

ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC SUSCEPTIBILITY PROFILE OF *SALMONELLA* SPECIES ISOLATED FROM ABATTOIR ENVIRONMENT

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ABSTRACT

In this study we investigated the antibiogram profile of *Salmonella* species isolated from abattoir environment. A total of 72 samples were collected from three different stations [station A (the drainage), station B (stagnant water in potholes and floors around the abattoir) and station C (water used for washing the meat slaughtered at the abattoir)] between January and June 2017. All samples were processed and analysed using standard culture-based and polymerase chain reaction (PCR) methods. The mean total heterotrophic bacteria population densities were: station A $2.0 \times 10^{10} \pm 0.02$ CFU/ml; station B $1.6 \times 10^{10} \pm 0.10$ CFU/ml and station C $3.9 \times 10^9 \pm 0.05$ CFU/ml. The mean total salmonellae population density were $2.4 \times 10^5 \pm 0.14$ CFU/ml, $1.3 \times 10^5 \pm 0.15$ CFU/ml, and $1.0 \times 10^4 \pm 0.06$ CFU/ml for station A, station B and station C respectively. A total of 50 confirmed salmonellae, using PCR identification system, were subjected to antimicrobial profiling using the disc diffusion method. The resistance profile of the isolates revealed that 24 (48%) of the isolates were resistant to piperacillin, 50 (100%) to gentamycin, 50 (100%) were resistant to tetracycline, while 22 (44%) of the isolates were resistant to aztreonam. Multidrug resistance (MDR) profile of the isolates revealed that 22 (44%) of the isolates were resistant to piperacillin, gentamycin, tetracycline, and aztreonam belonging to four antimicrobial class. Our findings revealed that abattoir environments could be a potential reservoir of multi-antibiotic resistant *Salmonella* species.

Keywords: Abattoir, Salmonellae, Antibiogram Profile, Multi-Drug Resistance, Effluent

INTRODUCTION

Salmonellosis is regarded as one of the most prevalent foodborne zoonotic infection in both developed and developing countries, even though the prevalence seems to vary between countries (Molla *et al.*, 2003). It is usually challenging to explain the state of salmonellosis in developing countries resulting from the very limited scope of studies, with lack of co-ordinated epidemiological surveillance systems. In addition, under-reporting of cases coupled with the presence of other diseases well-thought-out to be of high significance may have surpassed the problem of salmonellosis in some developing countries including Nigeria. The upsurge of global populace with mass production of animal and human food as well as the rapid international trade in aquaculture, agriculture and food products could further degenerate the problem (Dahshan *et al.*, 2010).

Food animals harbour a diverse range of salmonellae serovar and thus, could act as potential reservoir of contamination, which is of

significant epidemiological importance in non-typhoid human salmonellosis (Dallal *et al.*, 2010). Contamination of meat by *Salmonella* species may occur at slaughterhouses from animal faeces, symptomless animals, slaughter equipment, floors as well as abattoir personnel. Interestingly, this pathogen usually gains access to meat at any stage during butchering and processing. Cross-contamination of carcasses and meat products could continue during subsequent handling, processing, preparation and distribution (Igbinosa, 2015a). The safety and hygienic quality of meat can be determined by the presence or absence of disease-causing microorganisms. It has been reported that foodborne illnesses associated with the consumption of meat have continued to increase for the past 30 years (Akoachere *et al.*, 2009).

Salmonellae have been reported from abattoir settings in different parts of Nigeria such as Osun State, (Omololu-Aso *et al.*, 2017); Port Harcourt, (Ire *et al.*, 2017); Benue State, (Nseabasi-Maina *et al.*, 2017); Sokoto, (Bagudo *et al.*, 2014; Faleke *et al.*, 2017). Also from other Africa regions in

Nairobi, Kenya (Nyabundi *et al.*, 2017); Kampala, Uganda (Tinega *et al.*, 2016); Mansoura, Egypt (Sallam *et al.*, 2014) and Modjo and Bishoftu, Ethiopia (Kuma *et al.*, 2017). *Salmonella* are among organisms currently under public health surveillance for antimicrobial resistance (NARMS, 2013). An increasing number of primary sources of salmonellosis are considered to have been linked to food-producing animals as well as contaminated water sources (Kagambèga *et al.*, 2013). Therefore, this study was conducted to isolate, identify and determine the antibiogram profile of salmonellae from abattoir environment.

MATERIALS AND METHOD

Study area

The abattoir is situated at Ikpoba slope, located at latitude 6°21'0.5" longitude 5°38'34.98" Benin City, Edo State, Nigeria. Ikpoba slope is a community close to the Ikpoba River, a fourth order stream situated within the rainforest belt of Edo State, Southern Nigeria. The River rises from the Ishan Plateau in the Northern part and flowing in South Westerly direction in a steeply incised valley and through sandy areas before passing through Benin City and joining the Ossiomo River.

Sample collection

Seventy two (72) wastewater samples were collected within a six months period (between January and June 2017), from different sampling points/locations at the abattoir [station A (the drainage), station B (stagnant water in potholes and floors around the abattoir) and station C (water used for washing the meat slaughtered at the abattoir)]. All samples were collected using sterile glass container, labelled appropriately and transported to the Applied Microbial Processes & Environmental Health Research Group Laboratory, Department of Microbiology, Faculty of Life Sciences, University of Benin in cold ice pack and analysed within 24 h after collection.

Enrichment and isolation procedure

Serial dilution were carried out where 0.1 ml of each prepared dilutions (10^6 - 10^8) for all wastewater samples was spread inoculated into already prepared Nutrient Agar [NA] (Lab M, Lancashire, United Kingdom) plates in triplicates for heterotrophic bacteria enumeration; while 0.1 ml of each prepared dilutions (10^5) for all wastewater

samples was inoculated into sterile xylose lysine deoxycholate (XLD) (Lab M, Lancashire, United Kingdom) Petri dishes in triplicates for salmonellae enumeration. Aliquot of 100 µl each were spread on the respective agar plate and allowed to absorb before incubating at a temperature of 37 °C for 18-24 h. After incubation, discrete colonies were enumerated for total heterotrophic bacteria and salmonellae count. The colour of salmonellae on the XLD agar plate was observed as red with black centres.

In addition, an aliquot of 1.0 ml from each of the wastewater samples was inoculated into test tubes containing 9.0 ml of Selenite F-Broth (Lab M, Lancashire, United Kingdom) and incubated for 18-24 h at 37°C. Thereafter, streak plate technique was employed by streaking directly from the overnight culture from Selenite F-Broth into a fresh XLD agar plate and incubated at 37 °C for 18-24 h. Red colonies or red colonies with black centres was sub-cultured onto fresh XLD agar plates for pure culture. Thereafter, distinct colonies were purified repeatedly on nutrient agar plates. Pure isolates were stored on agar slants at 4 °C for further analysis.

Phenotypic identification of the Salmonella

The purified isolates from culture-based technique using XLD agar medium (colonies that appear red, and usually with a black centre) were streaked on nutrient agar plates to obtain pure cultures which were subjected to Gram-staining, oxidase, urease reactions, indole and motility tests. Organisms that appear as oxidase negative, urease negative, indole negative and Gram-negative rods were selected presumptively as Salmonellae (Cheesbrough, 2006).

DNA extraction procedure

The genomic DNA of the presumptive salmonellae isolates was extracted using boiling method. Distinct colonies of purified salmonellae isolates were inoculated into 200 µl of Tryptone Soy Broth (Merck, Darmstadt, Germany) and cultivated overnight at 37 °C. The cells were lysed with heat block (MK200-2, Shanghai, China) at 100 °C for 15 min. The boiled bacterial cells were centrifuged at 14,500 rpm for 10 min using micro-centrifuge to separate the cell debris from the supernatant which was then stored at -20 °C and

used as DNA template.

Polymerase chain reaction identification using salmonellae genus-specific primer

All PCR amplification assay were conducted in a reaction mixture (25.0 µl) with master mix (12.5 µl), forward (0.50 µl) and reverse (0.50 µl) primers, nuclease-free water (2.0 µl) and template DNA (5.0 µl). DNA templates of *Salmonella enterica* serovar Typhimurium ATCC 14028 was used as a positive control while nuclease-free water as a negative control in each PCR assay. All PCR reactions were carried out using a Peltier-based thermal cycler (BioSeparation System, Shanxi, China). The PCR primer pair [F-5'-TGT TGT GGT TAA TAA CCG CA-3' and R- 5'-CAC AAA TCC ATC TCT GGA-3'] amplicon size of 574 bp, for the salmonellae genus-specific (16S rDNA gene), and PCR reactions were as described by Lin and Tsen (1996). PCR products were electrophoresed using 1.5% agarose gel (Hispanagar, Burgos, Spain) stained with 0.5 mg/L ethidium bromide (Merck, Modderfontein, South Africa) at 100 V for 1 h, in 1 × TAE buffer and viewed under a UV trans-illuminator (EBOX VX5, Vilber Lourmat, France).

Antibiotic susceptibility testing

The *Salmonella* isolates that were confirmed using the PCR-based method were subjected to antibiogram characterization. Commercially available antibiotics disc, obtained from Mast diagnostics, Merseyside, United Kingdom, were used to determine the resistance patterns of the isolates against six different antibiotics (1 dose/disc) grouped into six different classes of antibiotics. Antibiotic discs with the following

drug contents: piperacillin (100 µg), gentamycin (10 µg), aztreonam (30 µg), imipenem (10 µg), ciprofloxacin (5µg) and tetracycline (30 µg) using the Kirby-Bauer disc-diffusion test, which conforms to the recommended standard of the Clinical and Laboratory Standards Institute (CLSI). These antibiotics were chosen based on the treatment practices for salmonellae in this area and from the literature (Besser *et al.*, 2000). The *Salmonella* were grown for 18 to 24 h on Mueller-Hinton broth (Merck, Darmstadt, Germany), harvested and then suspended in 0.85% normal saline solution adjusted to a 0.5 McFarland turbidity standard, corresponding to 10⁶ CFU/ml. The inoculum was streaked onto plates of Mueller-Hinton agar using a sterile cotton swab and aseptically impregnated with the appropriate antibiotics. The results were recorded after 24 h of incubation at 37 °C. The diameter of the zone of inhibition around each disc was measured and interpreted as resistance (R), intermediate (I) or sensitive (S) in accordance with the recommended standard established by the Clinical Laboratory Standard Institute (2014).

RESULTS

Population density of the bacterial isolates

The mean heterotrophic bacterial count are 2.0×10¹⁰ ± 0.02 CFU/ml from station A; 1.6×10¹⁰ ± 0.10 CFU/ml from station B; and 3.9×10⁹ ± 0.05 CFU/ml from station C. The mean salmonellae count are 2.4×10⁵ ± 0.14 CFU/ml from station A; 1.3×10⁵ ± 0.15 CFU/ml from station B; and 1.0×10⁴ ± 0.06 CFU/ml from station C (Table 1).

Table 1: Mean of total heterotrophic and salmonellae count recovered from abattoir environment

Population density	Stations	Min (CFU/ml)	Max (CFU/ml)	Mean ± SD (CFU/ml)
Total heterotrophic bacteria	Station A	1.9×10 ⁸	2.6×10 ¹¹	2.0×10 ¹⁰ ± 0.02 ^b
	Station B	1.3×10 ⁷	2.0×10 ¹⁰	1.6×10 ¹⁰ ± 0.10 ^b
	Station C	1.0×10 ⁶	4.5×10 ¹⁰	3.9×10 ⁹ ± 0.05 ^a
	p-value			0.013
Salmonellae	Station A	2.1×10 ⁴	2.5×10 ⁶	2.4×10 ⁵ ± 0.14 ^b
	Station B	2.1×10 ⁴	1.5×10 ⁵	1.3×10 ⁵ ± 0.15 ^b
	Station C	4.4×10 ³	1.0×10 ⁴	1.0×10 ⁴ ± 0.06 ^a
	p-value			0.015

Values are expressed in triplicates of mean ± standard deviation. The mean difference are represented with superscript lowercase alphabets across column. Means with significant difference carry different alphabets (p<0.05).

A total of 78 isolates of salmonellae were isolated using the culture-based methods. Further classical characterization of the isolates using morphological and biochemical reactions, revealed that 72/78 (92.31%) isolates were presumptively identified as salmonellae and subjected to polymerase chain reaction using salmonellae genus-specific primer, a total of 50/72 (69.44%) salmonellae were confirmed as *Salmonella* species.

Resistance profile of the bacterial isolates

The resistance profile of 50 *Salmonella* species identified from abattoir settings were further phenotypically characterized using six (6) antibiotics which are representatives of six (6)

different groups of antimicrobial agents. The resistance profile of the isolates revealed that 24/50 (48%) of the isolates were resistant to piperacillin; 50/50 (100%) were resistant to gentamycin; 50/50 (100%) were resistant to tetracycline; 4/50 (8%) were resistant to imipenem; 4/50 (8%) were resistant to ciprofloxacin; 22/50 (44%) of the isolates were resistant to aztreonam (Table 2). The distribution of antibiotics susceptibility profile of the isolates from abattoir showed that 16/18 (88.89%) and 8/16(50%) were resistant to the activity of piperacillin in station A, and station B respectively (Table 3). Also gentamycin and tetracycline were 100% resistant across the sampling stations during the study regimen (Table 3).

Table 2: Antimicrobial agents, disc content and zone diameter interpretative standards and resistance profile of *Salmonella* spp. from abattoir environment

Antibiotics Group	Antimicrobial Agent	Disc content	Zone diameter (mm)			Resistant Salmonellae n=50 (%)
			Resistant	Intermediate	Sensitive	
Penicillins	Piperacillin	100 µg	≤ 17	18-20	≥ 21	24/50 (48)
Aminoglycosides	Gentamycin	10 µg	≤ 12	13-14	≥ 15	50/50 (100)
Monobactams	Aztreonam	30 µg	≤ 17	18-20	≥ 21	22/50 (44)
Carbapenems	Imipenem	10 µg	≤ 19	20-22	≥ 23	4/50 (8)
Fluroquinolones	Ciprofloxacin	5 µg	≤ 20	21-30	≥ 31	4/50 (8)
Tetracyclines	Tetracycline	30 µg	≤ 11	12-14	≥ 15	50/50 (100)

Table 3: Distribution of the resistant profile of *Salmonella* spp. from abattoir environment

Antimicrobial agents	Station A n=18 (%)	Station B n=16 (%)	Station C n=16 (%)	p-value
Piperacillin (100 µg)	16 (88.89)	8 (50)	0 (0)	0.142
Gentamycin (10 µg)	18 (100)	16 (100)	16 (100)	0.041
Aztreonam (30 µg)	10 (55.55)	6 (37.5)	6 (37.5)	0.050
Imipenem (10 µg)	2 (22.22)	0 (0)	0 (0)	0.040
Ciprofloxacin (5 µg)	2 (11.11)	2 (12.5)	0 (0)	0.040
Tetracycline (30 µg)	18 (100)	16 (100)	16 (100)	0.213

Multidrug resistance (MDR) profile of the isolates revealed that 24/50 (48%) of the isolates were resistant to PTZ^R, GEN^R and TET^R; while 22/50 (44%) of the isolate was resistant to PTZ^R, GEN^R, TET^R, and ATM^R. Extensive drug-resistant profile of the *Salmonella* spp. revealed that 50/50 isolates

were resistant to GEN^R and TET^R (Table 4). Multiple antibiotic resistant (MAR) index revealed an index that ranged from 0.3 to 1; showing resistance to a minimum of 2 antibiotics and a maximum of 6 antibiotics.

Table 4: Multidrug-resistant and extensive drug-resistant profile of *Salmonella* spp. isolated from abattoir environment

Number of antimicrobial group	Number of antibiotics	Resistance phenotype	Number of isolates <i>n</i> =50 (%)	Multiple antibiotic resistant (MAR) index
6	6	IMI ^R , CIP ^R , ATM ^R , TET ^R , GEN ^R , PTZ ^R	4	1
4	4	PTZ ^R , GEN ^R , ATM ^R , TET ^R	22	0.6
3	3	PTZ ^R , GEN ^R , TET ^R	24	0.5
2	2	GEN ^R , TET ^R	50	0.3

Legend: PTZ: Piperacillin, ATM: Aztreonam, TET: Tetracycline, GEN: Gentamycin, IMI: Imipenem, and CIP: Ciprofloxacin.

DISCUSSION

Salmonellae infection can lead to invasive and focal infections that can be severe. The ability of *Salmonella* to cause invasive infection varies with the serovar. Good personal and environmental hygienic practices are instrumental in food safety. They are compulsory by law under the auspices of international and national hygiene protocols and are often described as prerequisites to food safety guidelines based on Hazard Analysis and Critical Control Point (HACCP). Poor hygiene in abattoir environment almost always results in dissemination and proliferation of pathogenic clones as well as food spoilage microbes on the environments of slaughterhouses. In the present study we examined the presence of antibiotic-resistant *Salmonella* species from abattoir environments using classical and PCR tools.

A significant difference in the heterotrophic bacteria as well as the salmonellae cell densities from the different sampling points was observed ($p < 0.05$). The high population of heterotrophic bacteria and salmonellae counts observed in this study could directly be attributed to the fact that the abattoir environments were contaminated due to poor personal and environmental hygiene of the butchers who slaughter meat at the abattoir; their waste discharged around the abattoir thereby making the meat unsafe for consumption; and favouring the proliferation of antibiotic-resistant *Salmonella* spp., as observed in this study. This could also be attributed to the poor water quality in the abattoir. The high rate of isolation of *Salmonella* from station A (drains) and station B (potholes and floors of the slaughterhouses) may be as a result of accumulation of excreta from cattle, prior to slaughter. There may also be sufficient nutrients in the moist environments to enable the proliferation of *Salmonella* viability for a

long duration of time. It was observed during this study that waste matter from cattle was not properly removed regularly from the abattoir environments, as there was no effective cleaning procedures put in place. The salmonellae in the environments could contaminate the cattle skin and hooves, neighbouring soils and water bodies resulting from poor environmental hygiene as a consequence of accumulated faeces (dung). Motsoela *et al.* (2002) reported that salmonellae were present in all sampled abattoir environments in Botswana. Gragg *et al.* (2013) reported the prevalence of *Salmonella enterica* subspecies enterica serotypes in cattle faeces among other sources in Mexico. Also, the occurrence of *Salmonella* at variable frequency from different samples obtained from goats (including faecal samples) in Ethiopia has been documented (Woldemariam *et al.*, 2005). There is every tendency that faeces from the carcass, mixing with the meat, could contribute to the contamination of the meat. This is also similar to the findings of Bello *et al.* (2011) in some other abattoir in North-Western Nigeria.

In Nigeria, many abattoirs dispose their effluents directly into streams and rivers without any form of treatment and the slaughtered animals are washed by the same water (Adelagan, 2002). This is the case in most abattoirs in Nigeria and we observed similarity during our sampling regime. Therefore, the occurrence of high total heterotrophic bacteria and salmonellae count in the effluent from this abattoir implies a lot for public health. Contamination of abattoir surfaces may emanate from the butchers, which could be as a result of using ungloved hands during dressing and processing of the carcass in the slaughtering hall. In addition, the footwears used by the butchers could function as a vehicle for faecal

contamination, since processing and dressing of the carcasses occurs on the open floor in the slaughterhouses. Moreover, in developing countries like Nigeria, water bodies such as river are used for drinking, bathing, washing, watering of crops and other domestic purposes which also indicate a high impact on the public health of the users (Nafarnda *et al.*, 2012). Similar study conducted by Mor-Mur and Yuste (2010) indicated that bacteria isolated from wastes and abattoir products include pseudomonads, salmonellae, klebsiellae and *Proteus*.

Salmonella isolates obtained in the study were multi-drug resistant. The isolates from the abattoir environments (station A, B and C) were found to demonstrate variable resistance patterns. Multi-drug resistance (MDR) has been described as acquired resistance to at least one antimicrobial agent in three or more antimicrobial classes (i.e. bacterial isolates remain resistant to ≥ 1 antimicrobial in ≥ 3 antimicrobial categories) while extensively drug-resistant (XDR) has been described as non-susceptibility to ≥ 1 antimicrobial agent in ≥ 2 antimicrobial classes (Magiorakos *et al.*, 2012). This formed the basis for MDR and XDR profile of isolates in this study. A significant regression ($r = 0.9378$) of the number of isolates on the resistance phenotype was observed ($p < 0.05$). This could be as a consequence of the dissemination of antimicrobial resistant genes within bacterial population via horizontal gene transfer. Similarly, introduction of such multidrug-resistant pathogens in the environment and subsequent accumulation upholds the spread of resistant pathotypes through transfer of integrons and plasmids within microbiota resulting in the formation of a pool of resistance reservoir.

It has been reported that antibiotics are widely used in food animal production for therapy and prevention of bacterial infection and for growth promotion (Igbinosa *et al.*, 2016). Studies have also shown that 50-90% of drugs administered to farm animals are excreted into the environment either un-metabolized or as metabolic intermediates which even though inactive, may undergo transformation to the active form in the environment (Dahshan *et al.*, 2010; Cabral *et al.*,

2017). In the course of this study, it was observed that discharge of faecal material from the processing carcasses was being released as effluent in the environment. This can consist of a lot of un-metabolized antibiotic residue (Kummerer, 2003). Findings from Prendergast *et al.* (2007) clearly demonstrate the potential for cross-contamination from equipment and meat contact surfaces in the cutting room environment. Multi-drug resistant *Salmonella* species have been elucidated by previous researchers (Gomes-Neves *et al.*, 2014; Sallam *et al.*, 2014; Nseabasi-Maina *et al.*, 2017). On some occasion, the same serotype from a single animal was isolated from more than one sample (i.e. faeces and rumen; faeces and carcass; rumen and carcass; faeces, rumen and carcass) (McEvoy *et al.*, 2003). Resistant microorganisms can persist in waste, soil, food and water with a number of consequences (Igbinosa, 2015a; 2015b).

The occurrence of multi-drug resistant (MDR) *Salmonella* spp., could pose major public health risks to consumers. Firstly, ingestion of products contaminated with MDR *Salmonella* species could cause salmonellosis which requires antibiotic therapy and therapy can then be compromised due to resistant strain (Omololu-Aso *et al.*, 2017). However, it should be noted that even if the products are cooked, it will not destroy genes responsible for the resistance. Secondly, resistant non-pathogenic bacteria can be transferred to human via consumption of contaminated food products and resistance genes are subsequently transferred to other bacteria in the gut through mobile genetic element such a plasmid, integrons and gene cassette, insertion sequence and transposon (Mbotu *et al.*, 2012; Bagudu *et al.*, 2014). Lastly, antibiotics which may remain as residues in animal products such as meat, liver and blood can also lead to the selective proliferation of resistant clones in the consumer of the product (Egan *et al.*, 2017). The isolating rate of salmonellae from abattoir environment is increasing with a multidrug resistance and an increasing tendency of their resistance rates. Therefore, it is necessary to improve bacterial resistance monitoring and antibiogram research to direct rational drug therapy.

CONCLUSION

We conclude from our study that effluents from the abattoir could be a potential source of reservoir and dissemination of antibiotic-resistant salmonellae between animal and human population as well as the environment. This calls for surveillance network system to track resistance patterns of salmonellae serovars circulating in abattoir and other environmental sources. Implementing suitable Hazard Analysis and Critical Control Points (HACCP) procedures to decrease the cross-contamination from these environmental sources as well as food animal handlers and the final consumers could decrease the occurrence of multiple antibiotic resistant salmonellae in the environment.

Conflict of Interest

The authors declare no conflict of interest

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