infections in humans are caused by more than one serovars of a given species, which may vary from country to country and over time. In the present work, seven distinct serovars of S. enterica together with a lone case of S, bongori have emerged. It suggests a huge diversity in the genomics and physiological adaptation of the microbe in the host. This has a huge implication in disease treatment and control because wider genetic make-up may widen the chances of multi drug resistance (MDR) and ability to develop different mechanisms for virulence. This view aligns with previous reports on salmonellosis as a bacterial infection caused by more than one Salmonella species with many subspecies and serotypes (8, 11). S. enterica, for example, is a pathogen that is currently divided into 2,587 serovars (2).

It is also likely that every serovar is pathologically distinct since there are evidences on the capability of every serovar to acclimate itself to the environment inside its host in a unique way (50). These adaptations are ascribed to copious virulence factors and other microbial physiognomies of a precise serovar which makes it survive in the host. This may account for why Salmonella originates a wide array of human diseases which include enteric fever, gastroenteritis and bacteremia. Similar observation was made in the work of Monteville and Mathew (51). It should be borne in mind that Salmonella may cause further intestinal infection like meningitis, osteomyelitis, pneumonia, colecystitis, peritonitis, pericarditis, vasculitis, pyelonephritis, endocarditis and chronic conditions like aseptic arthritis and Reiter's syndrome (52).

Apart from the epidemiological implication of diverse serotypes, the cost implications in the identity of broad spectrum of all the serovars affecting a given population is huge. This can only be consummated at the molecular level as consummated in this work thus changing the routine conventional method of diagnosis in public health, thus making treatment difficult. Serovar identification is an integral part of disease control because it determines the correct choice of antibiotics (6, 53). In this work, Salmonella infection depends on the type of serovar and it should be handled differently as such. Here, the predominant serovars are S. Typhimuriumand S. Enteritidis accounting for approximately 33% and 22% of the serovars respectively. S. Typhiand S. Heidelberg accounted for 11% each while S. Agona, S. Paratyphi B, S. Huaian and S.bongori accounted for approximately 6% each.

Though there might be slight variation in the composition of serovar types when compared with many studies, there is a unifying point of agreement, all reporting the predominance of S. Typhimurium and/or S. Enteritidis. From previous studies, the commonly reported serovars in some African countries include S. Enteritidis, S. Typhimurium, S. Concord and S. Isangi (54,55.). In the work of Fashae et al. (16) carried out in Ibadan South West Nigeria, S. Typhimurium and S. Enteritidis were the most predominant serovars among others such as S. Apapa, S. Dublin, S. Infantis, S. Jukestown, S. Monaschaui and S. Oritamerin. In Lagos, Akinyemi et al. (17) reported only S. Enteritidis from stools of children under five years. In Abuja, North Central Nigeria, three serovars: S. Zanziba, S. Brancaster and S. Enteritidis were recovered from children with acute gastroenteritis but the latter was the most dominant (56). Other

studies have also claimed that the major pathogenic serovars of Salmonella enterica that infect humans from a variety of different food products include the Enteritidis and Typhimurium serovars (57, 58 and 59).

Nevertheless, prevalence rate of each serovar is less than 5%. This result deviate from previous findings in Nigeria reporting higher prevalence rates based on serovar types. Some workers reported 45.0% prevalence of S.Typhi among other bacterial isolates in Northern parts of Nigeria (11). Also, Anejo-Okpobi et al. (37) published 27% for S. Paratyphi A, 25 % for S. Paratyphi B, 13.7% for S. Paratyphi C and 20% for S. Typhi among human subjects in Jos Plateau State, Nigeria. Umeh and Agbulu (20) reported 57.6% for S.Typhi, 26.3% for S. Paratyphi and 15% for the mixture of both serovars in Okpokwu Local Government Area of Benue State.

The total prevalence of Salmonella cases in Benue State was 18 (4.29%). Gboko and Katsina-Ala recorded the highest prevalence of 5.71% but all the six LGAs have equal chances of Salmonella infection. Two Salmonella species (S. enterica and S. bongori) and eight genetically diverse serovars were present in all samples but the dominant species one was S.enterica accounting for 94% of cases while the dominant serovars were S. Typhimurium and S. Enteritidis accounting for approximately 33% and 22% respectively. The use of Gene sequencing should be encouraged on all Salmonella isolates in future studies for precise strain identification.

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