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Evaluation of Extracellular Virulence and Antimicrobial Characterization of *Salmonella* pathogens from slaughterhouses in Benin City, Nigeria

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Abstract: Slaughterhouses are industries involved in the commercial slaughtering of livestock and processing of meat products. This study was aimed at evaluating the antimicrobial and extracellular virulence potentials of *Salmonella* isolates from slaughterhouses in Benin City, Nigeria. In total, 150 effluent samples from 6 different slaughterhouses were assessed in this study. The sampled effluents were screened for *Salmonella* species using standard culture-based techniques and polymerase chain reaction (PCR) using specific primer sets. The antibiotic resistance profile and biofilm formation potentials of isolated *Salmonella* were determined using the Kirby-Bauer disc diffusion technique and microtitre plate method respectively. Bacterial virulence factors were screened using classical microbiological culture-based procedures and molecular technique. From the 150 effluent samples assessed, 19(12.7%) were positive for *Salmonella* species. A total of 31 *Salmonella* isolates were screened from the positive samples using PCR. The biofilm formation profile was strong biofilm 8(25.81%), moderate biofilm 15(48.39%), weak biofilm 3(9.68%) and no biofilm 5(16.13%). Extracellular virulence profile of isolates was gelatinase production 17(54.84%), protease activity 14(45.16%), beta-haemolytic activity 21(67.74%) and DNA degrading activity 15(48.39%). The antibiotic resistance profile as revealed was ampicillin 31(100%), piperacillin 21(67.74%), aztreonam 22(70.97%), azithromycin 18(58.06%), tetracycline 14(45.16%), ciprofloxacin 16(51.61%) and chloramphenicol 20(64.52%). The multiple antibiotic resistance (MAR) index of the isolates range from 0.07 – 0.71. Findings from this study emphasized on importance of routine surveillance in curtailing the spread of multidrug-resistant *Salmonella* pathogens in slaughterhouse environments.

1. Introduction

Abattoirs, also known as slaughterhouses are enterprise of the food industry that are involved in the slaughtering of livestock (goats, cattle, sheep, etc.) and animal meat processing on a commercial scale. Livestock farming practices including the abattoir have been supplying meat and meat by-products to millions of Nigeria and also provide job for the rapidly growing Nigeria populace (Uzonwanne *et al.*, 2023). This industry is associated with characteristic highly organic and inorganic waste products mainly comprises of urine, blood, gut content, water and dissolved solids. These wastes could notably

influence environmental pollution either directly or indirectly when not properly managed (Mozhiarasi and Natarajan, 2022).

In Nigeria and several other developing nations, improper waste management in slaughterhouses and the exposure of the establishment to random solid wastes and untreated effluents has been of major public health concern (Omoni *et al.*, 2023; Ovuru *et al.*, 2024). This is unlike economically advanced nations with improved measures and strict regulations as regards waste management (Roy and Tarafdar, 2022). These slaughterhouses are often established close to water bodies, where water needed for processing is fully assured. However, the implications of contaminating the surroundings via the discharge of these wastes into receiving water body and terrestrial environment aren't usually considered properly. Poor water sanitation and improper waste management practices have notably contributed to the water scarcity (WHO. 2023). Consequently, the treatment of polluted water has received intensified attention globally in order to curtail the scarcity of safe water (Bouknana *et al*., 2014; El Abdouni *et al*., 2021; Kesari *et al*., 2021). However, waste management remains a major challenge in low- and middle-income nations, particularly in rural communities (Hassan *et al*., 2021).

Wastewater or effluents generated from the abattoir are also often associated with diverse pathogenic microorganisms, including *Salmonella* species which are of public health importance (Igbinosa *et al*., 2021). Aside from *Salmonella* species, other genera isolated from abattoir effluents and their environments include *Staphylococcus, Escherichia, Bacillus, Citrobacter, Streptococcus, Klebsiella, Proteus* and *Pseudomonas* (Gufe *et al*., 2021; Esemu *et al*., 2022). These pathogens are potential threat to public health as they could also infiltrate groundwater. Globally, gastroenteritis resulting from *Salmonella* has been implicated in about 93.8 million cases of illnesses and 155,000 deaths annually (Geresu and Desta, 2021). This study was aimed at examining the biofilm-forming capacity, extracellular virulence profile and the antimicrobial characterization of *Salmonella* pathogens isolated from slaughterhouses in Benin City, Nigeria.

2. Methodology

2.1 Sample collection

A total of 25 samples effluent samples were obtained and assayed from 6 different slaughterhouses where the animals were slaughtered in Benin City Nigeria using sterile plastic containers. These slaughterhouses include Lawal & Sons slaughterhouse, Vegetable Market slaughterhouse, Dynamic slaughterhouse, Association of Edo State Practicing Butchers slaughterhouse, Madam Sarah slaughterhouse, and Doctors House slaughterhouse. The sterile plastic pets were first rinsed with the samples three times before sample collection. The samples were immediately transported to Applied Microbial Processes and Environmental Health Research Laboratory, Faculty of Life Sciences, University of Benin, Nigeria for analysis within 24 h.

2.2 Isolation of Salmonella species

Isolation of *Salmonella* species was carried out based recommended protocols (International Organization for Standardization, 2017). The effluent samples were serially diluted and an aliquot of 100µL from 10^2 diluents was aseptically pipetted into 3mL of Rappaport Vassilanis broth (Merck, Darmstadt, Germany) and then incubated for 18-24 h. After incubation, a sterile wire loop was used to aseptically inoculate Xylose Lysine Deoxycholate agar (Lab M, Lancashire, United Kingdom) culture plates. The culture plates were incubated at 37 ^{o}C for 18 to 24 h. Distinct black colonies as observed after incubation were sub-cultured on Tryptone soy agar (Lab M, Lancashire, United Kingdom) using

the streak plate method and subsequently incubated for 18-24 h at 37^{o}C. Sub-cultured colonies were purified using nutrient agar (Lab M, Lancashire, United Kingdom) and preserved for further use in nutrient agar culture slants at 4^{o}C.

2.3 Identification of Salmonella species using polymerase chain reaction

The extraction of DNA was carried out using protocols previously described (Ziemer and Steadham, 2003). DNA extraction was conducted using peqGOLD Bacterial DNA Kit. The amplification assay of PCR was done in reaction mixture (25.0 µL) and master mix (12.5 µL), forward primers (0.50 µL), reverse primers (0.50 µL), nuclease free water (2.0 µL) and DNA (5.0 µL). The usage of *Salmonella enterica* serovar Typhimurium ATCC 14028 DNA template as the positive control and the nuclease free water as the negative control was employed in each PCR assay. The PCR reactions were conducted with the use of a Thermal cycler. The PCR primer pair [F-5'-TGT TGT GGT TAA TAA CCG CA-3' and R- 5'-CAC AAA TCC ATC TCT GGA-3'] of 574 bp amplicon size with a specificity for the genus *Salmonella* (16S rDNA gene).

2.4 Antimicrobial susceptibility profile for the Salmonella species

Antimicrobial susceptibility testing was carried out via the Kirby-Bauer disk diffusion technique following the recommended criteria of the Clinical Standards Institute (CLSI, 2020). The discs utilized include amoxicillin-clavulanate (20/10 µg), ampicillin (10 µg), meropenem (10 µg), piperacillin (100 µg), ampicillin-sulbactam (10/10 µg), imipenem (10 µg), aztreonam (30 µg), azithromycin (15 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg) and nitrofurantoin (300 µg). Diameter of inhibition zones were interpreted as intermediate (I), sensitive (S), and resistant (R) as measured.

2.5 Multiple antibiotic resistance index

Multiple antibiotic resistance (MAR) index was calculated based on previously employed protocol (Beshiru *et al*., 2022):

$$\text{MAR Index} = \frac{\textbf{Number of the antibiotics to which an isolate is resistant to}}{\textbf{Total number of antibiotics that the isolate were tested}}$$

2.6 Phenotypic virulence factors determination of Salmonella species

Phenotypic virulence factors assay was carried out accordingly as described (Smibert and Krieg, 1994). Distinct colonies on Tryptone Soy Agar (TSA) were made into a suspension using 3.0 mL Tryptone Soy Broth (TSB). The cell density of the suspended colonies was modified to 0.5 McFarland standards, which is the equivalently 10^{6}cells/mL. Using a sheep blood agar plate, a 5.0 mL of the modified suspension was cultured and incubated at 37 °C for 24 to 48 h. Thereafter, haemolytic reaction was denoted by colourless and clear zones encircling the grown colonies as an indication of red blood cells lysis. For lipase activity, the suspension of sample (5.0 mL) was inoculated on Tryptic soy agar (TSA) and incubated at 37 °C for 24 to 48 h. Lipase-producing organisms are indicated by a clear halo encircling the area of growth. For protease activity, a 5.0 mL suspension of the sample was inoculated on TSA plate supplemented using 1% casein and incubated at 37 °C for 24 to 48h. Positive casein hydrolysis was denoted via the appearance of cleared zone. For gelatine production, a 5.0 mL sample of the suspended inoculum was inoculated on gelatin medium and incubated at 37 °C for 24 to 48h. Proliferation of gelatin-liquefying microorganisms was denoted by the zones of clearance.

2.7 Biofilm formation potential for the Salmonella species

Purified colonies of *Salmonella* species were momentarily re-suspended in 10 mL of TSB then incubated at 37 ^{o}C for 18h, subsequently incubated for 18 h at 37^{o}C and undergo centrifugation at 12,000 rpm for 2 min. Rinsing of the cell pellets was carried out using phosphate-buffered solution (PBS) and the adherence potentials was denoted using sterile 96-welled polystyrene microtitre's well with sterile TSB as negative control. *Salmonella enterica serovar* Typhimurium (ATCC 14028) was employed as positive control. The microtitre plates were incubated at 37^{o}C for 24 h, rinsed with sterile PBS and air-dried at 28±2 ^{o}C which was then stained for 30 min using crystal violet. The wells were re-rinsed using sterile deionized water and air-dried at 28±2^{o}C. Cells with the crystal violet dye adherence were further suspended in 160 mL 99% ethanol. The optical density readings at 570 nm of the respective wells were conducted with the aid of a microtitre plate reader (Synergy MxBiotekR USA). In order to get the values in mean, the assay values were determined three times. The obtained values of biofilm formation were classified as moderate, weak, strong or negative producer according to previously described protocol (Basson *et al*., 2007).

2.8 Data Analysis

Data were evaluated via Microsoft Excel 2013 and statistical package (SPSS) 21.0 version. Descriptive statistics were used to calculate and express the values in percentages (%).

3. Results and Discussion

3.1 Occurrence of Salmonella species from Slaughterhouses in Benin City

In this study, 150 effluent samples were assessed and 19(12.7%) of the samples were positive for *Salmonella* species using PCR identification techniques. The total number of *Salmonella* isolates screened and obtained from the 19 positive samples after identification via PCR was 31 isolates (Table 1). In contrast to this study, a higher prevalence rate (45.3%) of *Salmonella* species from slaughterhouse effluents in Abia State, Nigeria has been reported (Edward *et al.*, 2021). Similarly, other comparable studies have reported the occurrence of *Salmonella* in untreated effluents of slaughterhouses (Igbinosa and Kadiri, 2018; Igbinosa *et al*., 2021).

Table 1. Occurrence of *Salmonella* species from slaughterhouses effluent in Benin City

Slaughterhouses	Number of samples assessed	Number of samples haboring *Salmonella* species
Slaughterhouse A	25	2
Slaughterhouse B	25	4
Slaughterhouse C	25	3
Slaughterhouse D	25	1
Slaughterhouse E	25	5
Slaughterhouse F	25	4
Total	**150**	**19 (12.7%)**

The contamination of surface water meant for household and irrigation purposes by pluvial discharge of effluents have notably intensified their influence along the food chain (González-López *et al*., 2022). Cross-contamination of *Salmonella* in meats and other food products meant for human consumption have also been reported (Ehuwa *et al*., 2021; Okon *et al*., 2022). The detection of *Salmonella* as observed could be due to haphazard release of animal and human faecal wastes in

slaughtering environment. Although *Salmonella* is habitually present in the intestinal tract of animals and human (Aljahdali *et al*., 2020), their presence in food processing environment has been a persistent pitfall to human and animal health as it could result in diverse food and waterborne infections (Galán-Relaño *et al*., 2023). Indiscriminate discharge of effluents could contribute to the contamination of water-bodies and surrounding agricultural soils when released without treatment or inadequate treatment procedures. Previous study has notably implicated untreated abattoir effluent as a potential reservoir for the transmission of pathogenic bacteria (Akpan *et al*., 2020). Similarly, other findings reported effluent from food processing environment as a major pathway for *Salmonella* related contamination in water which could subsequently triggers the manifestation of several gastroenteritis infections, enteric fever and bacteremia (Neupane *et al*., 2021; Mkangara, 2023).

3.2 Antibiotic susceptibility profile of the isolates

The antibiotic resistance profile of *Salmonella* species was illustrated in Table 2. The *Salmonella* species isolated in this study were found to have demonstrated significant resistance to most of the antibiotic agents utilized.

Table 2. Antibiotic susceptibility profile of the isolates

Antimicrobial Class	**Antibiotics**	***Salmonella* species (*n*=31)**		
		Resistant (%)	**Intermediate (%)**	**Sensitive (%)**
Penicillins	Ampicillin	31(100)	0(0)	0(0)
	Piperacillin	21(67.74)	9(29.03)	1(3.23)
B-Lactam combination agents	Amoxicillin-clavulanate	11(35.48)	4(12.90)	16(51.61)
	Ampicillin-sulbactam	12(38.71)	6(19.35)	13(41.94)
Monobactams	Aztreonam	22(70.97)	3(9.68)	6(19.35)
Carbapenems	Meropenem	0(0)	0(0)	31(100)
	Imipenem	0(0)	0(0)	31(100)
Macrolides	Azithromycin	18(58.06)	NA	13(41.94)
Tetracyclines	Tetracycline	14(45.16)	3(9.68)	14(45.16)
	Doxycycline	12(38.71)	5(16.13)	14(45.16)
Fluoroquinolones	Ciprofloxacin	16(51.61)	3(9.68)	12(38.71)
Folate pathway antagonists	Trimethoprim-sulfamethoxazole	12(38.71)	4(12.90)	15(48.39)
Phenicols	Chloramphenicol	20(64.52)	5(16.13)	6(19.35)
Nitrofurans	Nitrofurantoin	0(0)	0(0)	31(100)

Legend : NA: Not applicable

The antibiotic resistance profile showed that the highest resistance was demonstrated against ampicillin 31(100%), aztreonam 22(70.97%), piperacillin 21(67.74%), chloramphenicol 20(64.52%), azithromycin 18(58.06%) and ciprofloxacin 16(51.61). The least resistance observed was against nitrofurantoin (0%), meropenem (0%), imipenem (0%) and amoxicillin-clavulanate (35.48%).

The total resistance (100%) demonstrated by *Salmonella* against ampicillin in this study is similar to the resistance that has been previously reported (Okafor *et al*., 2020; Igbinosa *et al*., 2021). In further agreement with this study, no resistance was demonstrated by all the *Salmonella* isolates from abattoir effluents towards nitrofurantoin in a comparable study (Igbinosa *et al*., 2021). However, this study contrarily observed a higher level of ciprofloxacin (51.61%) and tetracycline (45.16%) resistance when compared to the 31.3% and 16.7% demonstrated towards ciprofloxacin and tetracycline respectively as previously reported (Masse *et al*., 2021). Previous study has emphasized on the high level of resistance demonstrated by *Salmonella* towards beta-lactam antibiotics which include the penicillins, phenicols and carbapenems (Edward *et al*., 2021). Based on the findings of this study, penicillins and phenicols demonstrated significant resistance but there was absence of resistance demonstrated against the tested carbapenems antibiotics (meropenem and imipenem). The elevated level of resistance demonstrated towards some antibiotics in this study remains a crucial health concern.

Table 3. Multiple antibiotic resistance profile of *Salmonella* species

Isolate code	Resistance phenotype	MAR Index
LS_1, DS_{5b}, AES_{11b}	AMP^R, PTZ^R, AUG^R, AZT^R, CIP^R, SXT^R, CHL^R	0.50
VM_1	AMP^R, PTZ^R, AMS^R, AZT^R, SXT^R	0.36
VM_{5a}, VM_{5b}, AES_{11a}	AMP^R, AUG^R, AMS^R, AZT^R, AZI^R, TET^R, DOX^R, CIP^R, SXT^R, CHL^R	0.71
LS_{3b}, MS_{1a}, MS_{1b}	AMP^R, PTZ^R, AMS^R, AZI^R, TET^R, DOX^R, CIP^R, CHL^R	0.57
VM_{7b}, AES_{10}	AMP^R, PTZ^R, AUG^R, AMS^R, AZI^R, CIP^R	0.43
VM_{4a}	AMP^R, PTZ^R, AZT^R, SXT^R, CHL^R	0.36
AES_{4b}	AMP^R, PTZ^R	0.14
DS_1, DS_{5a}, AES_{4a}	AMP^R, AZT^R, AZI^R, TET^R, DOX^R, CIP^R, CHL^R	0.50
AES_{7a}, AES_{6b}	AMP^R, PTZ^R, AZT^R, AZI^R, CHL^R	0.36
AES_{3a}	AMP^R, PTZ^R, AMS^R, AZT^R, SXT^R	0.36
VM_{7a}	AMP^R, PTZ^R, AMS^R, AZT^R	0.29
DS_4	AMP^R, AUG^R, AZI^R, SXT^R	0.29
AES_{3b}	AMP^R, AMS^R, AZI^R	0.21
VM_{4b}	AMP^R	0.07
AES_{7b}	AMP^R, PTZ^R, AUG^R, AZT^R, SXT^R	0.36
MS_2, MS_4	AMP^R, PTZ^R, AZT^R, TET^R, CIP^R, CHL^R	0.43
LS_{3a}, DS_3, AES_{7a}	AMP^R, PTZ^R, AZT^R, AZI^R, TET^R, DOX^R, CHL^R	0.50
DH_1	AMP^R, AUG^R, AZT^R, SXT^R	0.29

Legend: **AUG**: Amoxicillin-clavulanate 20/10 µg, **PTZ**: Piperacillin 100 µg, **AMP**: Ampicillin 10 µg, **AMS**: Ampicillin-sulbactam 10/10 µg, **AZT**: Aztreonam 30 µg, **ETP**: Ertapenem 10 µg, **MRP**: Meropenem 10 µg, **AZI**: Azithromycin 15 µg, **TET**: Tetracycline 30 µg, **DOX**: Doxycycline 30 µg, **CIP**: Ciprofloxacin 5 µg, **SXT**: Trimethoprim-sulfamethoxazole 1.25/23.75 µg, **CHL**: Chloramphenicol 30 µg, **NIT**: Nitrofurantoin 300 µg, **MAR**: Multiple antibiotic resistance index

Previous studies have emphasized that antimicrobial resistant bacteria strains notably affect the efficiency of infection management due to reduced efficacy of available antibiotics in humans and animals therapeutics with a subsequent increase in morbidity and mortality (Parmanik *et al*., 2022). The varying rate of resistance in this study and other related studies could be as a result of variance in location of study and different level of exposure to antibiotics.

3.3 Multiple antibiotic resistance profile of Salmonella species

The *Salmonella* species isolates were resistant to a minimum of 1 antibiotic and a maximum of 10 antibiotics (Table 3). The multiple antibiotic resistance (MAR) index range from 0.07 – 0.71 and

the multidrug resistance profile revealed that 29/31(93.55%) of the isolates were resistant to a minimum of 3 antimicrobial classes and a maximum of 8 antimicrobial classes (Table 3). According to prior account, an MAR index ≥ 0.2 connotes that the organisms must have emanated from an environment where antibiotics are used extensively (Shakir *et al*., 2021). Therefore, the 29/31(93.6%) isolates having MAR index which equals or more than 0.2 in this study indicates that they might have originated from region associated with extensive antibiotic exposure. The demonstration of high MAR index by isolates from slaughterhouses have also been reported in other studies (Igbinosa and Beshiru, 2017; Abdalla *et al*., 2021). The elevated resistance to antimicrobials could be ascribed to excessive and indiscriminate use of antimicrobials in farm animals.

3.4 Biofilm formation profile of the Salmonella isolates

The profile of biofilm formation in the isolates (Figure 1) revealed the following: strong biofilm formation 8(25.81%), moderate biofilm formation 15(48.39%), weak biofilm formation 3(9.68%), no biofilm formation 5(16.13%). In total, 26/31(83.9%) of the tested isolated demonstrated biofilm formation potentials while 5/31(16.1%) were non-biofilm formers. The association of most isolate in this study with biofilm has health implications because the structural properties of biofilm have been implicated as a factor that promotes antimicrobial resistance. The health risks resulting from the antimicrobial tolerance of biofilm-related infections has been reported in previous study (Pai *et al*., 2023).

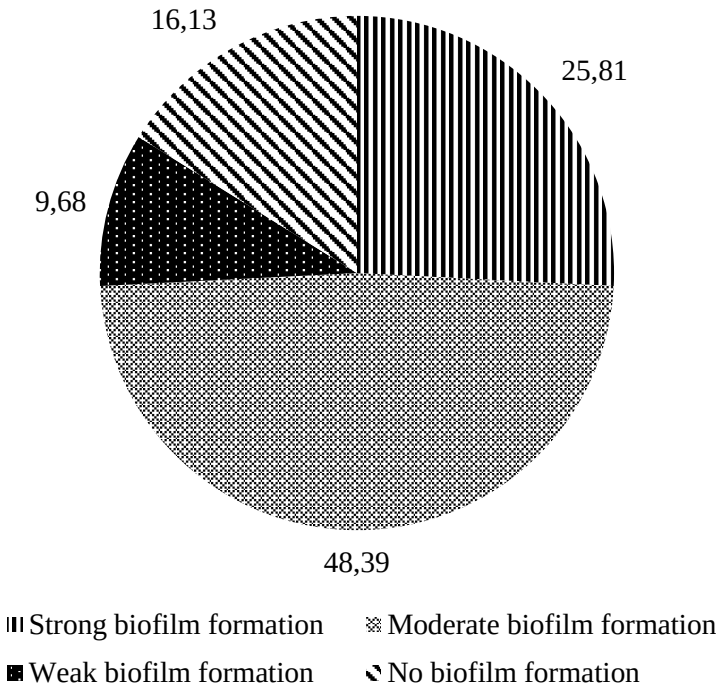

Figure 1. Biofilm formation profile of the *Salmonella* isolates

3.5 Extracellular virulence profile of Salmonella species from slaughterhouses

Extracellular virulence profile of *Salmonella* species in Figure 2 revealed the following: gelatinase production 17(54.84%), protease activity 14(45.16%), beta-haemolytic activity 21(67.74%), DNA degrading activity 15(48.39%).

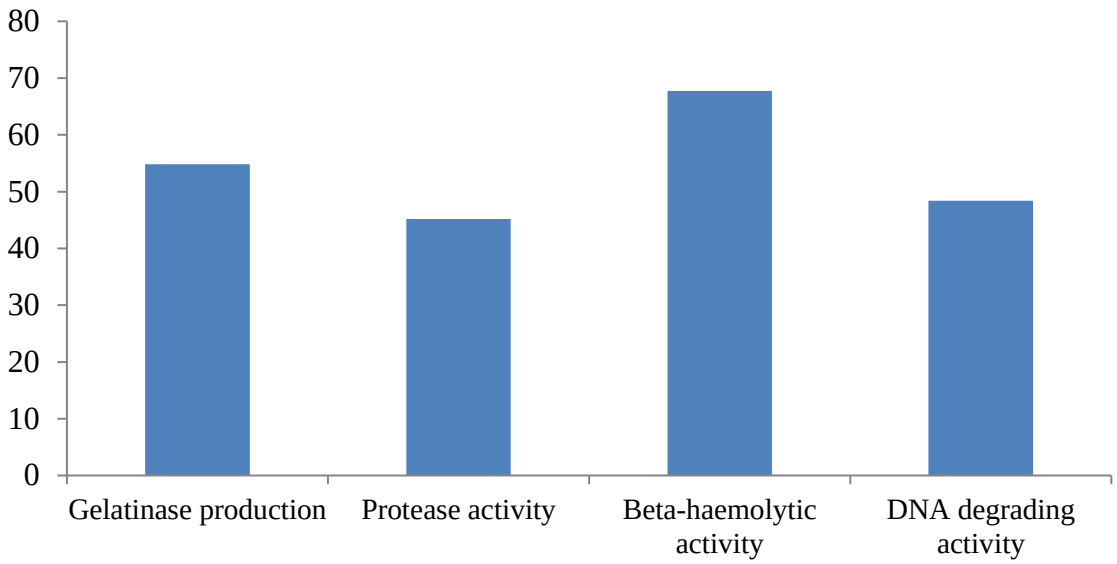

Figure 2. Extracellular virulence profile of *Salmonella* species from slaughterhouse

The impact of virulence factors in the promotion of pathogenicity and infection control has been reported (Soni *et al*., 2024; Isichei-Ukah *et al*., 2024). Therefore the *Salmonella* isolates that harbors extracellular virulence in this study indicates the possibility of difficulties in therapy should in case the bacterial activities lead to manifestation of infections.

Conclusion

The present study has elucidated the antibiotic-resistant of *Salmonella* species recovered from slaughterhouse effluents in Benin City, Nigeria. The apparently high presence of antibiotic-resistant *Salmonella* species in the effluents re-echoes it's potential to challenge public and ecosystem health as this may establish a reservoir of transferable resistance genes. These genes can be widely distributed within the slaughterhouse environment, animals and also humans through horizontal gene transfer. Hence, cogent adherence to good hygiene practices in slaughterhouses, appropriate water sanitation and the regulatory application of antibiotics is recommended in curbing the menace of antimicrobial resistance.

Disclosure statement: *Conflict of Interest:* The authors declare that there is no conflict of interest as regards the study.

Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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