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SHORT COMMUNICATION

Serogroups and antibiogram of *Salmonella* isolates from dairy cattle in Nkonkobe District, South Africa

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ABSTRACT

Aims: The use of antimicrobial agent for treatment or growth promotion has added burden to treat infection diseases caused by pathogenic bacteria as they can acquire resistance. *Salmonella* is one of the major zoonotic bacterial pathogens that acts as a potential reservoir of antimicrobial resistance elements. In this study, the presence of *Salmonella* serotypes and the antibiogram patterns of the isolates from fecal samples of healthy cows in some selected localities in Eastern Cape, South Africa were studied.
Methodology and results: Two hundred fecal samples were collected from healthy adult cows, of which 180 presumptive *Salmonella* isolates were recovered by conventional method. The isolates were identified using specific primer sets that are capable of detecting *Salmonella* spp. as well as delineating them into serogroups A, B, C1, C2, and D. Thereafter, antimicrobial susceptibility patterns of the identified isolates were determined by disk diffusion method against a panel of 12 antibiotics. From the molecular analysis of the isolates, 108 isolates were identified as *Salmonella* spp. and the confirmed isolates were further delineated into serogroup and the prevalence of the serogroups detected were 20%, 18%, 2%, 20% and 40% for serogroup A, B, C1, C2 and D respectively. Extremely high levels of antibiotic resistances were observed among the study isolates, while serogroup D was the most prevalent serogroup among the study isolates.
Conclusion, significance and impact of study: In conclusion, dairy cows could be considered as major reservoirs of antibiotic resistant *Salmonella* spp. that could be transmitted to humans via the food chain. This poses a significant public health risk especially to people living around the farms as well as those who consume poorly cooked meat and those who deal on raw cow meat.

Keywords: Antibiotics, fecal, resistance, *Salmonella*, serogroups

INTRODUCTION

Salmonella species are zoonotic bacterial pathogens that cause salmonellosis in animals and humans (Kemal *et al.*, 2015; Mohammadzadeh *et al.*, 2017; WHO, 2018). Two species *Salmonella enterica* and *Salmonella bongori* have been recognized in the current classification of the organism. *Salmonella enterica* has six subspecies, 2500 serovars and are regularly associated with warm-blooded animals and humans (Lamas *et al.*, 2018). Human infected with *S. enterica* normally will result in two major groups of diseases: gastroenteritis and typhoid fever (Smith *et al.*, 2019). Gastroenteritis is mainly caused by non-typhoidal serovars from foodborne sources (Asante *et al.*, 2019). Non-typhoidal *Salmonella* spp. (NTS) is estimated to cause 93.8 million cases of acute gastroenteritis and 155,000 deaths globally each year (Lo *et al.*, 2020). This makes *Salmonella* infections become a significant public health concern.

Animals such as swine, poultry and cattle are considered as the main sources of human salmonellosis as these animals are asymptomatic carriers with sporadic fecal shedding of the organism (de Freitas Neto *et al.*, 2010; Magwedere *et al.*, 2015; Hossain *et al.*, 2019). It is reported that fecal shedding of animals such as cattle is the leading source of water, food and environment contamination. Evisceration of animals during slaughter is another route of transmission or spread of *Salmonella* which the meats are contaminated with fecal materials and this practice is regarded as one of the most significant sources of carcass contamination at slaughterhouses (Kagambèga *et al.*, 2013).

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The uses of antibiotics in animal husbandry for prophylaxis, metaphylaxis and therapeutic purposes have been implicated as contributing factors for the development of antibiotic resistant bacteria that can be transmitted to humans through the food chain. Equally, antibiotics are sometimes used as growth promoters in an attempt to reduce bacterial pathogens which though do not cause serious infections but could retard the growth of farm animals. Globally, the indiscriminate use of antibiotics in veterinary practices is a major concern as this usually facilitates the evolution of multidrug-resistant bacteria. Antibiotics in animal husbandry are extensively used to prevent, control and treat bacterial infections as well as growth promoters (Seiffert *et al.*, 2013). These above-mentioned scenarios are more troublesome in resource constrained settings where abuse and lack of control over their usage is a public health crisis that needs to be mitigated by strong regulatory policy (Reardon, 2014). Antibiotics resistant bacteria could cause diseases in humans or transmit their resistance genes horizontally to pathogenic bacteria (Andersson and Hughes, 2014).

Human salmonellosis cases caused by antimicrobial resistant *Salmonella* spp. are increasing in many countries (Kuang *et al.*, 2015). Infection caused by resistant *Salmonella* spp. are more severe and difficult to control and the increasing prevalence of antimicrobial resistance in *Salmonella* spp. can limit treatment options available for clinical cases that need antimicrobial treatment (de Oliveira *et al.*, 2012). Additionally, in sub-Saharan Africa, NTS serogroups are regular bloodstream isolates in febrile or feverish patients. Nevertheless, little is known about these bacterial pathogens and their environmental reservoirs (Kagambèga *et al.*, 2013), yet serogroup prevalence continues to change and antimicrobial resistant isolates continues to increase. Continuous monitoring of *Salmonella* in livestock is mandatory especially in dairy cattle that serves as reservoir for *Salmonella* and have been implicated in human *Salmonella* infections (CDC, 2020). Hence, this study reports the prevalence of *Salmonella* serotypes and their antimicrobial resistance profiles isolated from apparently healthy cattle in Nkonkobe District of South Africa.

MATERIALS AND METHODS

Sample collection

Two hundred fecal samples were collected between July and September 2017 from healthy adult cattle in two commercial farms (Fort Beaufort 32.7663° S, 26.6200° E and Alice –32°46'59.99" S 26°49'59.99" E) within the Nkonkobe Municipality Eastern Cape Province, South Africa with sterile swab sticks. Approximately six samples each were collected weekly from the two farms and they were transported immediately in ice to the Applied Environmental Microbiology Research Group (AEMREG) Laboratory of the University of Fort Hare, South Africa for analyses.

Isolation of *Salmonella*

The method of Kagambèga *et al.* (2013) was adopted with little modification for the isolation of *Salmonella* species. Briefly, 25 g from each fecal samples were enriched in 225 mL of tryptic soy broth (TSB), incubated at 37 °C for 24 h. Thereafter, 0.1 mL of the enriched samples was added into 10 mL of Muller-Kauffman tetrathionate broth incubated at 42 °C for 24 h. After incubation, a loopful from the broth culture from each test tubes were plated onto xylose lysine deoxycholate (XLD) agar (Merck, South Africa) incubated at 37 °C for 22-24 h. The presumptive *Salmonella* isolates which were red colonies with black centers were purified on nutrient agar (NA) at 37 °C for 24 h and preserved it in 30% glycerol at –80 °C for further analyses.

DNA extraction

The DNA was extracted by boiling method as described by Iweriebor *et al.* (2015) with some modification. Briefly, glycerol stocks were resuscitated in nutrient broth and grown overnight from which 200 µL was aspirated into DNAase/RNAase free micro centrifuge tubes and centrifuged at 10,000 rpm. The supernatant was discarded and 200 µL of sterile nuclease free water was added into the tube. The suspension was vortexed, and the cells were lysed by heating at 100 °C for 15 min. The cell debris was centrifuged at 13,500 rpm for 10 min and the supernatants containing the genomic DNA templates were carefully transferred into another sterile Eppendorf tube and stored at –20 °C for further assays.

Molecular identification of *Salmonella* species and delineation of *Salmonella* serogroups

The presumptive isolates were screened for the confirmation of *Salmonella* species and *Salmonella* serogroups delineation into A, B, C1, C2 and D using polymerase chain reaction (PCR) technique. The PCR amplification was carried out in 25 µL reaction volume. The PCR primers and the conditions used are shown in Table 1. The amplified PCR products were resolved in 1.5% agarose gel stained with ethidium bromide in a TAE buffer at 100 V for 60 min and visualized under the UV transilluminator (Alliance 4.7). Nuclease free water was used as negative control, while previously confirmed *Salmonella* spp. from our laboratory served as positive control in the PCR confirmation of the presumptive isolates.

Antibiotic susceptibility testing

The phenotypic antibiotic susceptibility patterns of the identified *Salmonella* isolates were determined by the Kirby-Bauer disk diffusion technique (Hudzicki, 2009) against 12 antibiotics. The test was done by disk diffusion on Mueller-Hinton agar plates with each bacteria concentration equivalent to 0.5 McFarland standards, incubated aerobically at 37 °C for 18-24 h. The results of

Table 1: Primers used for detection of *Salmonella* spp. and serogroups delineation.

Target	Gene	Primer sequence (5' to 3')	Base pair	PCR condition	Cycles	Reference
Salmonella spp.	16S rRNA	F- AGCCAACCATTGCTAAATTGGCGCA R- GGTAGAAATTCCCAGCGGGTACTG	429	95 °C, 1 min; 94 °C, 15 sec; 57 °C, 15 sec; 72 °C, 30 sec; 72 °C, 8 min	30	(Aabo *et al*., 1993)
Serogroup A	*Prt*	F- TCACGACTTACATCCTAC R- CTGCTATATCAGCACAAC	720	94 °C, 1 min; 94 °C, 50 sec; 60 °C, 50 sec; 72 °C, 3 min; 72 °C, 10 min	35	(Luk *et al*., 1993)
Serogroup B	*RfbJ*	F- TGAAAGAATATGTAATTGTCAGTGG R- TTTCATTATCTCTTTGCTCTATCG	789	94 °C, 3 min; 94 °C, 50 sec; 60 °C, 50 sec; 72 °C, 50 sec; 72 °C, 10 min	35	(Franklin *et al*., 2011)
Serogroup C1	*wbaA*	F- TTGGCAGACTGGTACTGATTGG R- GCAGGAATCCGTGTAAAAATTC	976	94 °C, 3 min; 94 °C, 50 sec; 60 °C, 50 sec; 72 °C, 50 sec; 72 °C, 10 min	35	(Franklin *et al*., 2011)
Serogroup C2	*rfbJ*	F- GAACCCCTATATCTGAACAAT R- CTCGGCACTCCAACCTAATC	593	94 °C, 3 min; 94 °C, 50 sec; 60 °C, 50 sec; 72 °C, 50 sec; 72 °C, 10 min	35	(Franklin *et al*., 2011)
Serogroup D	*Prt*	F- AGTCACGACTTACATCCTAC R- ACCTGCTATATCAGCACAAC	703	94 °C, 3 min; 94 °C, 50 sec; 60 °C, 50 sec; 72 °C, 50 sec; 72 °C, 10 min	35	(Luk *et al*., 1993)

their susceptibility patterns were recorded according to Clinical and Laboratory Standards Institute guideline (CLSI, 2015) and all intermediate resistances were considered as resistant after measuring the zones of inhibition according to the limits set by CLSI (≤11 mm, ≤10 mm, ≤12 mm, ≤17 mm, ≤12 mm, ≤17 mm, ≤19 mm, ≤12 mm, ≤14 mm, ≤22 mm, ≤19 mm, ≤13 mm respectively for the antibiotics tested as listed below). The choice of antibiotics used in the susceptibility testing was based on the empiric antimicrobial agents frequently prescribed in the locality. The antibiotics used were as follow: cefuroxime (30 µg), erythromycin (15 µg), penicillin (10 µg), norfloxacin (10 µg), trimethoprim (1.25 µg), sulphametoxazole (23.75 µg), cefotaxime (30 µg), imipenem (10 µg), neomycin (10 µg) and ceftazidime (10 µg), meropenem (10 µg) and cephalothin (30 µg).

RESULTS

Analysis of serogroup distribution pattern of the *Salmonella* isolates

From the conventional culture technique, 180 presumptive *Salmonella* isolates were obtained and analyzed using PCR, of which 108 (60%) isolates were confirmed as *Salmonella* spp. (Figure 1). The 108 confirmed isolates were further delineated into five serogroups belonging to serogroups A 20% (22), B 18% (19), C1 2% (2), C2 20% (22) and D 40% (43). The representative gel images of the identified serogroups are shown in Figures 2 to 6. From the PCR analysis, serogroup D was the most prevalent and serogroup C1 was the least (Table 2).

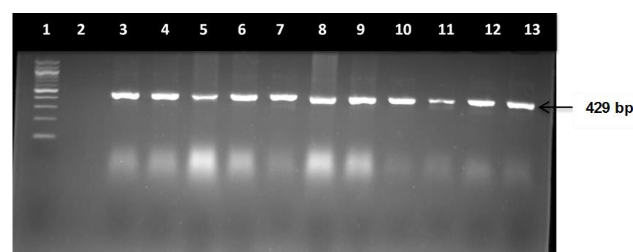

Figure 1: PCR products of some identified *Salmonella* isolates. Lane 1: 100 bp DNA molecular weight marker, Lane 2: Negative control, Lane 3: Positive control, Lanes 4-13: Confirmed study isolates.

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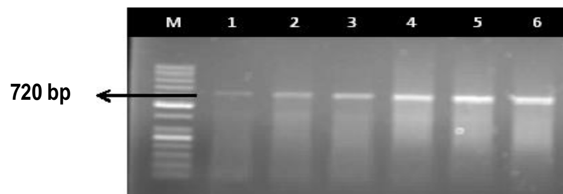

Figure 2: Electrophoretic gel of PCR products of *Salmonella* serogroup A amplification among confirmed isolates. Lane M: 100 bp ladder; Lanes 1-6: Some of the positive isolates.

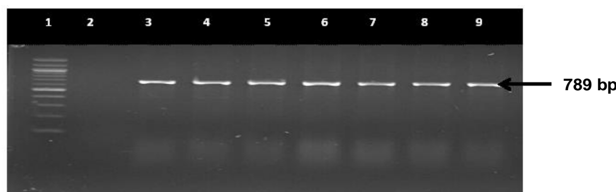

Figure 3: Electrophoretic gel of PCR products of *Salmonella* serogroup B amplification among confirmed isolates. Lane 1: 100 bp ladder, Lane 2: Negative control, Lanes 3-9: Some of the positive isolates.

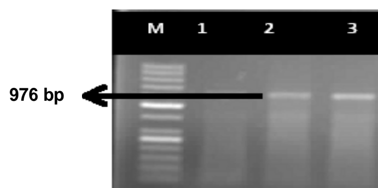

Figure 4: Gel image of *Salmonella* serogroup C1. Lane M: 100 bp ladder, Lane 1: Negative control, Lanes 2 and 3: The amplified serogroup C1.

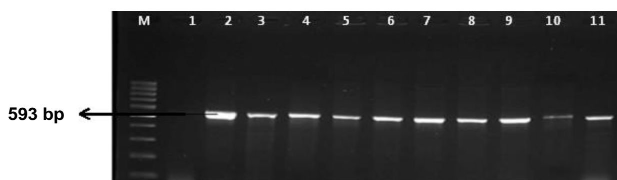

Figure 5: Gel image of *Salmonella* serogroup C2. Lane M: 100 bp ladder, Lane 1: Negative control, Lanes 2-11: The amplified serogroup C2.

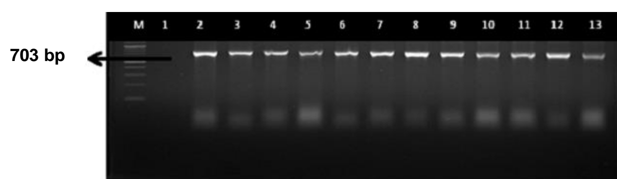

Figure 6: Gel image of *Salmonella* serogroup D. Lane M: 100 bp ladder, Lane 1: Negative control, Lanes 2-13: The identified serogroup D.

Table 2: Serogroups of *Salmonella* isolates obtained from dairy cattle.

Salmonella serogroups	No. of positive isolates
A	22 (20%)
B	19 (18%)
C1	2 (2%)
C2	22 (20%)
D	43 (40%)

Phenotypic resistance of *Salmonella* serogroups

From the 108 isolates belonging to the five serogroups, it was found that the isolates were highly resistant to most of the antibiotics, except for cefotaxime that had the highest sensitivity rate as shown in Table 3.

Table 3: Frequency of resistance against antimicrobial agents of 108 *Salmonella* isolates in this study.

Antimicrobial agents	Resistant % (n=10)	Zone of inhibition
Erythromycin	81 (75%)	≤11 mm
Trimethoprim	79 (73%)	≤10 mm
Neomycin	81 (75%)	≤12 mm
Cephalothin	70 (65%)	≤17 mm
Norfloxacin	69 (64%)	≤12 mm
Ceftazidime	74 (69%)	≤17 mm
Imipenem	53 (49%)	≤19 mm
Sulphamethoxazole	81 (75%)	≤12 mm
Cefuroxime	81 (75%)	≤14 mm
Cefotaxime	50 (46%)	≤22 mm
Meropenem	80 (74%)	≤19 mm
Penicillin	81 (75%)	≤13 mm

The percentage resistance among the confirmed isolates was higher to erythromycin, neomycin, sulfamethoxazole, penicillin and to the cephalosporin such as cefuroxime and cephalothin, while 46% of the isolates were resistant to cefotaxime. Similarly of the carbapenems (a class of antibiotics to which imipenem and meropenem belong) tested against the isolates, they exhibited comparatively lower percentage of resistance to imipenem (49%), but high level of resistance to meropenem (74%) among the study isolates as shown in Table 3. Serogroup C1 isolates were sensitive to neomycin and cephalothin, all isolates in serogroup A were resistant to erythromycin, cefuroxime and penicillin, while only 11 isolates were resistant to norfloxacin. Above 56% of all serogroup D isolates were resistant to all the antibiotics tested, while 53% of serogroup B isolates exhibited resistance to the antibiotics as shown in Table 4.

DISCUSSION

Salmonella originating from animals can infect humans through the food chain. Poorly pasteurized and contaminated milk could ultimately result in human salmonellosis (Awad *et al.*, 2020). Contamination of

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Table 4: Frequency of resistance patterns of the serogroups to the antibiotics.

Serogroups	Antimicrobial agents											
	Ery	Try	Neo	Ceph	Nor	Imi	Sul	Cefu	Mer	Pen	Cefo	Ceft
A (n=22)	22	20	20	16	11	15	21	22	21	22	12	17
B (n=19)	15	15	16	14	13	10	14	15	16	13	10	13
C1 (n=2)	2	1	0	0	1	2	2	2	1	2	2	2
C2 (n=22)	18	15	20	10	15	8	18	18	14	19	14	18
D (n=43)	24	28	25	30	29	18	26	24	28	25	20	24

Ery = Erythromycin, Try = Trimethroprim, Neo = Neomycin, Ceph = Cephalothin, Nor = Norfloxacin, Imi = Imipenem, Sul = Sulphamethoxazole, Cefu = Cefuroxime, Mer = Meropenem, Cefo = Cefotaxime and Ceft = Ceftazidime.

ground beef could arise during the slaughtering process; wherein contaminated whole cuts of meat trimmings are mixed. According to Centers for Disease Control and Prevention (CDC), approximately 50% of ground beef originating from culled dairy cows in USA are contaminated with *Salmonella* which is eventually transmitted through improper cooking to humans, thus resulting in salmonellosis (CDC, 2020). Similarly, drinking water that is contaminated with fecal materials from animals can equally result in outbreaks of human salmonellosis. Since *Salmonella* has the ability of spreading through several routes, a better knowledge of its prevalence in dairy cows is epidemiological importance.

Food producing animals such as cattle plays a principal role as one of the major sources of human salmonellosis, and a significant number of *Salmonella* serotypes often recovered from sick or healthy animals and humans. In addition, some human salmonellosis cases have been linked to direct exposure to cattle or consumption of contaminated beef (Hoelzer *et al*., 2011). From the PCR analysis, 36% (108/200) of the isolates recovered from cattle fecal samples were confirmed to be *Salmonella* and this result is in line with the study of Kagambèga *et al.* (2013) as they also detected 52% of *Salmonella* species in cattle fecal samples. This prevalence is high compared to 3.6% reported by Barilli *et al.* (2018) in Northern Italy and 2.46% by Deguenon *et al.* (2019). Differences in isolation rates could be attributed to collection seasons, culture and isolation methods and local environmental conditions such as overcrowding of animals which increases their contact and enhances transmission of pathogens within herds.

In this present study, serogroup D was the major serogroup detected compared to other serogroups profiled. The most probably reason for this observation is likely due to high endemicity of the serogroup in dairy cows within the study farms. Globally, serogroup D has been reported as the major cause of human salmonellosis (Mubita *et al*., 2020). Increase in gastroenteritis caused by serogroup D has been reported in previous studies in US and Europe (Elhadi *et al*., 2013). Detection of *Salmonella* in meat has been known as sources of human infection (Kagirita *et al*., 2017). Therefore, detection of *Salmonella* serotypes in cattle fecal samples raises public health concerns as this could cause direct or indirect meat contamination with resultant human infections as a result of consumption of undercooked meat.

There has been an upsurge in the incidence of antibiotic-resistant bacteria strains including *Salmonella* (Wang *et al*., 2019). Hence, *in vitro* antimicrobial susceptibility testing is significant to better understand the resistance patterns or trends in *Salmonella* species and to help guide in the choice of appropriate antibiotic for the treatment of human salmonellosis (Wang *et al*., 2019). The serogroups were highly resistant to a wide range of antimicrobial agents tested as shown in Table 4. This is probably due to the irrational use of antibiotics in disease management among food animals. High resistance in *Salmonella* from dairy cattle has increased in the last few decades globally (Eguale *et al*., 2016; Ketema *et al*., 2018). The usage of antibiotics in the control of *Salmonella* in farm animals often results in the evolution of antibiotic resistances in the organism and these resistant strains could ultimately be transmitted to humans through the food chain, water and vegetables cultivated using farm manure. One of the primary causes of antibiotic resistance is the indiscriminate use of antibiotics as growth promoters alongside treatments of animals and human bacterial infections. Therefore, it is very important to mitigate the inappropriate use of antibiotics in human and veterinary medicines through stringent regulatory policies.

Previous studies have shown that *Salmonella* serogroups isolated from farm animals overlapped with those that cause illnesses in humans (de Jong *et al*., 2009). In the Eastern Cape Province of South Africa, cows generally roam freely at pasture in the bush and this can contaminate river with their excreta. This movement potentialy contributes to the spread of antibiotic-resistant bacteria from animals to humans via the food chain (de Jong *et al*., 2009).

CONCLUSION

In this study, different *Salmonella* serogroups recovered from cattle fecal samples were identified and their antibiotic resistance patterns were determined. There were high antibiotic resistances among the isolates and one of the consequences of this finding is that it could contribute to treatment failures and limit the choice of antimicrobials agents in the treatment of human salmonellosis within the study areas. The current study highlights the importance of continued monitoring of *Salmonella* in farms and the prudent application of antimicrobials for veterinary usage in order to better

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address potential selection pressure for resistance to drugs of critical importance to human health. The limitation of the study is that molecular profiling of the resistance genes was not performed, and this could have added more information to the study.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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