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Research Article

Characterization and Antibiotic Resistance of *E. Coli* Recovered from Healthy Captive Non-Human Primates In Nigeria

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ABSTRACT

Escherichia coli is one of the members of the family Enterobacteriacea. The cells appear rod like in shape and are Gram-negative bacteria. It is part of micro flora of animals with non-human primates (NHPs) inclusive. *E. coli is pathogenic and is* causal organism of diarrhea all over the world. The aims of this study are to determine whether non – human primates are reservoirs for *E. coli*, to investigate the relatedness of *E. coli* with others in some regions and to determine the antibiotic sensitivity as well as resistance of the isolates. *Escherichia coli* were recovered from 5 (11%) out of the 43 NHPs. All the isolates appeared non-haemolytic. Findings of this study revealed that the isolates showed high level resistance to Amoxicillin/clavulanic (80%), Sulphamethoxazole/trimethoprim (80%), Gentamycin (60%), Cefoxitin (60%) and Ciprofloxacin (60%). Most of the isolates are multidrug resistant, showing resistance to two, three or more antibiotics. There are similar genetic backgrounds within *E. coli* isolates identified from *Cercopithecus mona* and *Cercopithecus sebaeus*. Clustering shows that isolates from *Cercopithecus mona* and *Papio Anubis* clustered together within the same clade. Wild monkeys usually interact with humans through activities such as domestication and tourism. Through these interactions, pathogenic bacteria are transmitted from humans and animals, particularly wild monkeys. This is a potential source of infections in man. Isolation of *E. coli* in this study shows that NHPs are natural reservoirs of the organisms, the isolates are genetically related to each other and are multidrug resistant.

Keywords: *E. coli*, non- human primates, antibiotics resistance, sequence.

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INTRODUCTION

Escherichia coli is a part of the intestinal microflora of both humans and animals. While their gut population is lesser than that of the obligate anaerobes, they dominate the gut of most animals including wild monkeys. (Bruce *et al*., 1989). Quite a number of *E coli* strain are nonpathogenic, but some strains are implicated in enteric disease through virulence factors plasmid-mediated. (Bruce *et al*., 1989). Apart of from their role in gut health maintenance, they can be very pathogenic (Bentley and Meganathan, 1982, Kaper *et al*., 2004). The Pathogenic forms are agents of diarrhea worldwide (Levine, 1987).

Some pathogenic strains of E. coli such as enteropathogenic *E. coli* (EPEC) are implicated in persistent diarrhea in many animals. (Carvalho *et al.*, 2003, Chen and Frankel, 2005). The identified pathotypes have differing virulence within hosts and related mammals. (Bueris *et al*., 2007, Rwego *et al*., 2008). A diarrhea outbreak caused by attaching and effacing E. coli was reported in marmosets maintained at the Primatology center. (Thomson and Scheffler, 1996). A simian immunodeficiency virus opportunistic infection in rhesus monkeys as well as ulcerative colitis in cotton-top tamarins was reported to be associated with enteropathogenic *Escherichia coli* (Mansfield *et al*., 2001). The aim of this study is to determine whether primates are reservoirs for *E. coli*, to investigate the relatedness of *E. coli* with others in some regions, to determine the sensitivity as well as resistance of the isolates to common antibiotics.

In this study, we examined *E. coli* isolates from 43 non - human primates from different zoos and gardens in Nigeria, investigated their genetic relatedness with a sequence based approach, phylogenetic tree and antibiotic susceptibility profiles.

MATERIALS AND METHODS

This study complied with protocols approved through the University of Ibadan Animal Care and Use Research Ethics Committee (UIACUREC) with the number UI-ACUREC/App/2015/54. A total of 43 primates were included in this study, they were from different accredited zoological and tourism gardens (Table 1). Fresh faecal samples were collected from the rectum using sterile cotton swabs, these were placed in ice packs and transported from the zoos or gardens to the laboratory for processing.

Bacteriological Processing: Sterile cotton swabs were used to collect faecal sample from the rectum of primates, transported to the laboratory and plated onto MacConkey agar. Suspected *colonies w*ere subcultured on eosine methylene blue (EMB) agar for *E. coli* confirmation. These were stored on tryptose soy agar for further use. Finally, the suspected colonies with greenish metallic sheen from the EMB agar were subjected to analytic profile index (API) and Polymerase Chain Reaction was used for final confirmation of the isolates as *E. coli*.

Determination of haemolytic activity: Heamolytic activities of the isolates were determined for by culturing each isolate on blood agar medium using 5% concentration. The cultured plates were incubated at 37°C for 24 hours.

DNA Extraction: Colonies grown on medium were transferred to 1.5 ml peptone broth and cultures were grown on a shaker for 48 h at 28 ºC. After this, liquid cultures (1-3 mL) were centrifuged at 4600x *g* for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hour at 37 ºC. After incubation, 5 M NaCl (100 µl) and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 x *g* for 20 min. The aqueous phase was transferred to a new tube, isopropanol (1:0.6) was added and DNA was precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 7200 x *g* for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours. Pellets were resuspended in 50 µl of TE buffer and kept at 4°C.

PCR Analysis: Primers used for the PCR analysis include
Forward primer: *27F (5'-AGAGTTTGATCMTGGCTCAG-3')*
Reverse primer: 1525R (5'-AAGGAGGTGWTCCARCC-3')
PCR conditions include 94°C for 2 mins, 30 cycles of 94°C for 30 secs, 50°C for 60 secs and 72°C for 90 secs, 72°C for 5 mins, Store at 4°C. Agarose gel was prepared by adding 1.5 g of agarose powder into 100 ml of 1X TAE Buffer. This was heated in a microwave for 5 minutes, allowed to cool briefly and 5 µl of GR Green® solution was added. It was then mixed briefly and poured into a gel tank with well combs. It was left to solidify while PCR products were loaded into each well. Electrophoresis was at 100V for one hour. Gel was viewed under UV light. Expected product size was 1,500 bp.

Sequence-based typing: A total of five *E. coli* isolates were subjected to partial sequence 16S ribosomal RNA using nBLAST on GenBank (Table 2). Partial sequences of 16S rRNA region of other worldwide *E. coli* isolates were retrieved from GenBank database and aligned with the sequenced samples using ClustalW multiple sequence alignment program with default parameters as implemented in BioEdit v.7.2.3 (Hall. 1999). Sequence pairwise identities occurring within the isolates were performed using SDT v1.2 (Muhire *et al*., 2014) with pairwise gap deletions. A phylogenetic tree was constructed using the maximum likelihood method based on Jukes-Cantor model in MEGA v.6.06 (Tamura et al., 2013) with bootstrap replicate values set at 1,000.

Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site.

Phylogenetic group typing: Partial sequences of 16S rRNA region of other worldwide *E. coli* isolates were retrieved from GenBank database from and aligned with the sequenced samples using ClustalW multiple sequence alignment program with default parameters as implemented in BioEdit v.7.2.3 (Hall, 1999). Sequence pairwise identities occurring within the isolates were performed using SDT v1.2 (Muhire *et al*., 2014) with pairwise gap deletions. A phylogenetic tree was constructed using the maximum likelihood method based on Jukes-Cantor model in MEGA v.6.06 (Tamura et al., 2013) with bootstrap replicate values set at 1,000.

Antimicrobial susceptibility testing: *E. coli* isolates (N - 05) were subjected to susceptibility with eight antimicrobial agents using disk diffusion method, according to the Clinical Laboratory Standards Institute (CLSI 2021). Antibiotic discs containing the following antimicrobial agents were used: amoxicillin/clavulanic acid 2:1 (AMC, 30µg Oxoid), Gentamicin (CN, 10µg Oxoid), cefoxitin (FOX 30µg Oxoid,), ceftazidime (CAZ, 30µg Oxoid), ceftriaxone (CRO, 30µg Oxoid), Ciprofloxacin (CIP, 5µg Oxoid) sulphamethoxazole/trimethoprim (SXT, 25 µg Oxoid) and ertapenem (ETP, 10 µg Oxoid).

RESULTS

A total number of 5 *E. coli* were isolated from NHP from different zoological gardens in Nigeria (Table 2). All of the isolates produced greenish colour on Eosine Methylene Blue agar, this is characteristic of *E. coli*. This study showed that all the isolates are non –haemolytic. Table 3 shows zone diameter of antibiotics for all isolates. The organisms showed high level resistance to Amoxicillin/clavulanic (80%) and Sulphamethoxazole/trimethoprim (80%) while there was 60% resistance to Gentamicin, cefoxitin and ciprofloxacin (Fig 1, Table 4). Antimicrobial susceptibility of the organisms showed that most of the isolates are multidrug resistant, showing resistant to three or more antibiotics (Table 5). Worthy of note is isolate number OS2 and AG1 showing resistance to 5 and 7 antibiotics respectively (Table 5). Fig. 2 showed Phylogenetic tree, this shows evolutionary relationships among the five partial 16S ribosomal RNA sequences of *E. coli* isolates obtained from NHPs in Oyo and Osun States, Nigeria with others from China, India and Australia. Further sequence analyses also showed similar genetic backgrounds within *E. coli* isolates identified from *Cercopithecus mona* and *Cercopithecus sebaeus* in Osogbo and Ibadan, respectively. The isolates from *Cercopithecus mona* and *Papio Anubis*, both from Ibadan, also clustered together within the same clade.

Table 1:
Non-human Primates included in this study

S/N	Identity	Location	Common Name	Zoological Name	Sex	Age (years)
1	1D	UI Zoo	Mona monkey	*Cercopithecus mona*	Male	6
2	2D	UI Zoo	Patas monkey	*Erythrocebus patas*	Female	4
3	3D	UI Zoo	mangabey monkey	*Cercocebus torquatus*	Female	Adult
4	4D	UI Zoo	mangabey monkey	*Cercocebus torquatus*	Female	Adult
5	5D	UI Zoo	Patas monkey	*Erythrocebus patas*	Male	10
6	6D	UI Zoo	White throathed monkey	*Cercopithecus nictitans*	Male	14
7	7D	UI Zoo	Patas monkey	*Erythrocebus patas*	Female	Adult
8	8D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Female	4
9	9D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Male	8
10	10D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Male	Adult
11	11D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Female	2
12	12D	Pet monkey at Jericho area, Ibadan	Green monkey	*Cercocebus sabaeus*	Female	3months
13	13D	UI Zoo	Patas monkey	*Erythrocebus patas*	Female	Adult
14	14D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Male	Adult
15	15D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Male	Adult
16	16D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Female	Sub adult
17	17D	UI Zoo	Patas monkey	*Erythrocebus patas*	Female	Youth
18	18D	UI Zoo	Green monkey	*Cercocebus sabaeus*	Female	Adult
19	19D	UI Zoo	Anubis baboon	*Papio Anubis*	Male	Adult
20	20D	UI Zoo	Anubis baboon	*Papio Anubis*	Male	Adult
21	21D	UI Zoo	Anubis baboon	*Papio Anubis*	Male	Adult
22	22D	UI Zoo	Anubis baboon	*Papio Anubis*	Female	Adult
23	23D	UI Zoo	Anubis baboon	*Papio Anubis*	Male	Adult
24	24D	UI Zoo	anubis baboon	*Papio Anubis*	Male	Adult
25	25D	UI Zoo	Mona monkey	*Cercopithecus mona*	Female	Adult
26	26D	Unilorin Zoo	Patas monkey	*Erythrocebus patas*	Male	Adult
27	27D	Unilorin Zoo	Patas monkey	*Erythrocebus patas*	Male	Adult
28	28D	Unilorin Zoo	Patas monkey	*Erythrocebus patas*	Female	Adult
29	29D	Unilorin Zoo	Patas monkey	*Erythrocebus patas*	Female	Adult
30	30D	Unilorin Zoo	Mona monkey	*Cercopithecus mona*	Female	Adult
31	31D	Unilorin Zoo	Green monkey	*Cercocebus sabaeus*	Male	Adult
32	32D	Unilorin Zoo	Green monkey	*Cercocebus sabaeus*	Male	Adult
33	33D	Unilorin Zoo	Green monkey	*Cercocebus sabaeus*	Female	Adult
34	34D	Unilorin Zoo	Green monkey	*Cercocebus sabaeus*	Male	Adult
35	OS1	Osun Osogbo Sacred grove	Mona monkey	*Cercopithecus mona*	Female	Adult
36	OS2	Osun Osogbo Sacred grove	Mona monkey	*Cercopithecus mona*	Male	Adult
37	OS3	Osun Osogbo Sacred grove	Mona monkey	*Cercopithecus mona*	Female	Adult
38	OS4	Osun Osogbo Sacred grove	Mona monkey	*Cercopithecus mona*	Male	Adult
39	AG1	Agodi gardens Ibadan	Green monkey	*Cercocebus sabaeus*	Female	Adult
40	AG2	Agodi gardens Ibadan	Green monkey	*Cercocebus sabaeus*	Female	Adult
41	AG3	Agodi gardens Ibadan	Patas monkey	*Erythrocebus patas*	Female	Adult
42	AG4	Agodi gardens Ibadan	Anubis baboon	*Papio Anubis*	Female	Adult
43	PET2	Pet monkey at Apata area, Ibadan	Patas monkey	*Erythrocebus patas*	Male	Adult

Table 2:
E. coli isolated from different zoos and gardens

S/N	Identity	Location	Common Name	ZoologicalName	Sex	Age (years)
1	OS2	Osun Osogbo Sacred grove	Mona monkey	*Cercopithecus mona*	Male	Adult
2	AG2	Agodi gardens Ibadan	Green monkey	*Cercocebus sabaeus*	Female	Adult
3	AG4	Agodi gardens Ibadan	Anubis baboon	*Papio Anubis*	Female	Adult
4	9D	UI Zoo	Green monkey	Cercocebus sabaeus	Male	8
5	AG1	Agodi gardens Ibadan	Green monkey	Cercocebus sabaeus	Female	Adult

Table 3:
Measured zone diameter of antibiotics for all isolates

S/N	Isolate Number	AMC (mm)	CN (mm)	FOX (mm)	CAZ (mm)	CRO (mm)	CIP (mm)	SXT (mm)	ETP (mm)
1	OS2	10	10	18	00	20	00	00	20
2	AG2	16	10	10	24	22	04	22	18
3	AG4	16	16	10	18	14	18	00	20
4	9D	00	20	18	20	20	16	00	12
5	AG1	04	10	11	15	12	00	00	10

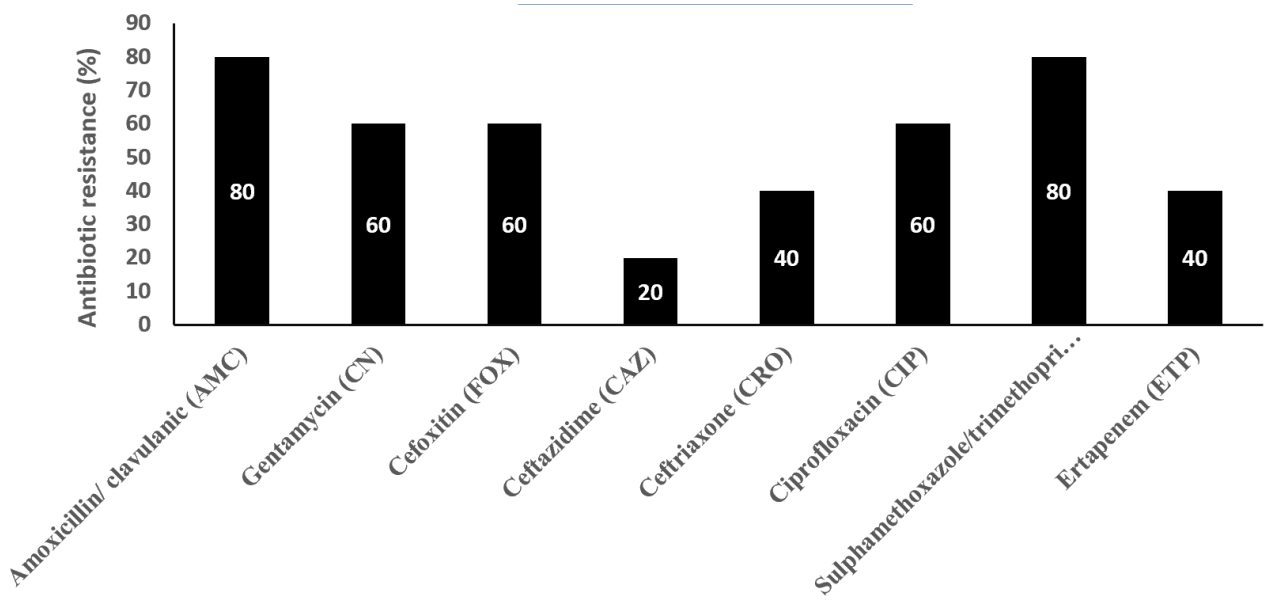

Figure 1:
Percentage Resistance Pattern of *E. coli* to selected antibiotics.
KEY: AMC: Amoxicillin/ clavulanic, CN: Gentamicin, FOX: Cefoxitin, CAZ: Ceftazidime, CRO: Ceftriaxone, CIP: Ciprofloxacin, SXT: Sulphamethoxazole/trimethoprim, ETP: Ertapenem

Table 4:
Percentage resistance/sensitivity of the organisms to different antibiotics

S/N	Antibiotics	Resistance %	Sensitive %
1	Amoxicillin/ clavulanic (AMC)	80	20
2	Gentamicin (CN)	60	40
3	Cefoxitin (FOX)	60	40
4	Ceftazidime (CAZ)	20	80
5	Ceftriaxone (CRO)	40	60
6	Ciprofloxacin (CIP)	60	40
7	Sulphamethoxazole/ trimethoprim (SXT)	80	20
8	Ertapenem (ETP)	40	60

Table 5:
Resistance Pattern of the *E. coli* isolates

S/N	Isolate Number	Resistance Pattern
1	OS2	AMC, CN, CAZ, CIP, SXT
2	AG2	CN, FOX, CIP,
3	AG4	FOX, CRO, SXT
4	9D	AMC, SXT, ETP
5	AG1	AMC, CN, FOX, CRO, CIP, SXT, ETP

Sequence-based typing: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.00420808 is shown in Fig. 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1429 positions in the final dataset.

Phylogenetic Analysis: Results of the sequence-based typing showed that isolates with similar genetic backgrounds were found
between Cercopithecus mona and Cercopithecus sebaeus.
There is similar identity between E. coli AG1 and strain CP049979 (Australia), CP049348 (China) and CP051222 (China). While E. coli AG4 shares identity with CP53720 (China). Similarly, E. coli OS2 shares identity with CP051222 (China) and AG2 shares identity with CP054940 (India).

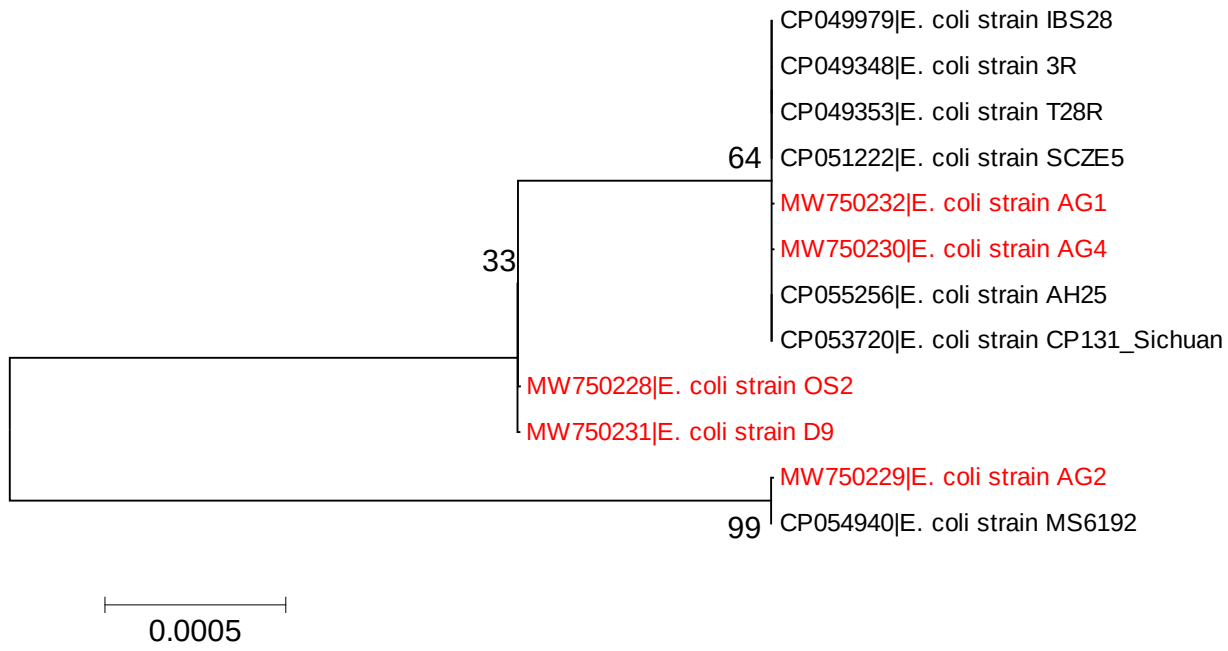

Figure 2:
Phylogenetic tree showing evolutionary relationships among the five partial 16S ribosomal RNA sequences of *Escherichia coli* isolates obtained from monkeys (in red) with others across different parts of the world.

DISCUSSION

A total number of 5 of 43 samples (11%) yielded *E. coli* in this study, this is lower compared to the work of Foster- Nyarko *et al* (2020) who reported 56% yield in 43 Gambian non- human primates. This study reveals the presence of *E. coli* known to be an agent of diarrhea being haboured by the NHPs. These agents though are gastrointestinal tract flora, their pathogenic strains are causative agents of diarrhea worldwide. Presence of *E. coli* in humans and non- human primates can be as a result of direct transmission from parent to offspring within the same host species or it can be from gut colonization of similar ancestors of humans and non- human primate species (Foster- Nyarko *et al.*, 2020). Transfer of strains from one host species to another is also a possibility (Lozupone et al., 2013). Results of this study reveals that non-human primates in Nigeria are usually exposed to humans through tourism activities, so there is possible bacteria transmission between humans and animals, especially wild monkeys. Drug resistance has constituted a great threat in the treatment of infectious diseases worldwide. Increase in resistance could largely be attributed to several reasons - including the migration of infectious people all over the world and misuse of antibiotics (Sarker et al., 2014). Other factors include, use of degraded, expired, poor quality, counterfeit and adulterated drugs (Cars *et al*., 2008). Horizontal gene transfer is a means through which antibiotic resistance in wildlife spreads. (Vittecoq et al., 2016). Though some of the isolates were sensitive to the antibiotics employed for this study, resistance especially to third generation cephalosporins recorded for the isolates is worrisome because these are the latest antibiotic groups employed in the treatment of diarrhea related infections. In essence humans that suffer clinical conditions after contact with the animals are at a high risk of infection.

Results of the sequence-based typing, targeting the bacterial 16S ribosomal RNA segment confirmed *E. coli*, although variations were observed within the nucleotides sequences of strain AG2. As suggested by Ahmed *et al* (2017), age plays a vital role in the genetic variation observed among gut *E. coli*, even though the mechanisms are not fully understood. However, findings from this study is in contrast to that of Ahmed *et al* (2017) who proposed that genetic diversity is predominant in young animals, variations recorded in this study is in adult non-human primates.

The similarities among the isolates ranged from 99.44% to 100% , this suggests evolution within the host after acquisition of the strain according to Foster- Nyarko *et al* (2020). The *E. coli* isolates AG1 and AG4 clustered together with strains from Australia (CP049979) and China (CP049348 and CP051222). Reasons for this maybe that there might have been a passage of some bacterial lineages via transmission from parents to offspring within the same host species. This might have arose from ancestral bacteria that colonized the guts of the most recent common ancestors of non-human primate species (Moeller et al., 2016). Similarly, *E. coli* isolate AG2 shared similar ancestor with isolate MS6192 from Australia.

Impacts of *E. coli* strains in enteric infections of captive non-human primates may be underestimated. In this study, *E. coli* were isolated, identified and antibiogram was performed. The results reported here shows that *E. coli* which are part of

normal flora can be pathogenic to both non - human primates and human. Apart from the effects that the isolates can have on the captive non- human primates, it poses a great threat to public health in that there is a high risk of transmission to humans. Since the organisms are pathogenic, they can serve as source of infection to humans through contact. Increasing antimicrobial resistance is a global phenomenon as it was observed in the present study. This is largely due to indiscriminate use of antibiotics even in non-human primates.

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