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ANTIMICROBIAL SUSCEPTIBILITY AND ESBLs PROFILES OF NON-TYPHOIDAL SALMONELLAE FROM POULTRY DROPPINGS IN KATSINA AND AKOKA, LAGOS, NIGERIA.

[2]Adeleye, I.A., *Abdulkadir, B[1]., Abdullahi, I[1]., Mujahid, H.,[1] Musa, I[3]., Ahmad, M. A[1]., Raubilu, I. A.[1] and Isah U[1].

[1]Department of Microbiology, Umaru Musa Yar'adua University, Katsina

[2]Department of Microbiology, University of Lagos Nigeria

[3]Department of Microbiology Kebbi State University of Science and Technology Aliero

*Corresponding Author: bashir.abdulkadir@umyu.edu.ng

ABSTRACT

Expression of extended-spectrum beta lactamases is a key property that confers on a microorganism ability to resist certain antimicrobials. Non-typhoidal salmonellae* (NTS) *commonly associated with poultry droppings are known to possess these enzymes. This study was aimed at determining the antimicrobial susceptibility and ESBLs profiles of non-typhoidal salmonellae isolated from poultry droppings obtained from Katsina and Akoka, Lagos, Nigeria. Nine (9) non-typhoidal Salmonellae, previously identified via the Kaufman-White serotyping scheme, and obtained from chicken, were received from University of Lagos, Akoka, Lagos State, in a transport medium; whereas, 15 chicken dropping samples were collected from Katsina. Each of the poultry droppings samples was enriched in Nutrient Broth for 24 hours. The isolates so obtained were inoculated onto Salmonella Shigella Agar, MacConkey Agar and Nutrient Agar for observance of colonial morphology and morphological characterization. Both the isolates from Katsina and Lagos were confirmed using Gram's stain and biochemical characterization. Subsequent to Gram's stain, the Gram negative rods observed were further subjected to biochemical tests such as the IMViC group of tests, triple sugar iron agar test, motility test and urease tests to identify them. A total of 6 non-typhoidal salmonellae were obtained from the Katsina chicken droppings samples. Antimicrobial susceptibility testing was done on both Lagos and Katsina isolates, using the disk diffusion method using the standard gram negative disk and the results were interpreted using the Kirby-Bauer chart. Phenotypic characterization of extended spectrum β-lactamases was done using the the double disk diffusion method, with beta-lactam antibiotics disks containing Amoxicillin-Clavulanic Acid, Ceftaxidime, Azetreonam and Cefotaxim used as the test antibiotics. Five out of the nine strains from Lagos were found to be positive for ESBLs production (55.55%). None of the non-typhoidal salmonellae from Katsina possesses ESBLs. The variegated resistance to the multitudes of beta-lactam antibiotics tested, coupled by the sensitivity to clavulanate phenotypically characterizes those ESBLs in the Salmonella isolates. The study highlights the growing menace of antibiotic resistance, particularly among the beta-lactams, and advocates for a paradigm shift towards solving this problem. Strategies to avoid zoonotic transfer of salmonellae are paramount in curtailing the spread of NTS to humans.

Key Words:- Non-typhoidal Salmonellae, Extended Spectrum Beta-Lactamases (ESBLs), antimicrobial and susceptibility profiles

INTRODUCTION

Development of antimicrobial resistance has been facilitated by the use of food additives in feeds of animals (Dierix, 2013). In the US, approximately 70% of all the antibiotics are sold for use in animals (CIDRAP, 2019). The motive behind these additions is to eliminate pathogenic microorganisms from these animals, and hence, boost their growth, inevitably creating a survival of the fittest scenario amongst the intestinal microbiota in these animals, with only those able to develop resistance capable of surviving (European Medicines Agency, 2015). One direct consequence of this is the increased value of minimum inhibitory concentration reported as able to prevent the growth of Salmonellae of animal origin (Greig and Ravel, 2009).

Beta-lactam antibiotics, agents for the treatment of Salmonella infection, act to inhibit bacterial cell wall biosynthesis (Bae *et al.*, 2015).

In response to these agents, bacteria have developed resistance to beta-lactams by synthesizing beta-lactamases, which cleave the beta-lactam ring and consequently inactivate the antibiotic. For example, Ceftriaxone, a third generation cephalosporin, has been widely used to treat infections by non-typhoidal Salmonellae. The resistance to these had already been documented. From 1996, when the prevalence rate of resistance to Ceftriaxone was at 0.1%, to 2003, there has been a significant increase in the resistance, and by 2011, the rate was at 4.4%. Non-typhoidal *Salmonellae* develop resistance to third generation cephalosprosporin antibiotics due to the acquisition of resistance gene associated with Extended Spectrum β-lactamases Enzymes (ESBL). *Escherichia coli* and also Salmonellae have already had their potential for production of extended spectrum β-lactamases evaluated. The problems posed by these enzymes have led to limited therapeutic options as well as sensitivity/resistance problems (Shin *et al.*, 2017). The fact that genes coding for ESBLs are located on mobile genetic elements are located on mobile genetic elements further aggravates the problem, and hence, previous reports have indicated that ESBLs were characterized from diverse members of the family: Enterobacteriaceae (Korzeniewska and Harnisz, 2013).

Hence, the need emanates for carrying out studies of this nature to determine the existence of extended spectrum beta-lactamases expressed by Non-Typhoidal Salmonella isolates. Besides contributing to existing literature about the subject in the study area, it will also shed more light on the prevalence of NTS and their antimicrobial susceptibility or resistance.

This study was aimed at isolating salmonellae from poultry droppings in Katsina, and comparing the prevalence of Extended Spectrum beta-lactamases between these salmonellae and other non-typhoidal salmonellae originating from chicken, obtained from Lagos, Nigeria, via the double disk diffusion test.

METHODOLOGY

Sample Collection and Isolation of Test Organisms

Three areas were chosen in Katsina Metropolis for the collection of chicken faeces. These include:- Fatima Baika Central Market, Katsina, Filin Polo and Babbar Ruga. A total of fifteen samples were collected in all, five for each chosen sample. Fifteen grams of the faeces were collected in a sterile polythene bag and transported to Microbiology Laboratory, Umaru Musa Yar'adua University, Katsina, for further analysis.

Additionally, nine strains of non-typhoidal salmonellae previously identified via the Kaufmanb-White Stereotyping Scheme were obtained from the University of Lagos, Akoka, Lagos State.

The chicken droppings samples from Katsina were first enriched using nutrient broth, which was prepared according to manufacturer's instructions, for 24 hours, at 37^0C. The isolates were then streaked onto nutrient agar plates and incubated for 24 hours at 37^0C, for observation of colonial morphology. Both the Lagos and Katsina NTS isolates were further plated on MacConkey Agar and Salmonella Shigella Agar, with their morphological characteristics such as lactose fermentation, H_2S production, colony pigmentation, margin, elevation, form, consistency, *et cetera* determined, as modified from Bell and Kyriakides, (2002).

Identification of the Salmonellae by Biochemical Characterization

The six non-typhoidal salmonellae from Katsina chicken droppings samples were identified by subjecting them to a battery of biochemical characterization as guided by the Cowan and Steel's Manual for the identification of medical bacteria (Barrow and Feltham, 2004). The tests conducted include Gram's staining, catalase test, the IMViC group of tests, determination of sugar fermentation and motility testing using triple sugar iron agar, and Urease test, and were performed as described by Hemraj *et al*. (2013). Additionally, the nine salmonellae were brought from the University of Lagos, Akoka, Lagos State. These were previously identified via the Kaufman-White Serotyping Scheme. However, the identity of the salmonellae was further confirmed and re-ascertained by following the protocols described above.

Antimicrobial Sensitivity Testing

This was carried out as described by Auf *et al*. (2009). Standardisation was carried out using 0.5 McFarland's standard. The cells were adjusted to match the turbidity of the McFarland standard, and contain approximately 1.5×10^8 cells (Cheesbrough, 2009). The test bacteria were cultured on nutrient agar by heavy inoculation, and the 24 hour growths were used to form a solution in distilled water. The turbidity of the solution was matched with that of the McFarland's standard, until they were even. The solution was heavily inoculated on already prepared (according to manufacturer's instructions) Mueller-Hinton Agar plates, using sterile swab sticks. Even streaking on the surface of the media was ensured by rotating the plates approximately 60^0 away for each fresh inoculation.

The plates were then incubated at 37°C for 24 hours, and the result of the susceptibility testing measured using a centimetre rule. Standard commercial gram negative disks were used for the testing. The antibiotics tested were: Augmentin/AU (30mcg), Septrin/SXT (30mcg), Streptomycin/S (30mcg), Ofloxacin/Tarivid/OFX (10mcg), Ciprofloxacin/CPX (10mcg) Pefloxacin/Reflacin/PEF (10mcg), Ampicillin/PN (30mcg), Gentamicin/CN (10mcg), Ceprox/CEP (10mcg) and Nalidixic Acid/NA (3mg) (Aouf *et al.,* 2009).

This was done according to the procedures described by Mukhtar*,* (2018). Triplicate of all the samples were done, after which the MARI was determined as follows:

$$\text{MARI} = \frac{\text{Number of antibiotics the bacterium resists}}{\text{Total number of tested antibiotics}}$$

Sensitivity Testing Using the Beta-lactam Antibiotics through the Double Disk Diffusion Test

A special beta-lactam disk was used for this assay. It contains the antibiotics: co-amoxiclav, i.e. amoxicillin/clavulanic acid. The rationale behind employing a beta-lactamase inhibitor, that is co-amoxiclav, in combination with an oxyimino-cephalosporin such as ceftazidime or cefotaxime in the Double Disk Diffusion Test is for the clavulanate to inhibit the ESBL, thereby reducing the level of resistance to the cephalosporin (Bradford, 2001), and hence, confirming the existence of the ESBLs in the tested strain.

In carrying out the test, the methodology of Jarlier *et al.,* (1988) was employed, the discoverer of the technique, with modifications from Aouf *et al* (2011) and Lertwoorapreecha *et al.* (2016) and Giwa *et al*. (2018). Non-typhoidal salmonella strains were tested using a combination disc diffusion method with combinations of Azetreonam (AZT), Ceftaxidime (CTX), Cefotaxim (CEF) and Amoxicillin-Clavulanate (AMC) all at concentrations of 30µg, i.e. AMC+AZT, AMC+CTX, and AMC+CAZ. The disks containing the individual antibiotics were placed within 20mm of the away from one another on the Mueller-Hinton Agar plate. ESBL-positive strains showed expansion of the zone of inhibition of the cephalosporins towards the amoxicillin-clavulunate, due to the inactivation of ESBL production in the strains by the clavulanate. Likewise, in ESBL positive strains, the zone of inhibition around the cephalosporins was smaller than 19mm (Giwa *et al*., 2018; Ziech *et al*., 2016).

Data Analysis

Comparison was carried out between the average zones of inhibition produced from each of the beta-lactam antibiotics, and all values were calculated at 5% confidence interval, using Microsoft Office Excel (2007) Data Analysis Tool Pak (DATP), specifically, standard deviation and two-tailed students' t-test were performed to compare results of zones of inhibition obtained when a combination of coamoxiclav and an oxyimino-cephalosporin inhibitor were used in the same plate, and when they were used individualistically. Charts were generated from the data for diagrammatical representation.

RESULTS AND DISCUSSION

Table 1 showed that the overall prevalence of non-typhoidal salmonellae from Katsina was 40%, ranging from the 20% obtained at Filin Polo to the 60% obtained from Fatima B'aika Central Market, Katsina. The identities of the nine salmonellae from Lagos, previously identified via the Kaufman-White serotyping scheme as being *Salmonella Carino, S. Budapest, S. Dasou, S. Agodi* and *S. Tennyson* were also presented in Table 2, including their code names.

The overall incidence of Non-Typhoidal Salmonellae isolates from chicken in Katsina Metropolis during this study was 40%, which was slightly lower than the 43.3% reported from Australia by Pointon *et al* (2008). It is also lower than the 50% prevalence reported by the previous study reported by CFSPH, obtained from Massachusetts, USA. It is also higher than the 18% prevalence reported by Lertworapreecha *et al* (2016). It is also lower than the 77% reported from the faeces by the same authors. Compared to prevalence from other food sources, such as beef, the study shows that chicken faeces have lower prevalence (40% vis-à-vis 57%), but also higher than that reported from young chicks in Iraq (Zhao *et al*., 2001; Zhao *et al*., 2009; Rehman *et al*., 2004; Ruban *et al*., 2010; Stojanov *et al*., 2005). The findings were also substantially higher than those reported from Egypt (14%), and in Canada (PHAC, 2019).

Table 1: Frequency of Isolation of Non-Typhoidal Salmonellae from Droppings of Poultry in Katsina

S/No	Sampling Area	Code Name	Number of Samples	Number Positive (%)	Number Negative (%)
1	Babbar Ruga	B/Ruga	5	2 (40%)	3 (60%)
2	Fatima B'aika Central Market	KCM	5	3 (60%)	2 (40%)
3	Filin Polo, Katsina	FP	5	1 (20%)	3 (80%)
	Total		15 (100%)	6 (40%)	9 (60%)

Table 2: Non-Typhoidal Salmonellae Isolated from Chiken from Lagos

S/No	Code Number	Isolate
9	A115	*Salmonella carino*
2	A152	*Salmonella budapest*
3	E114	*Salmonella budapest*
4	D6	*Salmonella agodi*
5	Cf3	*Salmonella budapest*
6	A2	*Salmonella dasou*
7	Fe	*Salmonella budapest*
8	A11	*Salmonella tennyson*
9	AF5	*Salmonella budapest*

The differences in the rates of the prevalence of non typhoidal salmonellae isolated from the sample areas can be explained by the variety in hygienic standards of the area, and other factors influencing microbial ecology.

The multiple antibiotic resistance indexes of both the Lagos and Katsina isolates were presented in Figure 1. The MARI gives a clear index about the status of an organism being multidrug resistant. The MARI values ranged from 0.3-0.5, for the Katsina Isolates, to 0.4-0.6 for the Lagos isolates, i.e. the organisms resist at least 3 and at most 6 antibiotics out of the ten tested.

The discrepancies between the MARI indexes of Lagos and Katsina isolates is explainable by the fact that the isolates from Lagos were obtained from chiken which had higher exposure to greater doses of multitudes of antibiotics, compared to Katsina isolates which received relatively lower doses of the antibiotic. The isolates can be termed as multidrug resistant, since some resist 6 out of the 10 tested antibiotics.

The high MARI Indices of the organisms coupled with the relatively low expression of synergistic interactions between the antibiotics can be ascribed to the possession of Extended Spectrum Beta-lactamases by the non-typhoidal salmonellae (Taneja *et al.*, 2014).

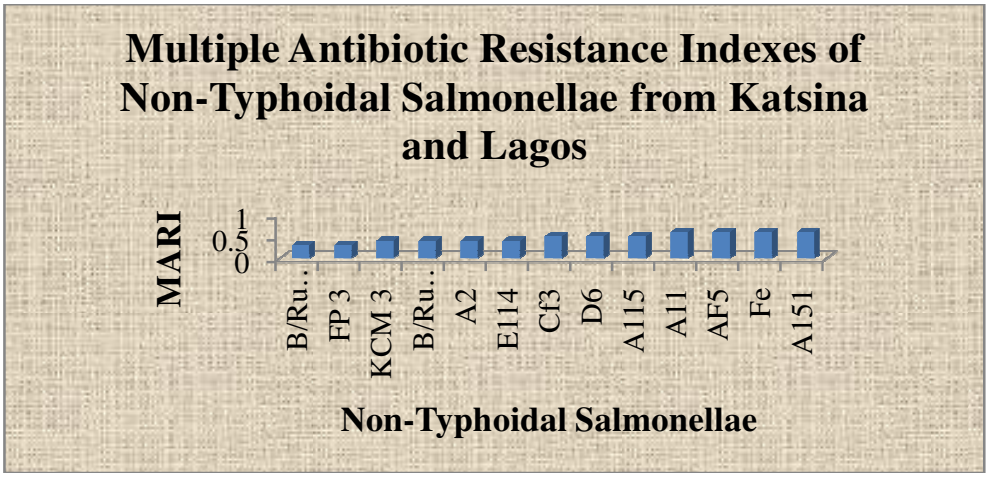

Figure 1: Multiple Antibiotic Resistance Indices of the Non Typhoidal Salmonella Isolates from Lagos and Katsina.

Key:- The Lagos isolates were: A11=*Salmonella Tennyson*, A115=*Salmonella Carino*, A151=*Salmonella Budapest*, A2=*Salmonella Dasou*, CF3=*Salmonella Budapest,* D6=*Salmonella Agodi*, AF5=*Salmonella Budapest*, AFG = *Salmonella Essem*, E114=*Salmonella Budapest* , Fe=*Salmonella Budapest.* The Katsina isolates were: B/Ruga 1, 2 = Non typhoidal Salmonella Isolated from Samples 1 and 2 from Babbar Ruga, FP3 = Non typhoidal salmonella isolated from sample 3 of Filin Polo, Katsina, KCM 1, 2, 3 = Non Typhoidal Salmonellae isolated from samples 1, 2 and 3 from Fatima Baika Central Market, Katsina.

The percentage resistance of both the Katsina and Lagos Isolates against the tested antibiotics was also evaluated (Figure 2), which showed that on the average, 47.3% resistance by the NTS against the antibiotics was observed. Some antibiotics have 100% efficacy, and 0% resistnace e.g. Ofloxacin and Ciprofloxacin, while others were resisted 93% of the time (Cephalexin, Nalidixic acid) and 87% (Augmentin/Amoxicillin-Clavulanic Acid and Ampicillin).

The dynamics of the resistance profiles of the organisms show that the most sensitive antibiotics to be used in the treatment of NTS-induced salmonelloses are Ofloxacin and Ciprofloxacin, to which the NTS strains are 100% sensitive. Pefloxacin is active against 93% of the tested organisms. These three antibiotics interfere with DNA replication, and are genrally highly effective against gram, negative organisms, especially members of the enterobacteriacae, and remain the popular alternatives for treating diseases by NTS. Septrin is also particularly effective. Only 13% of the tested NTS were resistant to cotromixazole/Septrin, and 87% were sensitive to it. Streptomycin showed good promise also, as it was effective against 80% of the tested strains. Streptomycin, an aminoglycoside, interferes with bacterial protein synthesis. The effectiveness of these drugs against the tested strains may be attributed to the low use of them in chicken feeds and in therapy of the diseases caused by the salmonellae.

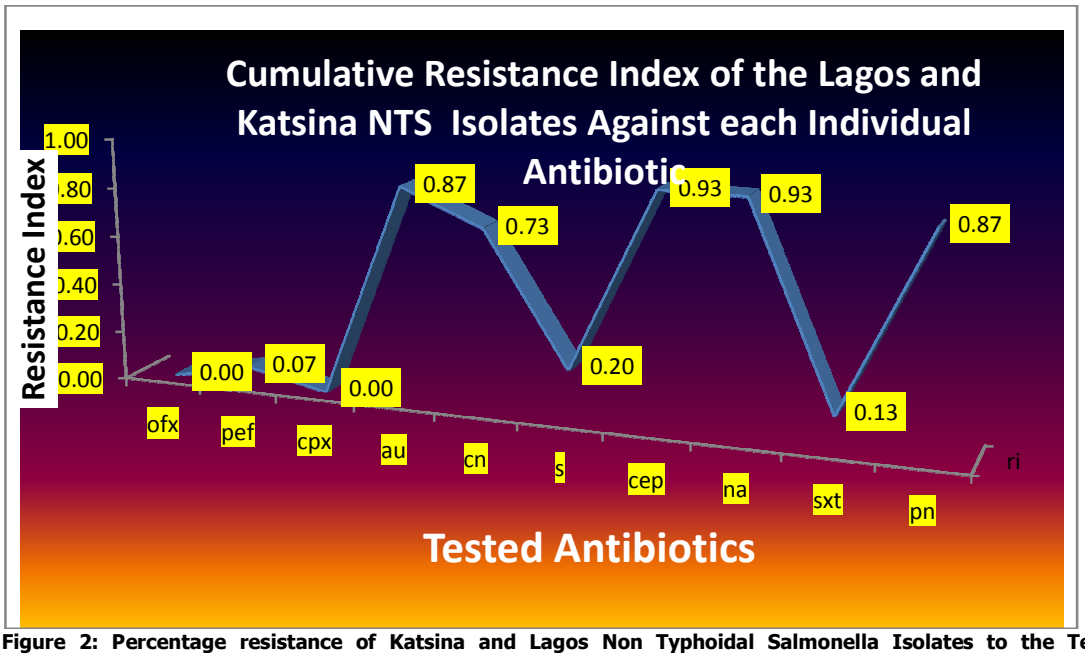

Figure 2: Percentage resistance of Katsina and Lagos Non Typhoidal Salmonella Isolates to the Tested Antibiotics.

Key:- AU = Augmentin (30mcg), SXT = Septrin (30mcg), S = Streptomycin (30mcg), OFX = Ofloxacin/Tarivid (10mcg), CPX = Ciprofloxacin/CPX (10mcg), PEF = Pefloxacin/Reflacin (10mcg), PN = Ampicillin (30mcg), CN = Gentamicin (10mcg), CEP Ceprox (10mcg) and NA = Nalidixic Acid (3mg).

The organisms exhibited the highest percentage of resistance to beta lactam antibiotics, and synthetic antibiotics containing a beta-lacatam/beta-lactamase inhibitor. Resistance to Cephalexin, a first generation cephalosporin, was 93%, the same as nalidixic acid, a first generation quinolone. Resistance to ampicillin was 87%, likewise augmentin (amoxicillin/clavulanic acid), which is a combination of a beta-lactam antibiotic (amoxicillin) and a beta lactamase inhibitor (clavulanic acid) was resisted 87%. Resistance to ampicillin, another beta-lactam antibiotic, was 87%. These may be associated with the possession of extended spectrum beta lactamases by the organisms. Similarly, resistance to Nalidixic acid, cephalexin, gentamycin and septrin points to the fact that the organisms are multidrug resistant.

However, the organism highly resisted other antibiotics, notably of the beta lactam group, ampicillin was resisted by 87% of the strains, cephalexin by 93%, also amoxicillin-clavulanic acid by 87% of the strains. This is explainable by the possession of beta-lactamase enzymes by the tested organisms, which enable them to counteract the effects of these drugs. Resistance to gentamycin was 73%, and this is attributable to the overuse of the drug against salmonellae, creating a selection pressure for resistant strains. Nalidixic acid was also resisted by 93% of the isolates. Nalidixic acid was commonly prescribed against salmonellae in hospitals for more than 50 years, and its ready availability may make it liable to being used in chicken feeds often, and this may facilitate the selection of organisms resistant to it (Giwa *et al.*, 2018).

The results of the double disk diffusion test which classify the organisms into ESBLs producers and Non-ESBLs producers are presented in Table 3. None of the samples from Katsina had ESBLs. However, the results indicate that five of the Lagos strains: A11, A115, A2, Cf3, E114 and Fe are positive for ESBL production, with the combinations of Strains A2 (*S. Dasou*) and A115 (*S. Carino)* exhibited resistance to both combinations of the beta-lactam cephalosporin and coamoxiclav (AMC + CTX and AMC + CAZ), as was shown in Figure 3, likewise, a sample plate exhibiting ESBLs producing NTS was shown in figure 4.

The use of the coamoxiclav produced higher zones of inhibition than the use of all the other coamoxiclav and the respective oxyimino respectively eliciting synergy with zones of the respective oxyimino-cephalosporins and where the zones were smaller than 19mm. Strains with zones greater than 19mm or those exhibiting resistance to both, i.e. the four remaining strains, are considered negative for ESBL production.

oxyimino cephalosporins alone, against the tested strains of non-typhoidal salmonellae, and statistically, there is a significant difference between the zones of inhibition at $p<0.05$, using the student's t-test, i.e. $2.37 > 1.86$.

Figure 4: Strain A115 (*S. Carino*) where resistance was exhibited against both coamoxiclav and Ceftaxidime

Figure 5: Synergy observed by the shifting of the zone of inhibition of the oxyimino cephalosporin towards the coamoxiclav disk

Table 4: Results of Double Disk Diffusion Test on Non-Typhoidal Salmonellae Isolated from Chicken from Lagos, Nigeria.

S/No	Isolate	Antibiotic	Zones of Inhibition Produced (mm)	Interpretation
1		AMC+ATM	20+14	
	A11	AMC+CAZ	18+18	ESBLs +ve
		AMC + CTX	19+18	
2		AMC+ATM	11+18	ESBLs +ve
	A115	AMC+CAZ	13+11	
		AMC + CTX	19+18	
3		AMC+ATM	19+14	ESBLs -ve
	A152	AMC+CAZ	17+19.5	
		AMC + CTX	17.5+17	
4		AMC+ATM	20+19	ESBLs -ve
	Af5	AMC+CAZ	19+18	
		AMC + CTX	20.5+16	
5		AMC+ATM	11+18	ESBLs +ve
	A2	AMC+CAZ	10.5+16	
		AMC + CTX	12+13	
6		AMC+ATM	22+11	ESBLs +ve
	Cf3	AMC+CAZ	21.5+10.5	
		AMC + CTX	22.5+18	
7		AMC+ATM	19+21	ESBLs -ve
	D6	AMC+CAZ	18.5+18.5	
		AMC + CTX	17.5+18	
8		AMC+ATM	17+16	ESBLs -ve
	E114	AMC+CAZ	16+22	
		AMC + CTX	17.5+10	
9		AMC+ATM	22+14	ESBLs +ve
	Fe	AMC+CAZ	20.5+13.5	
		AMC + CTX	21+10	

Key: AMC = Amoxicillin-Clavulanic Acid, ATM = Azetreonam, CAZ = Ceftaxidime, CTX = Cefotaxime, A11 *=Salmonella Tennyson*, A115 *=Salmonella Carino*, A152 *=Salmonella Budapest*, A2 *=Salmonella Dasou*, CF3 *=Salmonella Budapest,* D6 *=Salmonella Agodi*, AF5 *=Salmonella Budapest*, AFG *= Salmonella Essem*, E114 *=Salmonella Budapest*, Fe *=Salmonella Budapest.* Kirby-Bauer Interpretative Chart: AMC: Sensitive = ≥20, Intermediate = 16-19 and Resistant = ≤15; ATM: Sensitive = ≥20, Intermediate = 17-19 and Resistant = ≤16; CAZ: Sensitive = ≥19, Intermediate = 16-18 and Resistant = ≤15.

22% resistance to Amoxicillin-Clavulanic acid occurs, resistance to is Azetreonam is 55%, resistance to Ceftaxidime is 44% and resistance to cefotaxim is 88%, however, notably, only in 2 out of 9 cases does a combination of Amoxicillin-Clavulanic Acid and another antibiotic failed completely. The sensitivity of the isolates to clavulanate is a typical indication of the phenotypic characterization of extended spectrum beta-lactamases, as postulated by Brooks *et al* (2013).

The double disk diffusion synergy results showed that 5 out of the 9 tested strains of NTS were positive for ESBL production for a percentage of 55%. This percentage is higher than that reported by Giwa *et al*. (2018), which is 34.3% from uropathogens in Zaria; Akujobi and Enwuru (2010), who reported a rate of 23.6% from Nnewi, South Eastern Nigeria and Khurana *et al*. (2002) who reported a prevalence of 26.6% from India. The rate of ESBL positive strains from this study is higher than the prevalence reported by Mohammed *et al*. (2016), who reported a rate of 16% from Maiduguri, North Eastern Nigeria. This may be attributed to variation in the study area.

CONCLUSION

This study showed an occurrence of 40% of non-typhoidal salmonellae in chicken faeces from Katsina Metropolis. The double disk diffusion synergy results showed that Amoxicillin Clavulanic Acid and Ceftaxidime; and Amoxicillin Clavulanic Acid and Azetreonam, elicited synergistic activity, and could potentially be used in combination as therapeutic alternatives. Based on the MARI indices, the organisms exhibited resistance to augmentin, gentamycin, cephalexin, ampicillin and nalidixic acid, four out of which are susceptible to beta-lactamase activity. An important point to notice is the resistance exhibited to a combination of the third generation cephalosporins/betalactam antibiotic-beta lacatamase inhibitor, by some of the strains, such as *S. carino*. The prevalence of ESBL in the tested NTS strains was 22%. The study recommends the abolishing or minimizing of the use of ampicillin and its relatives in chicken feeds. Combined therapy is also advocated in the treatment of non-typhoidal salmonelloses, considering the efficacy of using beta-lactam and beta lactamase inhibitor in treatment, especially the use of Amoxicillin-Clavulanic Acid/Ceftaxidime. Employment of hygienic principles to avoid zoonotic transfer of salmonellae is also paramount in the control of the spread of NTS. The synthesis of antibiotics resistant to ESBLs is also a possible avenue to be exploited in curtailing the menace of ESBLs. This study advocates for a paradigm shift towards solving the problem of increased menace of antimicrobial resistance. Strategies to avoid zoonotic transfer of salmonellae are paramount in curtailing the spread of NTS to humans.

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