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RESEARCH ARTICLE

DETECTION AND MOLECULAR CHARACTERIZATION OF ESBL PRODUCING E. COLI ISOLATED FROM POULTRY DROPPINGS AND ITS ENVIRON.

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Abstract

Detection and molecular characterization of ESBL producing *E. coli* isolated from poultry droppings and its environ was investigated. Samples of poultry droppings (20), air (12), water (12) and soil (12) were collected from four randomly selected poultry farms within Calabar Municipal Local Government Area of Cross-River State. The samples were processed and *E. coli* isolates were identified using Standard Microbiological Techniques. ESBL phenotypic detection was carried out using Double Disc Synergism Test (DDST) while molecular detection of TEM, SHV and CTX-M genes in the *E. coli* isolates was carried out using Polymerase chain reaction (PCR). Results obtained revealed that 56 bacteria species were isolated from the samples, out of which 30 (53.57%) were *E. coli* while 12(21. 43%) were ESBL positive. A higher prevalence of ESBL producing *E. coli* was observed with samples of the poultry droppings (30%) as compared to that recorded with the air (8.33%), water (16.67%) and soil (25%) samples collected from the poultry farms. TEM, SHV, CTX-M, CTX-M-9 and CTX-M-15 genes were detected in 28.57%, 33.3%, 11.9%, 4.976% and 21.43% of the *E. coli* isolates while BLa OXA and CTX- M-2 genes were not present in the *E. coli* strains. However, the study has shown that ESBL producing *E. coli* circulate within poultry droppings and its environs. Hence, poultry farms are important source for ESBL-producing bacteria which causes difficult-to-treat infections in humans. Therefore, and integrated "one health" surveillance system becomes a necessity in monitoring transmission events and detection of resistant bacteria in a timely manner.

Introduction:-

External-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae are increasing in prevalence worldwide (Hetty *et a*l., 2019). ESBLs confer resistance to most beta-lactam antibiotics including third and fourth generation cephalosporin, which severely limits treatment possibilities for infections caused by these bacteria. Production of Extended- spectrum beta-lactamases (ESBLs) is the most common mechanism of resistance to third-generation cephalosporin among enterobacteriaceae including *Klebsiella pneumonia* and *Escherichia coli* (Paterson and Bonomo, 2005; Pitout and Laupland, 2008). ESBL determinants have been detected not only in clinical isolates but also in commensal bacteria from humans and animals and in isolates from products of the food chain and sewage,

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revealing distribution and suggesting the presence of environmental reservoirs for these resistance determinants (Brinas *et al*., 2003; Ewers *et al*., 2011).

The increase in antimicrobial-resistant bacteria of animal origin resembles the process in humans about two decades ago (Ogbulu *et al*., 2013). Since the late 1990s, extended spectrum beta-lactamases (ESBL) producing enterobacteriaceae in particular *E. coli*, have emerged globally. Initially, ESBL producing bacteria were only observed in human medical practice (Olowe *et al.,* 2012), but recent observations of these bacteria, first in companion animals and increasingly in livestock has initiated monitoring studies focused on livestock (Smet *et al*., 2010). ESBL producing *E. coli* are now being found increasing in numbers in food food-production producing animals leading to the hypothesis that animals might become infection sources or even reservoirs contributing to the spread of these bacteria (Carattoli *et al.,* 2009). Numerous reviews of extended-spectrum beta-lactamase (ESBL) involved in antibiotic resistance and in particular of *Escherichia coli* with ESBLs have been published recently (Chong *et al*, 2011; Woodford et al, 2011). However, the problem with ESBL producing *E. coli* in relation to poultry production have not been specifically addressed. Although, it is uncertain whether ESBL *E. coli* represent a direct threat to poultry production, Nevertheless, ESBL *E. coli* certainly represents a major problem to human clinical medicine.

Materials and methods:-

Study site and sample collection

The study was conducted in selected locations within Calabar Municipal Local Government Area of Cross River State. A total of four poultry farms with about 250-1000 birds (chickens) on each farm was visited. On each farm visits, 5 single faecal droppings and 3 samples of air, water and soil within the chicken housing were collected with an eswab^{TM} (Hain Life science, Nehren, Germany) and sterile universal containers. The samples were immediately transported to the laboratory for further analysis.

ESBL detection and antibiotics susceptibility testing

On arrival in the laboratory, each sample was inoculated on two selective MacConkey agar plates containing 1mg/l ceftazidime and cefotaxime respectively. Plates were incubated at 37^0c for 24-48h. All colonies with typical E. coli morphology were selected and confirmed biochemically by API 20E tests. For all E. coli, ESBL- production was confirmed by the combined disk test with cefotaxime and ceftazidime alone and in-combination with ceftriazone. Qualtiy control for each batch of cephalosporin-containing MacConkey agar plates was performed with E. coli ATCC 25922 and a bla CTX-M-15 Positive *E. coli* isolate.

ESBL typing using polymerase chain reaction (PCR)

The genes coding for the following beta lactamases ($bla_{TEM,, SHV,}$ CTX, OXA, CT-2, CT-9, CT-15) were detected by PCR amplification of genomic DNA in the cell lysates. The oligonucleotide PCR primers specific for beta lactamase genes, melting temperature and PCR products length are listed on Table 1.The gel was viewed in a gel doc system (Biorad Co. Ltd, USA) after electrophoresis. Standard Kilobase ladder used to confirm band sizes is shown on Table 2 Cycling conditions for polymerase chain reaction (PCR) is shown on Table 2. A concentration of each reagent in a mixture was 100-500ng of total genomic DNA, 10mM tris HCl (pH 8.3), 2mM $MgCl_2$, 0.5pM of each primer pair (forward and reverse). Two Hundred and fifty micro molar of each deoxynucleoside and triphosphate and 1µl of Taq DNA Polymerase for all the genes, blaTEM, SHV, CTX, OXA, CTX-2, CTX-9, CTX-15. The PCR products were separated in a 1% gel stained with ethidium bromide. The gel was run in an electrophoresis chamber at a voltage of 80mA for 2 hours. A standard 1KB DNA ladder was used to size the bands. The band sizes are as follows:

Table 1:-Primers guide used in the study

Primers	Nucleotide Sequence	Tm	Product Length	Ref
TEM	ATGAGTATTCAACATTCCGTG TTACCAATGCTTAATCAGTGAG	55	840	Gracia *et al*., 2008
SHV	ATTTGTCGTTCTTTACTCGC TTTATGGGCGTTACCTTTGACC	55	1051	Gracia *et al*., 2008
CTX-M	TTTGCGATGTGCAGTACCATAA CGATATCGTTGGTGGTGCCATA	51	544	Eldestem *et al.,* 2003
OXA	TTTTCTGTTTGGGTTTT TTTCTTGGCTTTTGTGTCTTG	52	427	Park *et al.*, 2006

CTX-2	AAATGTGCTGGCTCCTTTCGTGAGC AGGGTTCGTTGCAAGACAAGACTG	60	1122	Gracia *et al.*, 2008
CTX-9	GTGACAAAGAGAGTGCAACGC TAGATTCTCGCCCTAAGGCC	60	856	Sabata *et al.*, 2000
CTX-15	CACACGTGGAATTTAGGGACT GCCGTCTAAGGCGATAAACA	55	996	Muzaheed *et al.*, 2008

(Source: Sidjabat et al., 2009)

Table 2:-Standard band sizes used to confirm enzyme bands in PCR

Kilo base		Base Pairs
-	10	(10,000)
-	8	(8,000)
-	6	(6,000)
-	5	(5,000)
-	4	(4,000)
-	3	(3,000)
-	2	(2,000)
-	1.5	(1,500)
-	1.0	(1,000)
-	0.5	(500)

Table 3:-Steps of PCR process

Step	Procedure involved
1.	Initial denaturation at 95^0C for 4 minutes
2.	Denaturation at 95^0C for 30 seconds
3.	Primer annealing at 55^oC for 30 seconds
4.	Primer extension at 72^0C for 1 minute
5.	Final extension at 72^0C for 10 minutes
6.	Holding temperature 4^0C forever

Polymerase chain reaction (PCR)

This process was used to amplify and produce more genes. The Process involves three (3) major steps:

1. Denaturation
2. Annealing
3. Elongation

All of these steps take place in the PCR machine referred to as thermos-cyclers. Denaturation, this involves heating to about 90^0C-95^0C, while annealing takes place at a lower temperature of about 70^0C. The elongation step is made possible by the enzyme Taq DNA polymerase derived from the organism Thermus aquaticus.This enzyme attaches the dNTPs and the primers to the separated stands of the DNA at specific points which carry the target gene. In the PCR process, extreme care was taken to avoid contamination. All processes were carried out in a sterile hood or room free from nucleases to avoid waste of very expensive reagents.

Preparation of agarose gel

The agarose gel was used for electrophoresis after polymerase chain reaction. To prepare a 1% gel, 1g of agarose powder was weighed into 100ml of Tris-borate EDTA (TBE). This was placed in a microwave oven to melt and mix properly. Care was taken to ensure that the contents do not evaporate due to over-heating. It was allowed to cool to about 50^0C. Ethidium bromide was then added. The importance of this step was to allow for visibility of the amplicon bands under ultraviolet light. The mixture was allowed to cool further and then poured into gel tray with appropriate gel combs. The combs were used to create wells in the agarose gel where the PCR products will travel during electrophoresis. The gels were left to solidify (set) after which combs were removed.

Gel electrophoresis

The PCR products were introduced into the gel. They were then placed in the electrophoretic tank to separate the bands. DNA is negatively charged thus movement is from the negative to the positive end of the electrophoretic

chambers. The electrophoresis step lasted 1 hour 30 mins to 2 hours. All PCR processes were carried out in bowls containing ice cubes t prevent the development of primer dimers.

Results:-

ESBL producers from samples

Table 4 present the result of ESBL producers isolated from poultry droppings and it environ. it showed that 56 isolates from the samples were investigated for ESBL production. Of these, 30 (53.57%) were *E. coli* and 12(21.43%) were positive for ESBL production. The result of the percentage prevalence of ESBL producing *E. coli* isolated from the samples is presented in Table 5. It showed that 6(30%) of the 20 poultry dropping samples analyzed were positive for ESBL production, while 1(8.33%), 2(16.67%) and 3(25%) were positive for ESBL production as observed with the air, water and soil samples collected from each of the farms visited.

Results of ESBL typing using PCR

The results of the genotype typing of the ESBL producing E. coli isolates are presented in Fig. 1, 2 and 3. Figure 1 present the result of PCR for 12 Isolates of E. coli strains. Isolate A, N, R and T had bands in TEM and SHV, M had bands SHV, CTX-15. S had weak bands in TEM and SHV but two strong bands in CTX-15, U had weak bands in SHV and CTX. Isolates J, K, O, Q and V had no bands. Figure 2 present the results for the PCR test of the second set of E. coli isolates. Isolates A_5, A_6, $A_{8,}$ had bonds in TEM, X had bonds in SHV and CTX-15, A_{11} and A_{12} had bands in CTX-15 and SHV respectively. Isolates A_7, A_{13}, A_{14} had no bands. Figure 3 present the PCR results of the phenotypically negative strains. It revealed that Isolates B had CTX-M, CTX-M-15, G had amplification in TEM and a weak band in CTX-M-9, H had CTX-M-15, P had TEM, SHV. Only Isolate C, D and I had no bands.

The summary of the bla genes recovered in this study is presented in Figure 4. A total of 12 (28.57%) TEM; 14 (33.33%, SHV; 5 (11.90%) CTX-M; 2 (4.76%) CTX-M-9 and 9 (21.43%) CTX-M-15 were obtained. Bla OXA and CTX-M-2 were not found among the E. coli strains. 11 strains had no amplification with the primer sets used in this study

Table 4:-ESBL producers isolated from fecal droppings of poultry and its environ (air, water and soil)

Sample	Number of Isolates	Number of *E. coli* Isolated	Number of ESBl Positives (%)
Fecal Droppings of poultry and its environ (air, water and soil),	56	30(53.57%)	12(21.43%)

Table 5: **Prevalence of ESBL producing *E. coli* in the samples**

Environmental Samples	Number of ESBL positive *E. coli* isolates	% prevalence
Poultry Fecal droppings (n=20)	6	30
Air (n=12)	1	8.33
Water (n=12)	2	16.67
Soil (n=12)	3	25
	12	80

Fig 1: ESBL typing for 12 *E. coli* isolates (A-U) using primers: TEM = Lane1, SHV = Lane 2, CTX-M = Lane 3, OXA = Lane 4, CTX-M2 = Lane 5, CTX-M9 = Lane 6 and CTX-M15 = Lane 7

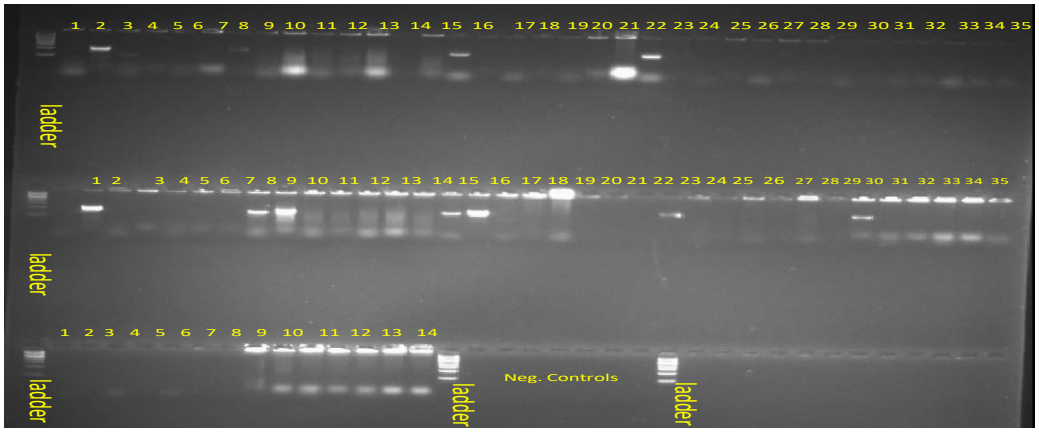

Fig 2: ESBL typing for 12 *E. coli* isolates (X - A_{14}) using primers: TEM = Lane1, SHV = Lane 2, CTX-M = Lane 3, OXA = Lane 4, CTX-M2 = Lane 5, CTX-M9 = Lane 6 and CTX-M15 = Lane 7

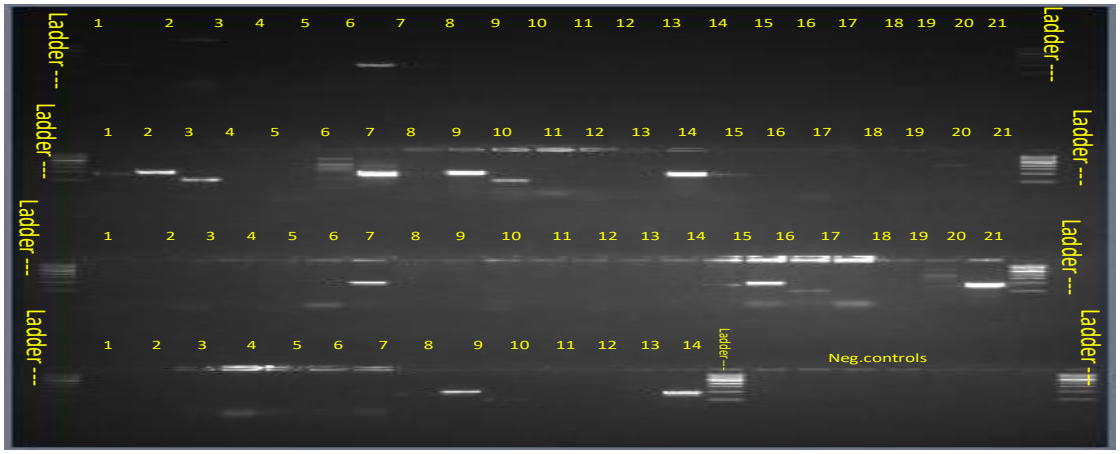

Fig 3: ESBL typing for phenotypically negative isolates using primers: TEM = Lane1, SHV = Lane 2, CTX-M = Lane 3, OXA = Lane 4, TX-M2 = Lane 5, CTX-M9 = Lane 6 and CTX-M15 = Lane 7

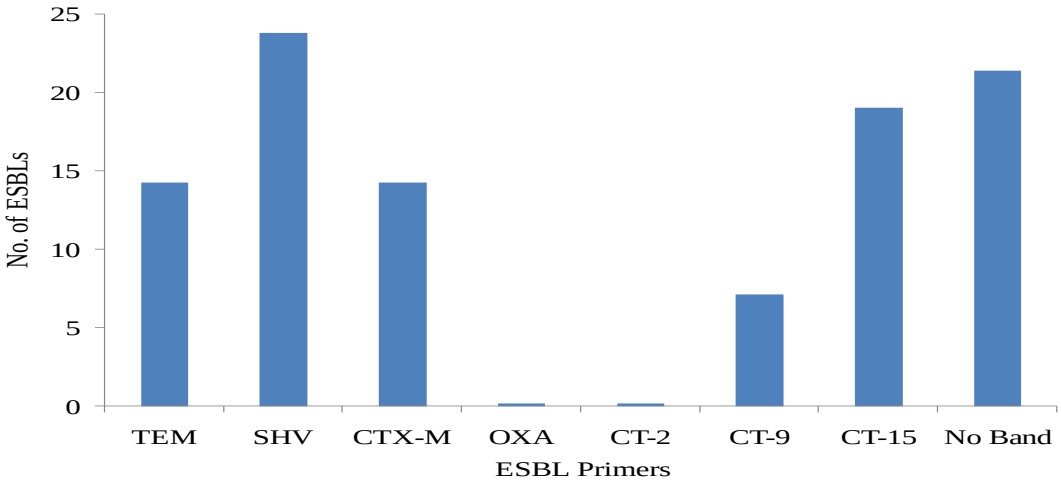

Fig 4: Bla genes recovered from study states

Discussion:-

Antibiotic resistance has continued to constitute serious problems not only in human medicine but also in animal husbandry, livestock management and vertinary medicine (Garrec *et al.,* 2011; Ajayi *et al.,* 2011). In this study, several methods of ESBLs detection were employed. Among 56 bacteria isolates from poultry droppings and its environ (air, water and soil), 30 (53.57%) were identified as *E.coli* while 12 (21.43%) were E5BL positive. This observation reiterates the finding in other studies that have reported antibiotic resistance among bacteria especially E. coli isolated from poultry, cattle and other animals at an alarming rate (Aibinu-*et al.,* 2004; kozak *et al.,* 2009; Ogunleye *et al*., 2009).

Extended- spectrum beta-lactamase indeed is a superbug of trouble to clinicians and microbiologist and is creating environmental stress to pharmaceutical pipeline in the development of new antibiotics. ESBL-producing *E. coli* are considered a major risk factor in human medicine, since infections with these bacteria are resistant to treatments with penicillin and cephalosporin. To reduce the risk, community originating ESBL *E. coli* either has to eliminated from poultry production or the occurrence has to be reduced to levels without risk to humans. Surveillance at farm level in this study clearly demonstrated a higher percentage prevalence (30%) of ESBL producing *E. coli* in poultry droppings as compared to that observed with air (8.33%), water (16.67%) and soil (25%) samples from the poultry farm environ. This observation was not surprising as similar study by Rikke *et al.,* (2014) reported to have isolated ESBL producing *Escherichia coli* from poultry farms in Denmark. The prevalence of ESBL producing *E. coli* in poultry droppings as well as in barn environment (air, dust) was reported by Hetty *et al* (2015). Also Dierikx *et al*., (2013) reported that in the outdoor farm environment , ESBL- producing E.coli were frequently detected in soil and surface water. They further reported that other farm animals present in the poultry farm were also carriers of ESBL-producing E. coli. Overall the prevalence of the ESBL producing E. coli in the poultry environs (air, water and soil) could have visibily be influenced by the poultry droppings. Indeed, ESBL-producing E. coli have been frequently identified worldwide in the chicken production chain including reports from Nigeria and Tanzania (Ojo *et al*., 2016, Linda *et al.,* 2019; Seni *et al.,* 2016). Interestingly, studies from Netherland, Sweeden and Vietnam detected ESBL-producing ST-10 E. coli not only in chickens but also in high numbers in the poultry environ (Huijbers et al., 2014; Ueda et al., 2015; Van Hoek *et al.,* 2015; Borjesson *et al.,* 2016). The PCR experiment is designed to detect ESBL-producers genotypically. Following the screening of the E. coli isolates for ESBL genes using polymerase chain reaction (PCR), 12 (28.57%) of blaTEM was recovered, while 14(33.33%), 5(11.90%), 2(4.76%) and 9(21.43%) of bla SHV, bla CTX-M, bla CTX-M-9, and blaCTX-M-15 were recovered respectively. Most amplification was observed with CTX-M. these findings suggest that blaCTX-M was more common among the ESBL genes in the E. coli isolates and this conforms to other studies by Olugbenga *et al.,* (2015) and Hetty *et al.,* (2019) . In this study,

the high prevalence of EBSL production among the *E. coli* isolates is alarming and worrisome, as ESBL have been reported to be plasmid encoded implying that these resistance determinants are found in our environment and can be transferred from one organization to another. However, the prevalence of these *E. coli* isolates with ESBL production potentials further buttresses the hypothesis that animals might become inspection source or even reservoirs (the natural persistent source of inspection) contributing to the spread of these bacteria.

Conclusion:-

The study has shown that ESBL – producing *E. coli* circulate within poultry droppings and its environ. Hence poultry farms are important source for ESBL – producing bacteria which causes difficult-to-treat infections in humans. Despite wide spread agreement that integration of human and animal data is desirable for antimicrobial resistance surveillance there is very little, if any, integration of data in most low and middle – income countries like Nigeria. Therefore, an integrated "One Health "surveillance system becomes a necessity, so as to monitor transmission events and detect resistance bacteria in a timely manner.

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