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Occurrence of Multi Drug Resistant Commensal *Escherichia coli* in Apparently Healthy Lambs and Kids from Maiduguri, Northeastern Nigeria

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Authors' contributions

This work was carried out with contributions from all authors. Author EYB designed the study, collected the samples and wrote the report. Author HIA designed and supervised the study also assisted in literature search. Author MMG performed the statistical analysis and prepared template for publication. Authors HBG and IAG carried out laboratory analysis and editing of manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to determine the occurrence of multi-drugs resistant commensal *Escherichia coli* (*E. coli)* in apparently healthy kids and lambs from Maiduguri, Northeastern Nigeria.
Study Design: A cross sectional study was conducted using convenience sampling method.
Methodology: In all, 200 fecal samples were collected from apparently healthy lambs and kids using sterile swaps (100 samples each). The lambs and kids were grouped into 3 categories based on age which include 0 to ≤ 1 month, >1 to ≤ 2 months and >2 to ≤ 3 months. A total of 90 (45%)

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commensal *E. coli* isolates were detected using Standard bacteriological and biochemical tests. Kids had significantly (P<0.05) higher isolation rate (70%) compared to lambs (20%). A significant association and linear trend in the proportions of commensal *E. coli* isolates between the age categories was observed. Lambs and kids up to 1 month old 12 (13.3%) had lower isolation rate compared to the other age groups 2 months old 39 (83%) and 3 months old 39 (56.5%). Antibiogram pattern of the isolated commensal *E. coli* was evaluated against 10 antibiotics which showed high resistance against Pefloxacin (100%), Amoxicillin (97.7%), Chloramphenicol (94.4%), Ceftriaxone (93.3%), Cefuroxime (92.2%), Nitrofurantoin (67.8%) and Streptomycin (51.1%). The isolates were highly susceptible to Ciprofloxacin (95.5%) and Ofloxacin (95.5%). All isolates of the commensal *E. coli* 90 (100%) showed varying multiple drugs resistance patterns ranging from 3 to 8 antibiotics.

Conclusion: These results indicated that commensal *E. coli* can be potential reservoirs for antibiotic resistance genes and there may be possibility of horizontal transmission to animals and humans. Using only effective antibiotics in management of *E. coli* infections is strongly recommended. Laws regulating prescriptions and dispensing of veterinary drugs ought to be fully implemented and there is need to create public awareness on dangers of indiscriminate use of antibiotics as growth promoters in animal feed.

Keywords: Commensal E. coli; resistance; susceptibility; antibiotics; lambs; kids.

1. INTRODUCTION

Commensal *E. coli* constitute a component of the normal flora of animals and humans and they have no known toxic, adhesive or invasive virulence attribute and should, ordinarily, not be associated with a specific disease caused by *E. coli* [1]. The organisms are highly versatile in nature and, as such, the most explored model organisms. The enormous diversity of *E. coli* is due to its high recombination potentials which usually occur through either acquisition or loss of genetic information by horizontal gene transfer [2]. The dynamics, development, and ways of evolution of resistance traits in *E. coli* populations may differ according to the hosts, and antimicrobials used [3]. Various environmental factors have been associated with the emergence of antimicrobial resistance in commensal *E. coli* strains, and such strains may serve as reservoirs of antimicrobial resistance [4]. The use of antimicrobials as growth promoters and as therapeutic agents targeted at pathogenic microbial strains have been incriminated as an important factor facilitating the acquisition of multi-drug resistance by commensal *E. coli* [1]. Studies in humans from various countries have also showed use of antibiotics in medicine and its occurrence in the environment as responsible for turning intestinal microorganisms into reservoirs of antibiotic resistance factors [4,5,6].

Although the mechanism by which commensal *E. coli* acquire multi-drug resistance is not fully understood, however, adaptation to a hostile niche created by antimicrobial treatment targeted at pathogens in the gut could be partly responsible. The non-target commensal organisms including *E. coli* are compelled to develop strategies to enable their survival and growth under such an unfavorable environment [1]. The most efficient and sophisticated defense mechanism used by these microorganisms is the acquisition of multidrug resistance, characterized by the complex interaction of different mechanisms (e.g., drug efflux, enzymatic inactivation, target protection) conferring simultaneous resistance to a wide range of older and/or new antimicrobial compounds or drug classes [1]. Recent epidemiological and genome-wide sequence analysis suggest that there is no clear line between the commensal and pathogenic *E. coli,* as a group they share most of the pathogenicity factors and belong to the same pathotypes and phylogroups and are host independent [7,8]. It has, therefore, becomes evident that the pathogenicity of *E. coli* is dependent on the regulation and interaction between a number of virulence factors and it is affected by environmental condition e.g. host species, host status, interaction with other bacterial species etc. [9]. Due to this under certain, yet unknown circumstances, any *E. coli* isolate carrying pathogenicity or antimicrobial resistance genes is potentially pathogenic and harmful to its host [9].

Livestock industry plays significant roles in the socio-economic wellbeing of Nigerians as well as the nation in terms of its contribution to gross domestic product, family income, employment,

festivities, nutrition and social status including utility of livestock by products (hides, skin, horns, hooves etc.) to the individual and nation at large [10]. Several surveys and studies of ruminants kept by the rural farmers, and even in the markets, across the country revealed that the animals are mostly infected with one form of diseases/pests or the other [11,12]. Most of the diagnosed livestock diseases in the country have been identified to be bacterial, viral, fungal and parasitic-caused diseases [13]. Of the bacterial diseases, gastroenteritis due to colibacillosis particularly caused by *E. coli* serotypes are commonly found in younger species or neonate [13]. *E. coli* is becoming an important gastro intestinal pathogen because it causes colibacillosis and this appears to be frequently reported in young calves, lambs and kids [13,14]. Domestic and wild animals are sources of *E. coli* but ruminants primarily sheep, goats and cattle have been identified as major reservoirs and source for human infection [15,16].

E. coli is pathogenic to both humans and animals resulting in intestinal and extra-intestinal problems. It has been isolated from wound infection, mastitis, respiratory infections and diarrhea in ruminants, wildlife and pets. It causes enteritis, arthritis, omphalitis, septicemia and complicated air sacculitis in birds [17] and is considered a major pathogen contributing significantly to economic losses in both turkey and chickens. Major virulence factors of *E. coli* are the toxin which is also known as the shiga toxins and are important in the pathogenesis of diarrhea whereby they inhibit protein synthesis of host cells, thus leading to cell death [18]. Other virulence factors include genes in the Locus of Enterocyte Effacement (LEE), responsible for the bacteria attaching tightly to mammalian epithelial cells. Additional virulence factors are the alpha-hemolysin and entero-hemolysin which act as pore-forming cytolysin on eukaryotic cells [19].

Verotoxin-producing *E. coli* (VTEC) including 0157:H7 have emerged as food-borne pathogens, they are a major cause of gastroenteritis that may be complicated by Hemorrhagic Colitis (HC) or Hemolytic Uremic Syndrome (HUS) which is the main cause of acute renal failure in children [20]. Transmission of these food-borne pathogens occurs through consumption of undercooked meat, unpasteurized dairy products, vegetables or water contaminated by ruminant feces, person to person transmission has also been documented [20]. Many serovars of EHEC associated with human infection like O91, O157, and O146 have been isolated from sheep [21,22]. O157 has also been isolated from goats [23]. The aim of this study is therefore to determine the occurrence of multi drug resistance in commensal *E. coli* isolates from apparently healthy kids and lambs in Maiduguri, Northeastern Nigeria.

1.1 Study Area

Maiduguri, the capital of Borno State, is located between the Northern Sudan savannah and Southern Sahel savannah vegetation zones. It has an average elevation of 300 meters and altitude of 700 km. Its geographical coordinates are 11°50′42″ North and 13°09′36″ East. It has 3 seasons which include cool dry (or Harmattan) from October to March, hot dry from April to June and rainy from July to September. Ambient temperature is high all year round with hot season temperatures ranging between 39°C to 40°C [24]. Livestock are among the most important sources of livelihood to many low-income earners in the area. The animals are kept in backyard mostly under semi-intensive system of management and they constitute a major source of small ruminants to the local livestock markets.

1.2 Study Population

Young small ruminants within Maiduguri formed the study population. Apparently health lambs and kids between 0 to 3 months of age were sampled.

1.3 Sampling Procedure

Samples were collected from lambs and kids at University of Maiduguri farm and other accessible farms and backyards flocks within the study area. Samples were collected from only the flocks or households who consent using convenience sampling method. This sampling method was used due to security challenges in the study area. In circumstances where many target subjects are present in the same area, a simple random method was used to select representative samples for that particular location.

1.4 Sample Collection

A total of 200 rectal swab samples were collected from lambs and kids (100 from each). After each animal is properly restraint, a sterile swab was used to collect fecal materials per rectum and then immediately returned into its tube and care was taken not to touch the tip of

the tube. Each sample was labelled appropriately and age of the animal was also noted accordingly. The samples were then transported in an ice packed container to Research laboratory of Department of Veterinary Medicine, University of Maiduguri where they were inoculated into already prepared media on agar plates for bacteriological examination.

1.5 Preparation of Culture Media

The procedure for the preparation of media, inoculation and bacteria identification were as described by Cheesbrough [25]. MacConkey agar, Eosin methylene blue agar and nutrient agar including the indole, methyl red, Voges Proskauer and citrate utilization (IMViC) test utilization test media were prepared according to manufacturer's instructions.

1.6 Inoculation and Isolation

All the fecal samples were inoculated on MacConkey agar using sterile wire loop. Streaks were made from the pools across the agar and then incubated at 37°C for 24 hours [25]. After inoculation, the plates were examined for evidence of bacterial growth. Suspected bacterial colonies (presumptive colonies) were then sub-cultured for purity onto Eosin methylene blue agar (EMBA) [26] and then preserved on nutrient agar for future use. The final pure cultures were confirmed to be *E. coli* using the standard biochemical indole, methyl red, Voges Proskauer and citrate utilization (IMViC) tests.

2. BIOCHEMICAL TEST

2.1 Indole Test

Presumptive *E. coli* strains were tested for indole by its ability to metabolize tryptophan by tryptophanase which leads to the formation of pyruvic acid, indole and ammonia. Colonies were inoculated onto tryptone water base and then incubated at 37°C for 24 hours. After incubation, 1 ml of Kovacs reagents (Para-dimethyl-amino-benzaldehyde in isoamyle alcohol) was added, the tube gently shaken and read immediately. A bright pink colour/red in the top layer indicates the presence of indole (indole positive) for *E. coli* [25].

2.2 Methyl Red (MR) Test

This is a biochemical test based on the ability of bacteria to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Methyl Red is a pH indicator, which remains red in colour at a pH of 4.4 or less. Glucose phosphate broth, which contains glucose and a phosphate buffer, was inoculated with the presumptive *E. coli* isolates and then incubated at 37°C for 48 hours. The pH of the medium was tested by the addition of 5 drops of MR reagent. Development of red colour is taken as positive. MR negative organisms produce yellow colour. *E. coli* is MR positive [27].

2.3 Voges Proskauer (VP) Test

This is a biochemical test that is used to detect butylene glycol producers. Presumptive *E. coli* isolates were inoculated into glucose phosphate broth and incubated for at least 48 hours, 0.6 ml of alpha-naphthol was added to the test broth and shaken. This was followed by the addition of 0.2 ml of 40% KOH to the broth which was shaken. The tube was allowed to stand for 15 minutes and appearance of red colour will be taken as a positive test. The negative tubes must be held for one hour because maximum colour development occurs within one hour after addition of reagents. *E. coli* is VP Negative [27].

2.4 Citrate Utilization Test

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Colonies of presumptive *E. coli* strains to be tested were picked up with a straight wire and then inoculated into Simmon's citrate (CT) agar slope. It was incubated at 37°C for 24 hours. If the organism has the ability to utilize citrate, the medium changes its colour from green to blue. *E. coli* is CT Negative [25].

2.5 Antibiotic Sensitivity Tests of Isolates

The antibiotic sensitivity profile of *E. coli* isolates was determined according to the method of Bauer-Kirby [28] by using commercially prepared discs (Polydisc®) with known concentrations of antibiotics. Ten antibiotics were used and they include; Nitrofurantoin (100 µg), Gentamicin (10 µg), Ciprofloxacin (10 µg), Chloramphenicol (10 µg), Ofloxacin (10 µg), Cefuroxime (10 µg), Pefloxacin (10 µg), Ceftriaxone (30 µg), Amoxicillin (30 µg) and Streptomycin (30 µg). Freshly sub cultured pure isolate of *E. coli* colonies from agar culture plates were emulsified in 3-4 ml of sterile normal saline. Nutrient Agar Medium was prepared and a sterile cotton swab

stick was dipped into the suspension. Excess fluid was removed by pressing and rotating the swab against the side of the tube above the suspension. The dried surface of the Nutrient Agar was inoculated by streaking the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60° to ensure even distribution [25]. The antimicrobial discs were placed on the inoculated plates and incubated at 35°C for 18 hrs-24 hrs. Zone of inhibition were measured in mm. The sizes of the zones of inhibition were interpreted based on the National Committee for Clinical Laboratory Standard criteria [29].

2.6 Data Analysis

Chi-squared test and Fisher's exact test were used to calculate Relative Risk at 95% Confidence Interval (C.I.) to determine the difference in proportions and statistical significance of commensal *E. coli* isolation between variables. The differences were considered to be significant when the *p* value is less than 0.05 ($P< 0.05$). GraphPad InStat version 3.10, (Graph Pad Software Inc.) was used to perform the analyses.

3. RESULTS

3.1 Isolation Rate of Commensal *E. coli* in Apparently Healthy Lambs and Kids from Maiduguri, Northeastern Nigeria

Out of the 200 sampled lambs and kids, 90 (45%) had feces positive for commensal *E. coli* (Table 1). A total of 20 (20%) lambs and 70 (70%) kids tested positive with a significant (P=0.0001) relative risk of 0.3056 at 0.2042-0.4573 C.I. (Table 1).

3.2 Age Distribution of Commensal *E. coli* Isolates in Apparently Healthy Lambs and Kids from Maiduguri, Northeastern Nigeria

Out of the 200 samples, 37 (18.5%) were collected from lambs and kids between the age of 0 to 1 month, 47 (23.5%) from>1 to ≤ 2 months and 69 (34.5%) from>2 to ≤ 3 months (Table 2). A total of 12 (32.4%) out of the 37 lambs and kids between 0 to month l were positive for commensal *E. coli*, 39 (83%) of the 47 lambs and kids between >1 to ≤ 2 months were positive whereas, 39 (56.5%) lambs and kids between >2 to ≤ 3 months were also positive (Table 2). The difference in commensal *E. coli* isolation rate was statistically significant (p=0.01) between the different age groups.

3.3 Antibiotic susceptibility pattern of Commensal *E. coli* Isolates in Apparently Healthy Lambs and Kids from Maiduguri, Northeastern Nigeria

Out of the 10 antibiotics tested against commensal *E. coli* in this study, all the 90 (100%) isolates have expressed resistance against Pefloxacin, 88 (97.7%), isolates were resistant to Amoxicillin, 85 (94.4%) resistant to Chloramphenicol, 84 (93.3%) were resistant to Ceftriaxone and 83 (92.2%) resistant to Cefuroxime (Table 3). Ciprofloxacin and Ofloxacin showed highest level of susceptibility amongst the tested antibiotics, both had 86 (95.55%) isolates showing susceptibility with 4 (4.4%) isolates showing intermediate susceptibility to both agents (Table 3). Sixty-one (67.8%) isolates were resistant to Nitrofurantoin, 20 (22.2%) isolates showed intermediate susceptibility and 9 (10%) isolates were susceptible to it. Streptomycin and Gentamicin were effective against 34 (37.7%) and 36 (40%) isolates respectively with 46 (51.1%) and 35 (38.9%) isolates showing resistance to them respectively (Table 3).

3.4 Multidrug Resistance Pattern of Commensal *E. coli* Isolates in Apparently Healthy Lambs and Kids from Maiduguri, Northeastern Nigeria

All the 90 (100%) isolates showed multi drug resistance with each isolate resistant to between 3 to 8 antibiotics (Table 4). A total of 20 (22.2%) isolates were found to be resistant to Chloramphenicol, Cefuroxime, Pefloxacin, Ceftriaxone and Amoxicillin; 16 (17.8%) isolates were resistant to Nitrofurantoin, Chloramphenicol, Cefuroxime, Pefloxacin, Ceftriaxone and Amoxicillin. Highest level of resistance was observed in 14 (15.6%) isolates which showed resistance to 8 antibiotics namely Nitrofurantoin, Gentamicin, Chloramphenicol, Cefuroxime, Pefloxacin, Ceftriaxone, Amoxicillin and Streptomycin (Table 4). There are 8(8.89%) isolates expressed resistance to 7 antibiotics which include Nitrofurantoin, Chloramphenicol, Cefuroxime, Pefloxacin, Ceftriaxone, Amoxicillin and Streptomycin.

Table 1. Isolation rate of commensal *E. coli* in apparently healthy lambs and kids from Maiduguri, Northeastern Nigeria

Animal	No. of samples collected	No. of samples positive	Percent positive	RR	p-value
Lambs	100	20	20%	0.3056	0.0001
Kids	100	70	70%		
Total	200	90	45%		

Table 2. Age distribution of Commensal *E. coli* isolates in apparently healthy lambs and kids from Maiduguri, Northeastern Nigeria

Age	Number of isolate tested	Number positive	p-value
0 – 1 month	37 (18.5%)	12 (13.3%)	0.0117
>1 to ≤ 2 months	47 (23.5%)	39 (43.3%)	
>2 to ≤ 3 months	69 (34.5%)	39 (43.3%)	
Total	200 (100%)	90 (45%)	

Table 3. Antibiotic susceptibility pattern of Commensal *E. coli* isolates in apparently healthy lambs and kids from Maiduguri, Northeastern Nigeria

Antibiotics	Resistance		Susceptible		Intermediate	
	Number of isolates	(%)	Number of isolates	(%)	Number of isolates	(%)
Nitrofurantoin (N)	61	67.8	9	10	20	22.2
Gentamicin (CN)	35	38.9	36	40	19	21.1
Ciprofloxacin (CIP)	0	0	86	95.5	4	4.4
Chloramphenicol (C)	85	94.4	0	0	5	5.5
Ofloxacin (OF)	0	0	86	95.5	4	4.4
Cefuroxime (CF)	83	92.2	5	5.5	2	2.2
Pefloxacin (PF)	90	100	0	0	0	0
Ceftriaxone (CT)	84	93.3	0	0	6	6.7
Amoxicillin (AX)	88	97.7	0	0	2	2.2
Streptomycin (ST)	46	51.1	34	37.7	10	11.1

4. DISCUSSION

E. coli are a normal flora of the GIT of warm blooded animals, including small ruminants, and are thus shed via feces. Zschock et al. [30] have demonstrated *E. coli* shedding in small ruminants. The low recovery of commensal *E. coli* from lambs could possibly be associated with the method used in sample collection. Fecal sampling using swaps in some instances don't yield good result especially, for instance, if the animal in question had an empty rectum. It might also be caused by lack of enrichment of the samples which are usually collected on dry cotton tipped swaps and, in most cases, are kept overnight in an ice packed container before submitting to the Laboratory the next day for analysis. It is therefore highly probable that some of the samples must have lost viability before inoculation. Although not clearly understood, the higher *E. coli* isolation rate recorded in kids as against lambs from this study could possibly be indicative of early onset of rumen activity in kids compared to lambs. Previously, a study [31] has reported higher *E. coli* isolates in lambs (57.8%) than in kids (42.4%). The higher load of fecal *E. coli* observed in the kids and lambs between 2 to 3 months old age category, as compared to the younger ones, is suggestive of the facts that rumen microbial activity is a function of age. It was reported in a study [32] that as starter concentration intake and some small pieces of forage increases in kids, the quicker their rumen bacterial community becomes more similar to those in adults. More recently, it has been reported that the colonization by rumen epithelial bacteria is age related and is achieved at 2 months of life [33].

Based on the antimicrobial resistance data in this study, exceptional resistances were observed in isolates tested against Pefloxacin (100%) followed by Amoxicillin (97.7%), Chloramphenicol (94.4%), Ceftriaxone (93.3%), Cefuroxime

(92.2%), Nitrofurantoin (67.8%) and Streptomycin (51.1%). This means that, these groups of antibiotics are no longer suitable for use in the control of diseases caused by *E. coli*. Moemen et al. [34] have reported similar findings where he founds 100% *E. coli* isolates showing resistance against gentamicin, Pefloxacin, amoxicillin, and Enrofloxacin in Egypt. This implies that, the high levels of antimicrobial resistance exhibited by *E. coli* strains to clinically valuable antimicrobials, suggest that *E. coli* from lambs and kids may play a considerable role as reservoirs for resistance genes. Ciprofloxacin and Ofloxacin are the drugs with potentials in the control of *E. coli* where both showed (95.5%) sensitivity. Similarly, in a study [35], both Ciprofloxacin and Ofloxacin were reported with 90% sensitivity to *E. coli*. Another study [36] reported 88.89% sensitivity to both Ciprofloxacin and Ceftriaxone, 72.23% sensitivity to Cefuroxime and 36.11% resistant to Gentamicin.

Table 4. Multidrug resistance pattern of Commensal *E. coli* isolates in apparently healthy lambs and kids from Maiduguri, Northeastern Nigeria

Resistance pattern	Number of isolates
C, CF, PF	2
C, CF, PF, AX	1
N, PF, CT, AX	1
N, C, CF, PF, AX	2
C, CF, PF, CT, AX	20
N, C, PF, CT, AX	1
N, CF, PF, CT, AX	1
CN, C, CF, PF, AX	1
N, C, CF, PF, CT, AX	16
CN, C, CF, PF, CT, AX	3
N, CF, PF, CT, AX, ST	1
N, CN, C, CF, PF, AX	1
CN, C, CF, PF, AX, ST	1
N, C, PF, CT, AX, ST	1
N, CN, C, PF, AX, ST	1
N, C, CF, PF, CT, AX	1
CN, C, CF PF, CT, AX	1
N, C, CF, PF, CT, AX, ST	2
N, CN, C, CF, PF, CT, AX	2
N, CN, CF, PF, CT, AX, ST	2
N, CN, C, CF, PF, AX, ST	2
N, CN, C, CF, PF, CT, AX	4
N, C, CF, PF, CT, AX, ST	8
N, CN, C, CF, PF, CT, AX, ST	14
N, CN, C, PF, CT, AX, ST	1

N=Nitrofurantoin, CN=Gentamicin, CIP=Ciprofloxacin, C=Chloramphenicol, OF=Ofloxacin, CF=Cefuroxime, PF=Pefloxacin, CT=Ceftriaxone, AX =Amoxicillin and ST= Streptomycin

Expression of multi-drug resistance by all the *E. coli* isolates in this study is a matter of serious public health concern. Although, investigating the rational for such high occurrence is not within the scopes of this study, however, some previous studies have highlighted frequent use of antibiotics as animal feeds supplement and abuse of veterinary drugs as the possible factors [31,37]. The problem is further aggravated by the transfer of *E. coli* from livestock to poultry to human [38,39]. Hinton et al. [39] reported that the use of drugs does not induce resistance, but rather provides an intense selection pressure which eliminates the susceptible normal flora in the host and spares the resistant ones.

5. CONCLUSION

Commensal *E. coli* has been isolated from apparently healthy lambs and kids and the organism's load increases with age. Ciprofloxacin and Ofloxacin are drugs of choice in the control of *E. coli* infections. All the isolates were multi-drug resistant strains showing resistance to between 3 to 8 out of the 10 antibiotics used in this study. Awareness should be created amongst the general public on the danger of indiscriminate use of antibiotics in food animals' production as growth promoters and veterinary practice. Studies should be carried out to evaluate the possible mechanism by which commensal *E. coli* in lambs and kids acquire multi-drug resistance.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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