Prevalence, antibiotic susceptibility and extracellular virulence factors of *Salmonella* Species Recovered from Abattoir Wastewater in Benin City, Nigeria

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ABSTRACT

The indiscriminate disposal of abattoir wastewater into the environment could result in the spread of potential pathogenic microorganisms which constitute public health challenges. The aim of the study was to determine the antibiotic susceptibility profile of *Salmonella* spp isolated from abattoir wastewater, and screen for extracellular virulence factors. A total of 48 abattoir wastewater samples from various locations in Benin City were assessed. Standard culture-based techniques and Analytical Profile Index 20E were used to identify the isolates. Antibiotic susceptibility profile was done using the Kirby-Bauer disc diffusion method. While bacterial extracellular virulence factors were examined using the standard bacteriological and microtiter plate method. Results revealed occurrence of *Salmonella* species on the samples was 14(29.2%). The distribution pattern was; Government abattoir 4(25%), Victory abattoir 8(50%), and UBTH co-operative abattoir 2(12.5%). The resistance profile of the *Salmonella* species includes ampicillin and tetracycline 8(57.1%), azithromycin 10(71.4%) nitrofurantoin 7(50%), and chloramphenicol 6(42.9%). The *Salmonella* species were also sensitive to gentamicin 12(87.5%), ciprofloxacin 11(78.6%), trimethoprim-sulfamethoxazole 10(71.4%), fosfomycin 9(64.3 %) and amoxicillin-clavulanate 7(50.0%). A total of 13 species (92.9%) were resistant to a minimum of 1 antibiotic while 10(71.4%) of the isolates were resistant to a minimum of 3 antibiotics. A total of 10(71.4%) species exhibited multiple drug resistance (MDR) with a multiple antibiotic resistance index of 3.0. The virulence factor formation for *Salmonella* species is as follows: gelatinase production (68.9%), protease activity (65.5%), *β*-haemolytic activity (68.9%), and DNA degrading activity (68.9%). The recovery of *Salmonella* species with virulence potential which also exhibited multiple antibiotic resistance calls for concern from the appropriate authorities to ensure responsible use of antimicrobials and to monitor the appearance of antimicrobial resistance to safeguard public health.

Keywords: Abattoirs, Drug Resistance, *Salmonella* Profiling, Analytical Profile Index 20E, Benin City, Susceptibility Profile.

Introduction

Salmonella is a natural inhabitant in the gastrointestinal tract of many animals, including birds, reptiles, livestock, and humans (Whiley *et al*., 2017). It is estimated that *Salmonella* species causes 93.8 million cases of gastroenteritis worldwide annually with 155,000 deaths (Majowicz *et al*., 2010). The causative source for salmonellosis has traditionally been attributed to animal origin (Haley *et al*., 2009), and untreated wastewater from abattoir, hospitals and industries. Abattoir wastes with large quantities of animal feces are often channeled directly into water bodies, used for domestic purposes by human beings.

Contamination of river body and land from wastewater could constitute a significant environmental health hazard (Coker *et al.,* 2001; Yaji *et al.,* 2006; Osibanjo *et al.,* 2007). Potential health risks from waterborne pathogens can exist in water contaminated by abattoir effluents which has attracted considerable regulatory attention and enormous mitigation efforts (Brandl *et al*., 2013).

In Nigeria, adequate waste management is lacking, such that large solid wastes and untreated effluents are common sites (Adeyemo, 2002; Adebowale *et al*., 2010) unlike in developed countries where these facilities are adequately provided (Ogbonnaya, 2008).

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These could be a source of embarrassment since conventional methods of waste management have been grossly neglected (Adedipe, 2002; Adeyemi and Adeyemo, 2007). These untreated wastewaters, are often emptied into surface water bodies, which include ponds, lakes, rivers, and streams, used for domestic purposes by human beings (Onuoha *et al.,* 2016). Rivers have been widely used as an irrigation source for agricultural practice. River water, however, has been reported to be one of the largest reservoirs of viable *Salmonella*. *Salmonella* present in water can be traced back to its animal origins. This pathogen may directly be transported from faeces or exudates of wild animals by rainwater runoff to rivers or ponds used for irrigation (Haley *et al*., 2009). Manure of domesticated animals has long been used to fertilize soil because it is economical and beneficial to the environment.

However, studies have indicated that *Salmonella* in manure can survive as long as 231 days and could eventually contaminate produce by rainwater splashing or by surface irrigation water (Islam *et al*., 2004). The average concentration of *Salmonella* in waste water can reach as high as 2.7×10^2 CFU/100 ml (Rose *et al*., 2001; Howard *et al*., 2004), which could become a major source of contamination if discharged directly or with inadequate treatment. *Salmonella* and other bacteria in wastewater can be effectively reduced to very low levels with modern treatment methods, but it is not practical to eliminate all the bacteria. When discharged, this will pose another contamination source of *Salmonella* to surface waters (Kay *et al*., 2008; Naidoo and Olaniran, 2014) as reported in the proposed mode of *Salmonella* transmission in the environment (Li *et al*., 2015).

Salmonella gastroenteritis is generally a self-limiting disease, but severe cases in very young or elderly persons, immune-compromised individuals, or patients with systemic infections may require effective chemotherapy (Lee *et al*., 2009). Quinolone and fluoroquinolone are used against a broad spectrum of bacterial pathogens in human and veterinary medicine, and with increased use, the resistance of *Salmonella* spp. to these antibiotics has increased (Crump *et al*., 2015). In Enterobacteriaceae, resistance to nalidixic acid, an elementary quinolone, correlates with decreased susceptibility to ciprofloxacin (MIC $\geq$ 0.12 µg/ml) and possible fluoroquinolone treatment failure.

Ceftiofur is a third-generation cephalosporin used in food animals in the United States; resistance to ceftiofur among Enterobacteriaceae correlates with resistance to ceftriaxone (MIC $\geq$ 4 µg/ml) (CDC, 2010). Recently, the increasing prevalence of multi-drug resistant (MDR) *Salmonella* and resistance to clinically important antimicrobial agents such as fluoroquinolones and third-generation cephalosporins have been an emerging problem worldwide (Chen *et al*., 2007). Additionally, isolated MDR *Salmonella* strains are of many serotypes such as Agona, Anatum, Choleraesuis, Derby, Dublin, Heidelberg, Kentucky, Newport, Pullorum, Schwarzengrund, Senftenberg, Typhimurium, and Uganda (Zhao *et al*., 2008; Pan *et al*., 2009). Therefore, of particular concern is the degree of MDR in *Salmonella*.

In Nigeria, a number of studies have been carried out on abattoir waste (Adeyemo, *et al.*, 2002; Nwanta, *et al*., 2010; Nafarda *et al.,* 2012; Obire and Ariyo, 2021) and it was found that several bacteria such as, *Escherichia coli* O157:H7, *Salmonella* spp, and *Shigella* spp. were present in the waste. Also, several studies in Africa have been done by different researchers on abattoir wastes and different pathogenic bacteria were recovered from untreated abattoir effluent (Abiade-Paul *et al*. 2005; Nyamboya, *et al.,* 2013; Ariyo and Obire, 2021). The contribution of abattoirs and associated wastewaters is rarely considered and yet abattoirs are potential sources of enteric bacteria that could possess antibiotic resistance genes (Onuoha *et al*, 2016). Little or no data have been reported from the study area, hence the study was designed to isolate and determine the antimicrobial resistance of the *Salmonella* isolates in abattoir waste in Benin City.

This study was therefore, aimed at isolating and identifying *Salmonella* species from wastewater in abattoirs in Benin City, to determine the antibiotic susceptibility and screen for extracellular virulence factors of the isolated *Salmonella* species.

Materials and Methods

Study area

This study was conducted in Benin City, Edo State, Nigeria between the months of January, 2019 and June, 2019. The City comprises of about five (5) Local Government Areas and located in the South-South region of Nigeria with an estimated average population of 3,206,531 in the 2006 general census (NBS, 2011).

Benin City lies between latitude 6° 11 and 6° 29 North and between longitude 5° 35 and 6° 47 East of the Greenwich Meridian which is found within the Sub humid tropical region. It has temperature of about 27 °C and an annual rainfall of over 2000 mm (Omoregbe and Omorede 2024). The study sites or designated abattoirs in Benin City from where wastewater samples will be collected include: Government Abattoir at Ikpoba slope which is a public abattoir, Victory private Abattoir Ikpoba slope and the University of Benin Teaching Hospital co-operative abattoir also regarded as a public abattoir.

Sample collection

Abattoir wastewater samples were collected from the three (3) designated abattoirs in Benin City, namely; Government Abattoir Ikpoba slope, Victory private Abattoir Ikpoba slope and University of Benin Teaching Hospital (UBTH) co-operative abattoir. Samples were collected using sterile plastic containers in the morning between 8:00 am and 9:00 am from three different abattoirs. The samples were collected from the point where it is discharged to the environment without treatment and conveyed to the Applied Microbial Processes and Environmental Health Research Laboratory, Faculty of Life Sciences, University of Benin for analysis within 24 h after collection. A total of 48 wastewater samples 16 each from Government, Victory and UBTH co-operative abattoir were collected and assessed in this study.

Isolation, Identification and Characterization of *Salmonella* species

Isolation of *Salmonella* species was carried out in accordance with the International Organization for Standardization (2017). The samples were serially diluted (10^1 - 10^{10}). An aliquot of 100 μL from 10^{-2} diluent was aseptically pipetted into 3mL Rappaport Vassiliadis Soya broth (Merck Darmstadt, Germany) and incubated for 18-24 h.
Salmonella was isolated by streaking the enriched culture broth on Xylose Lysine Deoxycholate agar (Lab M, Lancashire, United Kingdom) plates which were subsequently incubated at 37°C for 18-24 h. After incubation, distinct black colonies were categorized as presumptive *Salmonella* isolates. The presumptive isolates were sub-cultured on Tryptone Soy agar (Lab M, Lancashire, United Kingdom) and incubated at 37°C for 18-24 h.

The colonies were purified on nutrient agar (Lab M, Lancashire, United Kingdom) and thereafter stored on nutrient agar slants at 4 °C until ready for further use. Presumptive *Salmonella* colonies were further identified by Gram's reactions using 3% potassium hydroxide (3 % KOH), catalase test, oxidase test and indole activity. The isolates were further characterized biochemically using Analytical Profile Index (API) 20E. The tests were performed according to the manufacturer's instruction for use. Data interpretation was performed using the Analytical profile index (API) database (V4.1) with the apiwebTM identification software.

Phenotypic virulence factors determination

The phenotypic virulence factors of the *Salmonella* species were determined according to the method previously described by Smibert and Krieg (1994). Gelatinase production was determined by inoculating a 5.0 mL suspension of isolates on gelatin medium and incubated for 24-48 h at 37°C. Zones of clearance in the media indicated the proliferation of gelatin-liquefying microorganisms. The extracellular protease activity was assayed by growing the isolates on TSA plates that have been supplemented with 1 % casein and incubated for 24-48 h at 37°C. Zone of clearance due to casein hydrolysis was considered a positive result. For haemolytic activity, colonies grown on tryptone soy agar (TSA) (Merck, Darmstadt, Germany) were suspended in 3 mL of Mueller-Hinton broth (Lab M, Lancashire, United Kingdom). The density of this suspension was adjusted to 0.5 McFarland standards. A 5.0 mL sample of this suspension was inoculated on sheep blood agar plate and incubated at 37°C for 24-48 h. Thereafter, beta haemolysis was indicated by clear colourless zones surrounding the colonies indicating that there has been total lysis of the red blood cells. The DNA degrading activity was determined by inoculating a 5.0 mL suspension of the isolates on DNase agar plates and subsequently incubated for 24-48 h at 37°C. When DNA is hydrolyzed, it results in the release of methyl green which turns the medium colourless around the test organism.

Antimicrobial susceptibility profile of the *Salmonella* species

Isolates were subjected to antimicrobial susceptibility screening using Kirby-Bauer disc diffusion method.

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Suspension of the test isolates with an approximated turbidity 0.5 McFarland's standard was obtained and aseptically spread on Mueller-Hinton agar plates (Lab M, Lancashire, United Kingdom). The respective antibiotics discs were placed on the Mueller-Hinton agar plates aseptically. The antibiotics tested include gentamycin (10 µg), ampicillin (10 µg), nitrofurantoin (300 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), amoxicillin-clavulanate (30 µg), tetracycline (30 µg), azithromycin (15 µg), trimethoprim-sulfamethoxazole (25 µg) and fosfomycin (200 µg) (Oxoid, Basingstoke, Hampshire, United Kingdom). The plates were incubated for 18 - 24 h at 37°C. Diameter of zones of inhibition were measured and interpreted according to Clinical Laboratory Standards Institute (CLSI, 2020).

Statistical analysis

Data obtained were analyzed using descriptive statistics such as frequency counts, percentages, charts and tables to present the distribution and prevalence of fungal isolates. Comparative analysis between different sample types was carried out to highlight the most contaminated site.

Results

The occurrence of *Salmonella* species from abattoir waste water in Benin City (Table 1) varies from 50% in Victory (a private manage) abattoir to 25% and 12.5% for Government and UBTH cooperative abattoir. Table 2 shows the Antibiotic susceptibility profile of the *Salmonella* species isolated which revealed various degree of sensitivity with the isolates most sensitive to gentamicin (87.5%) while azithromycin and ampicillin are the agent the isolates are least sensitive to (28.6%).

The result also revealed isolates high resistance to azithromycin and mildly intermediate to some of the selected antibiotics. 68.9 % of the isolates showed gelatinase production, beta haemolytic activity and DNA degrading activity as virulent factorsas shown in figure 1.

A total of 13 (89.6 %) of the *Salmonella* species were resistant to a minimum of 1 antibiotic while 10 (68.9 %) of the isolates were resistant to a minimum of 3 antibiotics as shown in Table 3.

Table 1: Occurrence of *Salmonella* species from samples

Wastewater Source	Number of Samples Assessed	*Salmonella* positive samples (%)
Government abattoir (public)	16	4(25)
Victory abattoir (private)	16	8(50)
UBTH co-operative abattoir (private)	16	2(12.5)

Table 2: Antibiotic susceptibility profile of the *Salmonella* species

Antimicrobial class	Antibiotics	Susceptibility profile of *Salmonella* species (*n*=14)		
		Resistance (%)	Intermediate (%)	Sensitive (%)
Aminoglycosides	GEN	2(12.5)	0	12(87.5)
Penicillins	AMP	8(57.1)	2(14.3)	4(28.6)
Nitrofurans	NIT	7(50)	2(14.3)	5(35.7)
Fluoroquinolones	CIP	1(7.1)	2(14.3)	11(78.6)
Phenicols	CHL	6(42.9)	1(7.1)	7(50.0)
Tetracyclines	TET	8(57.1)	1(7.1)	5(35.8)
Macrolides	AZM	10(71.4)	NA	4(28.6)
Folate pathway antagonists	SXT	4(28.6)	0	10(71.4)
Fosfomycins	FOS	5(35.7)	0	9(64.3)
β-lactam combination agents	AUG	6(42.9)	1(7.1)	7(50.0)

Legend: GEN: gentamicin, AMP: ampicillin, NIT: nitrofurantoin, CIP: ciprofloxacin, CHL: chloramphenicol, TET: tetracycline, AZM: azithromycin, SXT: trimethoprim-sulfamethoxazole, FOS: fosfomycin, AUG: amoxicillin-clavulanate.
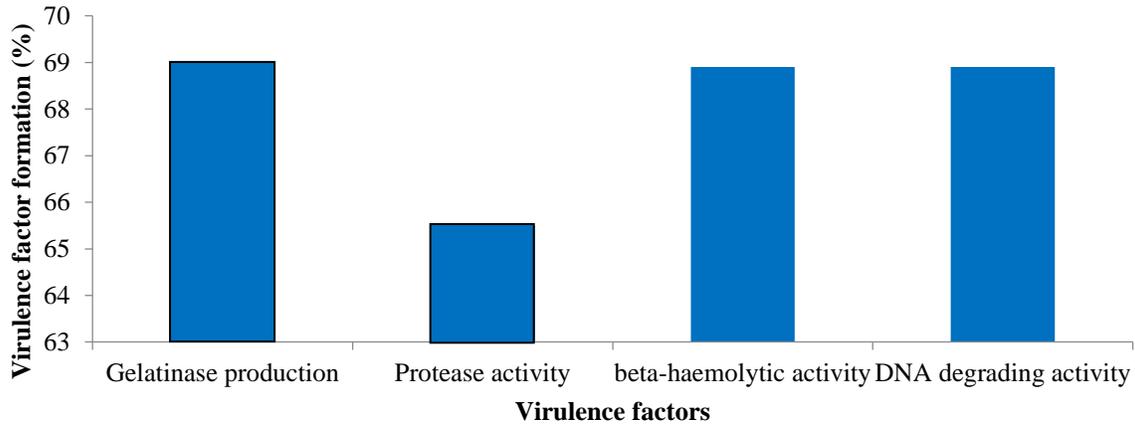
Figure 1: Virulence factor formation of *Salmonella* species

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

Isolate Code	Resistance Phenotype (Antibiotics)	MAR Index
Cii,	AMP/NIT/TET/AZM/FOS/AUG	0.6
A5ii	AMP/NIT/TET/AZM/FOS/AUG	0.6
B3i,	AMP/TET/AZM/SXT	0.4
B5iii	AMP/TET/AZM/SXT	0.4
A4i	CHL/TET/AZM/SXT	0.4
A3i	AMP/NIT/CHL	0.3
B4i	CHL/TET/AZM	0.3
C2iii	AMP/CHL/AUG	0.3
C4i	NIT/TET/AZM	0.3
B4ii	NIT/TET/AZM	0.3
B6i	AZM/AUG	0.2
C5i	AZM/AUG	0.2
B6i	TET/AZM	0.2
D6i	AZM/AUG	0.2

Discussion

The study revealed the prevalence, antibiotic susceptibility and extracellular virulence factors of *salmonella* species recovered from abattoir wastewater in Benin City. Every year millions of *Salmonella* cases are reported worldwide and the disease results into thousands of deaths (Pui *et al*., 2011; Tadesse and Tessema, 2014). In Nigeria, the meat industry has experienced a rise in the rate of consumption of its product. Unfortunately, quite a lot of slaughterhouses hardly adhere strictly to recommended standard operation procedures which can lead to cross contamination from the wastewater.

The occurrences of *Salmonella* spp isolated from the Government abattoir, Victory and UBTH Cooperative abattoirs, based on API 20E identification, was 4/16 (25 %), 8/16 (50 %) and 2/16 (12.5 %) respectively. The overall prevalence of the *Salmonella* isolates is 14/48 (29.2 %). These numbers reveal a high prevalence of *Salmonella* in the abattoir wastewaters. This prevalence according to Onuoha *et al*. (2016) can be attributed to the poor sanitary and hygienic practices of the abattoirs' management and workers, the poor state of health of the slaughtered cows and contamination from the ruminal content of the slaughtered animals.

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Studies by Kupriyanoy *et al*. (2010) revealed that *Salmonella* can persist in farm surrounding for long period of time owing to movement of animals and humans inside the farm and from livestock excrement, soil and plants. *Salmonella* is also known to be the most extensively spread organism that has the ability to cause foodborne infections and can be transmitted to man through water and cow (Eguale, 2018). The prevalence of Salmonella in this study was found to be higher than 16.04 % that was recorded by Gautam *et al*. (2019), and however support the 22.64 % and 30.19 % recorded by Omeregbe *et al*. (2017) from abattoir effluents and lower than 45.3 % recorded by Edward *et al*. (2021). Ayogu *et al*. (2018) reported a high prevalence rate of 60 % from meat market and Ogoja Road abattoir effluents in Abakiliki metropolis. However, lower level of prevalence was observed in studies reported by Mthembu *et al*. (2019) 5 % [South Africa], Ejo *et al*. (2016) 5.5 % [Ethiopia], Sjölund-Karlsson *et al*. (2013) 5.7 % [United States and Canada]. The variation in the level of prevalence could be due to difference in product sampled, sampling techniques, variant locations and different hygiene practices.

The susceptibility profile of the *Salmonella* species in Table 2 showed that all isolates were susceptible to the10 drugs used at different degree. Gentamycin, Ciprofloxacin and trimethoprim-sulfamethoxazole are the drug the different species of *Salmonella* were most sensitive to (87.5 %, 78.6 % and 71.4 %) respectively and are suggested as best drug for the treatment of the isolates obtain from this study. This finding corroborates with the findings of Edward *et al.,* (2021). Who found Gentamicin to be (89.5 %) sensitive to *Salmonella* species from abattoir waste water in Abia state Nigeria. Igbinosa *et al*. (2016) also in their study found Gentamicin to be (89.5 %) sensitive and Ofloxacin (89.3 %) of their isolated *Salmonella* species.

The findings of this study (Table 2) revealed that 71.4 % of the *Salmonella* isolates were resistant to Azithromycin, 57.1 % were resistant to Tetracycline and Ampicillin, and 50% were resistant to Nitrofurantoin. Nair *et al*. (2016) attributed the resistance to azithromycin to a macrolide repressor protein produced by *Salmonella* spp. which inactivates the antibiotics. This study agrees with previous study by Mthembu *et al*. (2019) in which resistance was demonstrated by *Salmonella* spp. isolated from livestock to ampicillin (64%) and tetracycline (63%).

According to Edward *et al*. (2021), antimicrobial resistance associated with livestock might be a consequence of excessive use and indiscriminate dumping of antimicrobials. This can also be as a result of abusive use of these antimicrobial agents by herders, even without formal prescription by a veterinary doctor. This study however contracts the previous studies by Igbinosa *et al*. (2016) who recorded 100% resistance to Ampicillin and Chloramphenicol, 92.3% to Augmentin and 85.2 % to Sulfamethoxazole. This study also agrees with previous study by Mthembu *et al*. (2019) in which resistance was demonstrated by *Salmonella* spp. isolated from livestock to ampicillin (64%) and tetracycline (63%). ***The MAR index*** range in this study (0.2-0.6) does not correlate with the report of Naik *et al*. (2015) [India]; Adzitey *et al*. (2020) [Ghana]; Igbinosa and Beshiru, (2017) [Nigeria] and Ayogu et al. (2018) [Nigeria] in which the MAR index range from 0.00-0.50, 0.00-0.63, 0.3-1 and 0.5-0.9 respectively. Furthermore, in this study 88.46 % of the isolates were found to have MAR index equal to or more than 0.2, thus incriminating the origin of these isolates to have been exposed to extensive antibiotics usage. Earlier reports by Chrinius *et al*. (2014) and Hammuel *et al*. (2015) stated that MAR index ≥ 0.2 indicate that the organisms must have originated from an environment where antibiotics are used frequently. A total of 13 (89.6 %) of the *Salmonella* species were resistant to a minimum of 1 antibiotic while 10 (68.9 %) of the isolates were resistant to a minimum of 3 antibiotics as shown in table 3. A total of 10 (68.9 %) of the *Salmonella* species were multidrug resistance (resistance to ≥1 antibiotics in ≥3 antimicrobial class) and also possess multiple antibiotic resistance index of 3.0.

The findings of this study (Figure 1) also revealed the virulence factor formation for *Salmonella* species as follows: gelatinase production 10 (68.9 %), protease activity 9 (65.5 %), beta haemolytic activity 10 (68.9 %), DNA degrading activity 10 (68.9 %). *Salmonella* virulence potentials detected in this study was also reported earlier in a study by Beshiru and Igbinosa. (2018). Previous report by Turki *et al*. (2014) has reported positive correlation of *Salmonella* virulence with antibiotic resistance phenotypes. Beshiru *et al*. (2019) also affirm the involvement of virulence determinants in the pathogenicity of bacteria; therefore, their occurrence in *Salmonella* can result into salmonellosis.

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Conclusion

In conclusion, this study has demonstrated the presence of multidrug resistant *Salmonella* species with virulence potentials in abattoir wastewaters in Benin City and these calls for concern. The occurrence of *Salmonella* in abattoir wastewater should be given serious attention by the relevant authorities as it signals towards public health hazard and potential food borne intoxication. On this note, abattoir workers and meat retailers should be enlightened on the adverse effect of using contaminated water and equipment in meat processing. It is also of vital importance that meat handlers should observe strict hygienic measures in order to prevent cross contamination. The extensive use of antimicrobials in livestock production has already resulted in acquisition of resistance by *Salmonella* species. Therefore, the prudent and judicious use of antimicrobials in the agriculture and public health sectors is very mandatory in order to subdue the ever- increasing rate of antimicrobial resistant *Salmonella* strains. The resistance profiles of *Salmonella* strains and other potential pathogenic factors must be monitored carefully and continuously as this will minimize public health risks.

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