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Phenotypic and molecular characterization of *Salmonella* serotypes in cow raw milk and milk products in Nigeria

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The presence of *Salmonella* and human pathogens in unpasteurized milk remains a public health hazard. The study reported the phenotypic and molecular characterization of Salmonella serotypes in cow raw milk, cheese and traditional yoghurt marketed for man's consumption in Nigeria. Isolation of *Salmonella* was done on Salmonella and Shigella agar by pour plate method at 37°C. Susceptibility to antibiotics was determined by the disc diffusion method. Molecular characterization of the *Salmonella* serotypes and detection of some target genes was done by the polymerase chain reaction. All the isolates were sensitive to ofloxacin and nalidixic acid. Resistance to antibiotics was in varying proportions with amoxicillin (95.2%), tetracycline (90.4%) and augumentin (28.5%). Ten (10) different multiple antibiotic resistance patterns were observed among the serotypes. The serotypes identified include *Salmonella typhi*, *Salmonella typhimurium* and *Salmonella enteritidis*, and the serogroups include D (28.6%), B (52.4%), D_1 (28.6%), respectively. The *invA* genes were amplified in all the *Salmonella* serotypes while *fliC* and *fliB* genes were absent. The *rfbJ* gene was detected only in *S. Typhimurium.* The recovery of multiple antibiotic resistant pathogenic *Salmonella* serotypes in cheese and yoghurt samples in the study is of great health concern.

Key words: *Salmonella* serotype, molecular characterization, virulence genes, antibiotics.

INTRODUCTION

The presence of *Salmonella* and other microorganisms in milk underscore its importance as a vehicle of human infection. For this reason, the presence of *Salmonella* and human pathogens in unpasteurized milk remains a

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Abbreviations: NAL, Nalidixic acid**; COT,** cotrimoxazole; **AMX,** amoxicillin; **NIT,** nitrofurantoin; **GEN,** gentamicin; **AUG,** augmentin; **TET,** tetracycline; **MAR,** multiple antibiotic resistance; ***InvA,*** invasion gene; ***RfbJ, FliC, FljB,*** adherence genes.

public health hazard. Milk-borne illnesses have been recognized since the beginning of the dairy industry.

Many diseases are transmissible via milk products; raw or unpasteurized milk has been a major vehicle for transmission of pathogens (Vasavada, 1988). The use of unclean teats, milking and transporting equipment contributes to the poor hygienic quality of traditional milk products (Altug and Bayrak, 2003). Milk products such as yoghurt, ice cream, and cheese are widely consumed and market for them has existed in many parts of the world for many generations. There are 16 million annual cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths worldwide due to *Salmonella* (Bhunia, 2008). *Salmonella* is the most frequent pathogen associated with outbreaks of foodborne illnesses (80.5% of the outbreaks), followed by *Escherichia coli* (10.1%) (De Buyser et al., 2001). Cooked food products and raw milk were most commonly contaminated with food borne pathogens and many of them were resistant to different antibiotics (Walsh et al., 2005; Normanno et al., 2007; Okpalugo et al., 2008 and Novakova et al., 2010). Contamination of dairy foods with virulent pathogens renders them to be a source of public health hazard. The possible contamination sources are either mastitis in dairy cow or the milk itself (Carter, 1995). Ubiquitous serotypes such as *Salmonella Enteritidis* or *Salmonella Typhimurium* which affect both man and animals generally cause gastrointestinal infections usually less severe than enteric fever. However, they also have the capacity to produce typhoid-like infections in mice and in humans or asymptomatic intestinal colonization in chickens (Velge et al., 2005). Growing concerns over food safety among the consumers call for the manufacturing and processing of foods under extremely hygienic conditions to avoid possible health challenges (Farzana et al., 2009). According to Pui et al. (2011), epidemiologic classification of *Salmonella* is based on the host preferences. The first group includes host-restricted serotypes that infect only humans such as *S. Typhi*. The second group includes host-adapted serotypes which are associated with one host species but can cause disease in other hosts serotypes such as *S. Pullorum* in avian. The third group includes the remaining serotypes. Typically, *S. enteritidis*, *S. typhimurium* and *Salmonella* Heidelberg are the three most frequent serotypes recovered from humans each year (Gray and Fedorka-Cray, 2002; Boyen et al., 2008). Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (Vi) capsular K antigens. More than 99% of *Salmonella* strains causing human infections belong to *Salmonella enterica* subspecies *enterica*. The infectious dose of *Salmonella* depends upon the serovar, bacteria strain, growth condition and host susceptibility. However, single-food-source outbreaks indicate that as little as 1 to 10 cells can cause salmonellosis with more susceptibility to infection (Yousef and Carlstrom, 2003; Bhunia, 2008). Pui et al. (2011) in their review article implicated poultry, eggs and dairy products as the most common vehicles of salmonellosis; including contaminated environment with moving animals (swines, cows and chickens) as vectors hence, constituting important risk factor for infection. Salmonellosis continues to be a major public health problem worldwide and contributes to negative economic impacts thus, making research on *Salmonella* gained great interest and concern from scientists.

The present study reported the molecular characterization of *Salmonella* serotypes in cow raw milk, cheese and traditional yoghurt samples in Nigeria and their consequent health hazards in man.

MATERIALS AND METHODS

Collection of samples

Thirty samples comprising each of cow raw milk, yoghurt and cheese were collected. Raw milk samples were collected from individual cows marketed at different markets in Ile-Ife, Modakeke, Edun-abon, and Akinlalu, Osun State, Nigeria. The raw milk was collected in a sterile container by placing the container underneath the cow for milking. Yoghurt samples were bought from the hawkers at various markets within the same localities while local cheese (wara) was equally purchased from the same market as above in transparent polythene bags. All cheese and yoghurt samples were transported to the laboratory under refrigeration (4 to 6°C) in thermal boxes containing ice packs and were analyzed bacteriologically within one hour of collection. Raw milk samples were transported to the laboratory at ca.4°C. All samples were collected between June and August, 2011.

Isolation and characterization of *Salmonella* isolates

Isolation of *Salmonella* species was done on MacConkey agar and *Salmonella-Shigella* agar (SSA) (Oxoid Ltd., Hampshire, England) plates. One milliliter of a five- fold dilution of raw milk and yoghurt was cultured on the sterile agar plates by pour plate technique. For cheese sample, 10 g was aseptically transferred into a sterile mortar and pestle, and homogenized in 90 ml sterile distilled water using stomacher. One milliliter of the 10 fold dilutions of the homogenized cheese was cultured appropriately as above. All plates were incubated at 37°C for 48 h. The isolates were differentiated first on the basis of colonial morphology and microscopic examination. The identity of isolates was confirmed by various biochemical tests with reference to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1994).

Antibiotic susceptibility

Antibiotic susceptibility of isolates was performed by antibiotic disc diffusion technique on Mueller Hinton agar (Hi Media, Vadhani, India) (Clinical and Laboratory Standards Institute (CLSI) 2008). The antibiotics (oxoid, UK), augmentin (30 µg), nitrofurantoin (200 µg), ofloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), amoxicillin (20 µg), cotrimoxazole (25 µg), and nalidixic acid (30 µg) were firmly placed on the agar plates previously seeded with the

Table 1. Percentage distribution of *Salmonella* sp. in raw milk, yoghurt and cheese.

Sample	Occurrence (%)
Yoghurt (n= 10)	9 (90)
Cheese (n= 10)	10 (100)
Raw milk (n=10)	1 (10)

Table 2. Antibiotic resistance profile of *Salmonella* sp in raw milk, cheese and yoghurt samples in Nigeria.

Antibiotic (µg)	Resistant	
	Isolates (n)	(%)
Nalidixic acid (30 µg)	0	0
Cotrimoxazole (25 µg)	13	61.9
Amoxicillin (20 µg)	20	95.2
Nitrofurantoin (300 µg)	7	33.3
Gentamicin (10 µg)	11	52.3
Ofloxacin (5 µg)	0	0
Augmentin (30 µg)	6	28.5
Tetracycline (30 µg)	19	90.4

test organisms and incubated at 37°C for 24 h. Sensitivity of the isolates to different antibiotics was indicated by the clear zones of inhibition which were measured in milliliter using a calibrated ruler. The diameters of zone of inhibition were compared with the interpretative chart of zone sizes of susceptibility to antibiotics (CLSI, 2008). Resistance to three or more antibiotics by isolate was defined as multiple antibiotic resistance (MAR).

Polymerase chain reaction and amplification of genes

The target genes selected for *Salmonella* sp. were invasive- *invA* (*Salmonella*-specific), adherence- *rfbJ*(B) (serogroup B), *fliC* (l, v) and *fljB* (e, n and z15) using appropriate primers. Oligonucleotide primers were used for the amplification of *invA*, *rfbJ*(B) and *fljB* genes.

A 50 µl reaction mixture contained 1 µl of template DNA (approximately 50 ng), 25 Pmol of each primer, 200 µM deoxyribonucleotide triphosphate mixture, 8 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase was used. The standard cycling condition after the initial denaturation step of 5 min at 94°C, were 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C for 35 cycles and final extension step of 5 min at 72°C. For some strains, the annealing temperature was reduced to 54 to 55°C to increase the amount of product that was amplified. PCR products (10 µl) were separated by electrophoresis in 0.8% agarose gel in Trisborate buffer (0.089 M Tris-base,0.089 M boric acid, 2.5 mM EDTA-Na_2, pH 8), with the 1 kbp DNA ladder as a molecular size marker.

Statistical analysis

Comparisons of the final values were compared statistically using Chi-square Tests of the statistical analysis system software (SPSS 16.0 version). P value of < 0.05 was considered statistically significant for all the comparisons.

RESULTS

Salmonella sp. were recovered in all the cheese and yoghurt samples but found in only one of the raw milk samples analysed. The percentage occurrence of *Salmonella* sp. in the samples includes raw milk (10 %), yoghurt (90%) and cheese (100%) (Table 1). The antibiotic susceptibility profile of the isolates from cow raw milk, yoghurt and cheese samples is shown in Table 2.

Generally, all the *Salmonella* serotypes were sensitive to ofloxacin and nalidixic acid. Resistance to antibiotics was in varying proportions with 95.2% developing resistance to amoxicillin, 90.4% to tetracycline and 28.5% to augumentin. Resistance was generally higher to amoxicillin than other antibiotics ($p>0.05$).

Ten (10) different multiple antibiotic resistance (MAR) patterns were displayed by the isolates with 41.17% showing resistance to three different classes of antibiotics (Table 3). All the *Salmonella* serotypes were multiple antibiotic resistant.

Table 4 shows the result of the polymerase chain reaction for the detection of *Salmonella* serotypes. The serotypes identified include *S. typhi*, *S. typhimurium*, *S. Enteritidis*, and the serogroups were D, B, D_1 respectively. Out of the 21 strains of *Salmonella* species, 52.4% belong to B serogroup while 20.0 and 28.6% belong to D and D_1 serogroups respectively. The *invA* gene was amplified in all the *Salmonella* serotypes while *rfbJ* was detected only in *S. Typhimurium.* The genes *fliC* and *fliB* were absent in all the serotypes. Figure 1 presents the PCR amplification of *invA* and *rfbJ* genes in *Salmonella* serotype obtained from cow raw milk and milk products.

DISCUSSION

The detection of *S. typhi*, *S. typimurium* and *S. enteritidis* in cheese and yoghurt samples analysed may probably be due to contaminated water in processing traditional dairy products, infected people who produce dairy products, contaminated equipment, and lack of public and individual hygiene. In this study, only 4.8% of the raw milk samples yielded *Salmonella* isolate. This may likely be due to observance of simple hygiene during collection. The findings of this present studies agree with the reports of earlier researchers who reported the presence of pathogenic bacteria in cow milk in Czech Republic, cow foremilk in Botswana, pasteurized milk and soya milk in Nigeria (Adeleke et al., 2000; Schlegelova et al., 2002; Guta et al., 2002; Okpalugo et al., 2008).

Previous studies by Fontaine et al. (1980) reported the isolation of *S. typhi* as the causative agents of mastitis in dairy animals which perhaps might have led to the contamination of milk from the udder of infected animals.

Salmonella sp. also reside in the intestinal tract where

Table 3. Multiple antibiotic resistance patterns among the *Salmonella* isolates in raw milk, cheese and yoghurt samples in Nigeria.

No. of antibiotic	MAR patterns	Isolates	Total (%)
3	COT, AMX, TET	1	7 (41.17)
	AMX, AUG, TET	2	
	AMX, NIT, TET	2	
	AMX, GEN, TET	2	
4	COT, AMX, AUG, TET	1	7 (41.17)
	COT, GEN, AUG, TET	1	
	COT, AMX, GEN, TET	4	
	COT, AMX, AUG, TET	1	
5	COT, AMX, NIT, GEN, TET	1	1 (5.88)
6	COT, AMX, NIT, GEN, AUG, TET	2	2 (11.76)

NAL,Nalidixic acid; COT, Cotrimoxazole; AMX, Amoxicillin; NIT, Nitrofurantoin; GEN, Gentamicin; AUG, Augmentin; TET, Tetracycline; MAR, multiple antibiotic resistance.

Table 4. Detection of *Salmonella* serotypes by Polymerase Chain Reaction.

Salmonella serotypes	Serogroup	Antigenic formular	Percentage number (%)	Number of strains positive for target gene			
				InvA	***rfbJ***	***fliC***	***fljB***
Typhi	D	9,12 (Vi):d	4 (20.0%)	4	-	-	-
Typhimurium	B	4,(5),12:i:1,2	11 (52.4%)	11	11	-	-
Enteritidis	D_1	9,12:g,m	6 (28.6%)	6	-	-	-

-, Not found.

they cause gastro-enteritis in animals and may have occurred in milk as a result of faecal contamination.

All the *Salmonella* serotypes (*S*. *typhi*, *S. typimurium* and *S*. *enteritidis*) were resistant to more than three classes of antibiotics used. Resistance by isolates to antibiotics may however, have been acquired from over exposure to antibiotics particularly in the veterinary sector or to a lesser extent from natural sources especially when some of the antibiotics tested in this study are commonly used in both the health and veterinary sectors. Studies had reported that of the more than 1 million tons of antibiotics released into the biosphere during the past years, approximately 50% are estimated to flow into the veterinary and agricultural channels (Mazel and Davies, 1999). The application of these antibiotics at sub-therapeutical levels for increased growth and feed efficiencies in farm animals is an integrated part of modern agriculture worldwide, and this leads to the emergence of antibiotic-resistant microbes (Prescott, 2000). When the same antibiotics are used in humans as in animals, resistant microbes which passed on to humans from animal sources could then lead to treatment failure in humans with serious public health implications. The present study recorded high resistance to amoxicillin, cotrimoxazole and tetracycline, which could be due to misuse of these antibiotics in disease conditions. This study also reveals that all the multi-resistant strains showed a characteristic tetracycline resistance trait. This may account for the indiscriminate use of the antibiotic invariably resulting to selection by the organism. Reduced susceptibility to tetracycline has been reported common in *S. typhimurium* isolates from healthy breeder and broiler flocks in Portugal (Clemente et al., 2014). The multiple resistance recorded in our study was against those antibiotics frequently employed in public health and veterinary sectors. The sensitivity of all the *Salmonella* serotypes to nalidixic and ofloxacin may account for the non- abuse of these antibiotics.

Pui et al. (2011) in their review reported that *Salmonella* strains resistance to one or more antibiotics have increased in the Saudi Arabia, United States, United Kingdom and other countries of the world. This is due to the increased and uncontrolled use as well as easy accessibility to antibiotics in many countries of the world

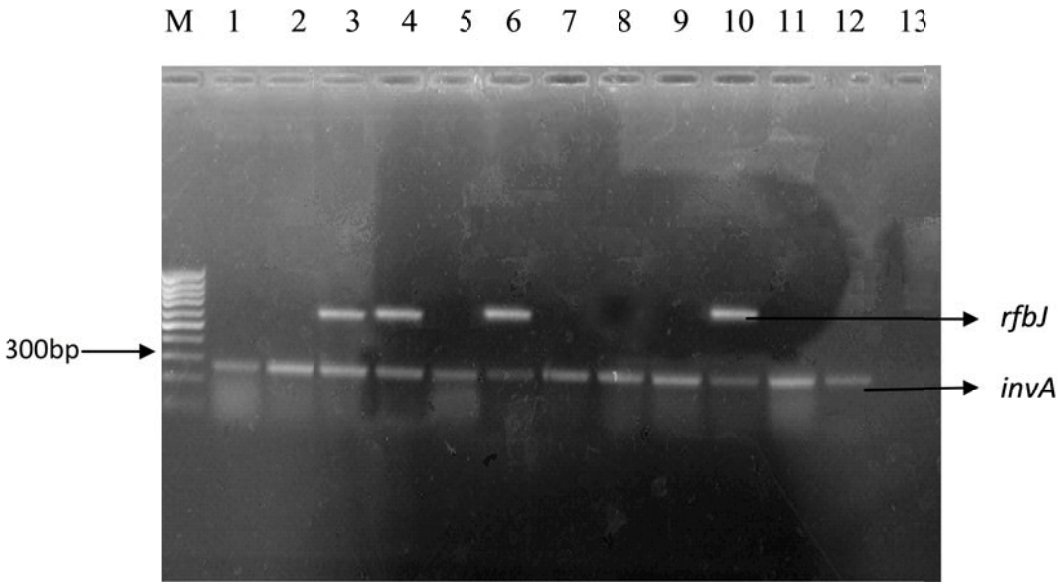

Figure 1. The PCR amplification of *invA* and *rfbJ* genes in *Salmonella* serotype obtained from cow raw milk and milk products. M, 100-bp DNA ladder; lanes 3, 4, 6, 10, *S. typhimurium* isolates; lanes 1, 2, 5, 7, 8, 11; *S. typhi* isolates; lane 9, *S. Enteritidis;* lane12, positive control; lane 13, negative control.

(Grob et al., 1998; Yoke-Kqueen et al., 2007). In this study, *S. typhi* and *S. typhimurium* were multiresistant to the antibiotics tested, thus confirming the earlier reports in Africa. Emerging resistance in *S. typhi* has been described especially in Africa and Asia and the appearance of *S. typhimurium* DT104 in the late 1980s raised main public health concern, thereby threatening the lives of infected individuals (Grob et al., 1998). Evidence exists to suggest that not only are such antibiotic resistant strains more difficult to control in terms of human infection, they may also be more resistant to heat processes (Davidson and Henson, 1995). This is of great concern because majority of infections with MAR *Salmonella* are acquired through the consumption of contaminated foods of animal origin such as swines and chicken eggs.

Asai et al. (2010) mentioned that cephalosporin and fluoroquinone-resistant strains of *S. Choleraesuis* have been identified in swines in Taiwan and Thailand. Previous study by Prapas et al., (2008) reported that *S. typhimurium* and *Salmonella* Heidelberg ranked first and second, respectively in multidrug resistance, and is among the most commonly isolated serovars from dairy products.

Due to the use of antibiotics for the promotion of growth and prevention of disease in food animals, there is an increase of human salmonellosis cases caused by food-borne MAR *Salmonella* nowadays (Yang et al., 2010). This indiscriminate and injudicious use of antibiotics in any setting especially in food animals worldwide should be monitored to reduce the transfer risk of MAR *Salmonella* to humans. Finally, there is a need for continuous surveillance and sharing of antimicrobial susceptibility data for Salmonella among countries worldwide to ensure the effectiveness of control programmes.

The amplification of *invA* and *rfbJ* genes by polymerase chain reactions in the *Salmonella* serotypes revealed that the isolates are pathogenic. *S. typhimurium* was the most predominant occurring serotype (52.4 %). The presence of *invA* and *rfbJ* genes may cause *Salmonella* infections to become invasive and result in bacteremia and serious extra intestinal disease since it is the genes code for invasion and attachment. *Salmonella* serotypes have been implicated in several diseases, including enteric or typhoid fever (primarily *S. typhi* and *S. paratyphi*), bacteremia, endovascular infections, focal infections (osteomyelitis), and enterocolitis (typically *S. typhimurium*, *S. enteritidis*, and *S. heidelberg*) (Brenner et al., 2000).

Conclusion

The recovery of MAR pathogenic *Salmonella* serotypes in cheese and yoghurt samples in the study areas calls for great health concern as these organisms have been associated with salmonellosis and other grave diseases. Good quality raw materials for product processing, adoption of Good Manufactured Practices (GMP) and strict personal hygiene will ensure safety and high quality dairy products.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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