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Extended spectrum beta lactamase producing *Escherichia coli* and *klebsiella* species isolated from layer chicken farms in Jalingo,Nigeria

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----------***ABSTRACT***----------

This study investigated the extended spectrum beta lactamase(ESBL) producing Escherichia coli and Klebsiella species isolated from layer chicken farms. A total of one hundred and fifty six isolates of E.coli and Klebsiella species were tested for beta lactamase production while the betalactamase producing strains were further tested for the ability to secrete ESBL .The antibiotic susceptiblity profile, the plasmid sizes and the curing rates were determined.The results obtained revealed that the occurren ce of E.coli,K.pneumoniae and K.oxytoca in the farms were 86.6%,8.9% and 4.5% re spectively while the occurrence of the ESBL producers were respectively 16%,0.6%,a nd 0%.The antibiotic susceptibility profile showed that the proportion of the ESBL pr oducing E.coli resistant to ampicilin,ceftazidime and ceftriaxone was100%.Others we re ciprofloxacin(72%), chloramphenicol(48%) and tetracycline(60%),gentamicin(24 %)and nitrofurantoin(0%).All the ESBL positive isolates investigated harboured plasmids of 23130bp molecular weight. The proportion of the ESBL producing E.coli that their resistance plasmids was eliminated was 30.8%.The properties of ESBL producing E.coli and K.pneumoniae revealed that they are of public health importance.

KEY WORDS: curing,E.coli, ESBL, Layer chickens, Third generation cephalosporins.

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I. INTRODUCTION

Antibiotic resistance ,a menace to the society, threatens the life of all.Secretion of enzymes is one of the mechanisms bacteria use to resist attack from antibiotics and extended spectrum beta lactamase(ESBL) is one of such enzymes.ESBLs are rapidly evolving group of beta lactamases which share the ability to hydrolyse third generation cephalosporins and aztreonam but are inhibited by clavulanic Philippon *et al*.,(1989).

The chromosomal enzymes are believed to have evolved from penicillin binding proteins(PBPs) with which they show same-sequence homology.This was probably as a result of the selective pressure exerted by betaLactam producing soil organisms found in theenvironment(Bradford, 2001).The first plasmid mediated beta lactamases in Gram negative bacteria TEM 1 was described in the early 1960s (Bradford,2001).Being plasmid mediated and transposon mediated,TEM 1 enzymes spread worldwide and are now in many different species of the family Enterobacteriaceae.Over the

years the use of newer beta lactam antibiotics has enabled selection of new variants of beta lactamases.In the early 1980s,the third generation,or oxy-imino,cephalosporins were introduced into clinical practice in response to the increasing prevalence and spread of the beta lactamases.Resistance to these extended spectrum cephalosporins emerged quickly and the first report of an SHV-2 enzyme which was capable of hydrolyzing these antibiotics was published as early as 1983 from Germany(Bradford,2001).ESBLs are effective against betalactam antibiotics likeceftriaxone,cefotaxime,oxyimino and monobactam(Bradford,2001).ESBLs are encoded by genes that can be exchanged between bacteria(Paterson and Bonomo,2005).They are mostly produced by Gram negative Enterobacteriaceae which *E.coli* and *K.pneumoniae* are the chief culprits(Anago,*et al*.,2013).

This strain of *E.coli* and *K.pneumoniae* limit therapeutic options as a result of their multidrug resistance(Anago *et al.*,2013).Plasmids responsible for ESBL production tend to be large and carry resistance to several agents.The most frequent coresistances found in ESBL organisms are aminoglycosides,fluoroquinolones,tetracycline,chloramphenicol and sulfamethoxazole-trimethoprim(Nathisuwan *et al.*,2006) .

Patients with infections caused by drug resistant bacteria are generally at increased risk of worse clinical outcomes and death and consume more healthcare resources than patients infected with the same bacteria that are not resistant(Tumbarello *et al.*,2006).The achievements of modern medicine are put at risk by antimicrobial resistance.Without effective antimicrobial for prevention and treatment of infections,the success of organ transplantation,cancer chemotherapy and major surgery would be compromised(WHO,2014)

There is abundance of ESBL genes in the food chain and this may have a profound effect on future treatment of infections caused by Gram negative organisms(Grave *et al*.,2010).Drug resistance in animals is caused mainly by the large amount of antimicrobial drugs used in food production(Grave *et al*.,2010). ESBL producing bacteria can be found almost everywhere.It can be found in chicken feeds (Oyinloye and Ezekiel, 2011).It can be isolated from waters(Gao *et al.*,2014),from humans(Folasage *et al*.,2014).This study therefore investigated the extended spectrum beta lactamase producing *E.coli* and *klebsiella* species isolated from layer chicken farms.

II. Materials And Methods

Study Area

Jalingo local government area and the capital of Taraba State located in the North East geo political zone and situated between latitude 8^0 47′ North and $90^0$1′ North; longitude $11^0$9′, East and $11^0$30′ East was the study location.

Collection of Samples

Samples were collected from two poultry farms which comprised of Egg laying stage healthy brown and black chickens.Layer chickens are those chickens reared solely for the purpose of egg production. The samples were cloacal swabs,stool of the chicken rearers and floors swabs. A swab stick was soaked in sterile distilled water and was inserted into the cloaca of each of the randomly selected chickens in each of the poultry farms.while still in the cloaca, the swab stick was rotated three times before it was finally put back into its container.Swab sticks were soaked in sterile distilled water and were used to swab strategic areas on the floor of the poultry . Each swab stick was then put back into its container.A sterile wide mouthed container was used to collect the stool specimen of the chicken rearers

Bacterial Isolation and Identification

Samples collected were cultured within 2 hours of collection on MacConkay agar and EMB agar (Oxoid CM 516, UK) and incubated at 37°C for 18–24 hours.Then, MacConkey agar culture was examined.The distinct colonies were examined for their morphology and ability to ferment lactose. Lacstose fermenting colonies were further subcultured on Eosin methylene blue (EMB) agar and incubated for 18h at 37^0C.EMB cultures were also examined for the appearance of metallic green sheen colonies. All the isolates both lactose and non lactose fermenters) were individually gram stained and subjected to biochemical tests Deoxyribonucleic acid (DNA) sequencing using sangar sequencing technique was used for the molecular characterization of each of the representative isolate.

ESBL Detection

Isolates were tested for beta-lactamase production using acidimetric method as described earlier by(Cheesbrough,2010).All positive *β*-lactamase isolates were screened for ESBL production by double disk synergy test(DDST) according to Liofichem(2014). Briefly,four milliliter of 0.5 Mc Farland equivalent standard of the test organisms were spread on the surface of a sterile Mueller Hinton agar plate using a sterile swab stick.After 20 minutes, Augmentin disc (30µg) (Amoxicillin 20µg/clavulanic acid 10µg combination) was placed 15mm apart from the center of ceftriaxone disc(30µg) and ceftazidime disc(30µg) .This was incubated for 18 hours at 37^{o}C.

Antimicrobial Susceptibility Testing

This test was done using the modified Kirby-Bauer disc diffusion method on Mueller-Hinton agar as described by the Clinical and Laboratory Standards Institute guidelines(CLSI,2012). Briefly,a suspension was made from a 24 h growth of the organism in saline to match the 0.5 McFarland turbidity standard. This was spread on the entire surface of a Mueller-Hinton agar plate using a sterile swab stick. The following antibiotic discs were used: ceftazidime (30µg), ceftriaxone (30 µg), ampicillin (10 µg), nitrofurantoin (300 µg), gentamicin (10 µg),chloramphenicol(30 µg),tetracycline(30 µg) and ciprofloxacin (5 µg). The Mueller-Hinton agar plate was then incubated at 35°C for 18–24 h, after which the diameter of the zones of growth inhibition around the discs was measured with a ruler.The results were further interpreted using the Performance Standards for Antimicrobial Susceptibility Testing(CLSI,2012).

Plasmid Profile of Extended Spectrum Betalactamse (ESBL) Positive Isolates

Plasmid profile analysis of the ESBL positive isolates was carried out according to the modified alkaline lyses plasmid extraction protocols described in, Dagan *et al.*(,2006);Charles *et al.*(2010). A 1% Agarose gel was prepared and loaded into electrophoresis chamber containing 8 wells; buffered with 40 mM Tris, 20 mM, 2 mM EDTA and adjusted to pH 7.8 with acetic acid. Electrophoresis was allowed to proceed at room temperature for 20 min. After electrophoresis, gels were stained with ethidium bromide (1 µl/ml) and viewed under UV transillumination. The molecular marker that was used was the *Hind Phl III* digest and extrapolations were made from the Electropherogram obtained Piglansky *et al.*,(2003).

Plasmid Curing

ESBL positive isolates were selected and subjected to acridine orange as described by Iroha,(2010).Each tested organism was grown in a solution of 5ml double strength nutrient broth sup plemented with *0.1ng/ml* acridine orange and incubated at 37^{0}C for 24 hours. After incubation the test organisms were retested for ESBL production using DDST.

III. Results

A total of 156 isolates was obtained from chicken layers farms.The frequencies of *E.coli,K.pneumoniae and K.oxytoca* were respectively 86.6%,8.9% and 4.5% respectively as presented in table 1.

Table 1:Frequency of Isolates from Layers chickens

Farms	Samples	No of isolates	*E.colii*	Frequency (%)	*K.pneumoniae*	Frequency (%)	*K.oxytoca*	Frequency (%)
Black	chickens	89	79	50.6	6	3.8	4	2.6
Layers	Floor	9	9	5.8	0	0	0	0
	Stool	4	4	2.6	0	0	0	0
Brown	Chickens	36	29	18.6	4	2.6	3	1.9
Layers	Floors	13	10	6.4	3	1.9	0	0
	Stool	5	4	2.6	1	0.6	0	0

Total	156	135	86.6	14	8.9	7	4.5

Clavulanic acid inhibited the action of the ESBL produced by *E.coli* thereby making the zone of inhibition around disc 1(ceftazidime) and 2(ceftriaxone) on the area closer to the disk 4(augmentin ,which contained clavulanic acid) to augment towards disc 4 as shown in plate 1.

Plate 1:Synergyof Clavulanic acid (containing disk) with ceftazidime and ceftriaxone in Double Disk Synergy Test(DDST) exhibited in ESBL positive *E.coli*

Clavulanic acid inhibited the action of the ESBL produced by *Klebsiella pneumoniae* thereby making the zone of inhibition around disk 1(ceftazidime) and 2(ceftriaxone) on the area closer to the disk 4(augmentin ,which contained clavulanic acid) to augment towards disk 4 as shown in plate 2.

Plate 2:Synergyof Clavulanic acid (containing disk) with ceftazidime and ceftriaxone in Double Disk Synergy Test(DDST) exhibited in ESBL positive*k.pneumoniae*

The occurrence of ESBL producing *E.coli* and *K.pneumoniae* in layers farms were respectively 16.0% and 0.6%.No ESBL producing *K.oxytoca* was obtained as is presented in table 2

Table 2: Occurrence of ESBL producing E.coli,K.pneumoniae and K.oxytoca in chicken Layers

Farms	Samples	No of Isolates	No of ESBL positive *E.coli*	Freq. (%)	No of ESBL Positive *K.pneumoniae*	Freq. (%)	No of ESBL positive *K.oxytoca*	Freq. (%)
Black	Chickens	89	79	50.6	6	3.8	4	2.6
Layers	Floors	9	9	5.8	0	0	0	0
	Stool	4	4	2.6	0	0	0	0
Brown	Chickens	36	29	18.6	4	2.6	3	1.9
Layers	Floors	13	10	6.4	3	1.9	0	0
	Stool	5	4	2.6	1	0.6	0	0
Total		156	135	86.6	14	8.9	7	4.5

The ESBL producing *E. coli* isolates were 100% resistant to ampicillin, ceftriaxone and ceftazidime. The isolates were 48%,72%, 0% 24% and 60% resistant to chloramphenicol, ciprofloxacin, nitrofurantoin gentamincin and tetracycline respectively as presented in table 3 below.

Table 3: Antibiotic Susceptibility Profile of ESBL Positive *E. coli*

Antibiotics	(Ug/Disk)	S	%	I	%	R	%
Ampicillin	10	0	0	0	0	25	100
Chloramphenicol	30	7	28.0	6	24.0	12	48.0
Ciprofloxacin	5	6	24.0	1	4.0	18	72.0
Nitrofurantoin	300	23	92.0	2	8.0	0	0.0
Gentamicin	10	17	68.0	2	8.0	6	24.0
Tetracycline	30	6	24.0	4	16.0	15	60.0
Ceftriaxone	30	0	0	0	0	25	100
Ceftazidime	30	0	0	0	0	25	100

S-Sensitive, I-Intermediate, R-Resistant

Plasmid profiling showed that all the 17 ESBL positive isolates harboured only one plasmid of molecular weight 23130bp each as shown in fig 1.

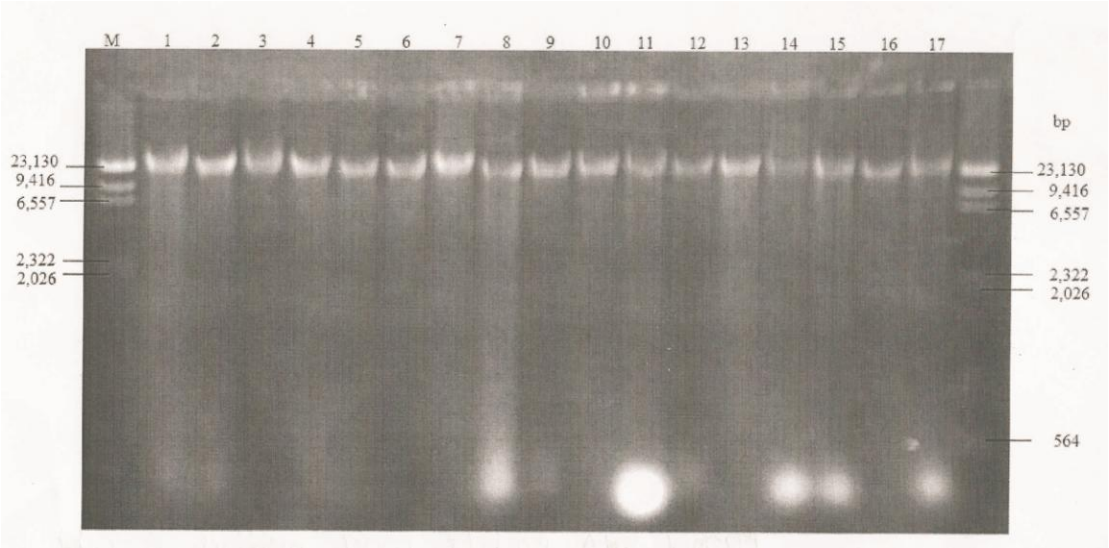

Fig 1:Plasmid Profile of ESBL Positive Isolates

Lanes
1-6 –*E.coli* from Floor
7-*K.pneumoniae* from Floor
8-15-*E.coli* from chickens
16-17-*E.coli* from Stool

Eight(30.8%) of the ESBL producing isolates subjected to acridne orange were cured as presented in table 4.

Table 4:Plasmid Curing Rate of the ESBL positive Isolates

farms	Bacteria	No of ESBL Positive Isolates	No of Isolates without Plasmids after curing	Percentage of isolates Cured
Black Layers	*E.coli*	13	4	15.4
	K.pneumoniae	0	0	0
	K.oxytoca	0	0	0
Brown Layers	*E.coli*	12	4	15.4
	K.pneumoniae	1	0	0
	K.oxytoca	0	0	0
Total		26	8	30.8

IV. DISCUSSIONS

.The occurrence of the isolates was investigated,and it was revealed that 86.6% were *E.coli* while 8.9% were *K.pneumonia*e and 4.5% were *K.oxytoca* (Table 1).This is because more than 50% of the samples were cloacal swabs and *E.coli* are coliforms.The high occurrence(86.5%) of *E.coli* is lower than the high occurrence 100% reported by Dadheech *et al*.,(2016).The 8.9% occurrence of *K.pneumoniae* is higher than 5.8% reported by Hassan *et al*.,2015.

The synergy of clavulanic acid containing disc with ceftazidime and ceftriaxone in double disk synergy test (DDST) was investigated.The observations made revealed that the enzyme, ESBL,produced by *E.coli* in plate 1and *K.pneumoniae* in plate 2 was inhibited by clavulanic acid which is a componenent of the antibiotic disc 4(augmentin).This made the zone of growth inhibition around antibiotic disc 1(ceftazidime) and 2(ceftriaxone) to extend towards antibiotic disc 4.This synergistic effect observed in Fig 1a and 1b supports a previous report by Oyinloye and Ezekiel,(2011).

The occurrence of ESBL positive *E.coli*,*K.pneumoniae* and *K.oxytoca* was studied and the observations made revealed that the occurrence rates of *E.coli*,*K.pneumoniae* and*K.oxytoca* were respectively 16.0%,0.6% and 0% (Table 2).The occurrence rate of ESBL producing *E.coli was the* highest as a result of the source of the samples.The 16.0% occurrence of *E.coli* is lower than 65% detected by Blaak *et al*.,(2015) in Netherland .Hiroi *et al*.,(2012) reported 5.9% prevalence rate from rectal samples.A low occurrence(0.6%) of *K.pneumoniae* obtained in this study agrees with a low occurrence reported by Hiroi *et al*.,(2012).The observed difference in the occurrence rates of ESBL positive *E.coli*,and *K.pneumoniae* in brown and black layers chickens farms is significant($p<0.05$). This is because of the differences in the management practices(such as observance of aseptic techniques) and in the breed of the chickens. Dahms *et al*.,(2012) also reported differences in the occurrence rates of different farm animals.

The antibiotic susceptibility profile of the ESBL positive *E.*coli was investigated.The findings revealed that the ESBL positive *E.coli* strain showed absolute resistance to ampicillin,ceftriaxone and ceftazidime.The strain also showed high resistance to Chloramphenicol(48%),ciprofloxacin(72%) and tetracycline(60%).It also showed moderate resistance(24%) to gentamicin(Table 3) .ThisThis ESBL producing bacteria showed resistance to other antibiotics aside third generation cephalosporins. This is because the plasmids responsible for ESBL production carry resistance to several antibiotics.The multidrug resistance observed in this study agrees with the report by Motayo *et al*.,(2013);Afunwa *et al*., (2011).

The plasmid sizes of ESBL producing *E.coli* and *K.pneumoniae* were investigated and the observations made revealed that all the isolates investigated harboured high molecular weight(23130bp) plasmids(Fig.1). Folasage *et al*.,(2014) had also previously reported heavy molecular weight (24.3kbp) plasmids among ESBL positive *E.coli* and *K.pneumoniae*.

Finally,the curing rate of the ESBL positive isolates was observed and the findings made revealed that 30.8% ESBL positive isolates were cured while 69.2% were not(Table 4).The isolates that were cured did not have the resistance characteristics as a genetic trait.Some other researchers like Duru *et al*., (2013) and Folasage *et al*.,(2014) reported curing rates of 0% and 13.5% respectively.

V. CONCLUSION

ESBL producing *E.coli* and *K.pneumoniae* can be obtained from Layers chicken farms . They are multidrug resistance They harbor heavy weight molecular plasmids which can be cured depending on whether it is a genetic trait .

Conflict of Interest:The authors declare no conflict of interest

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