Antimicrobial Susceptibility Profiles of Diarrheagenic *Escherichia coli* Strains Isolated in Children Below Five Years and Food Animals Kisumu County, Kenya

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Abstract

Background: Diarrheagenic *Escherichia Coli* (DEC) are the major cause of diarrhoea in children in developing and undeveloped countries. Domestic food animals act as reservoirs of DEC that are transmitted to children below five years through inadequate hygiene practices.

Aim: To determine the antimicrobial susceptibility patterns of DEC strains isolated from children below five years and food animals in Kisumu County.

Methods: A total of 250 samples, were collected and analyzed using polymerase chain reaction. The antimicrobial susceptibility was determined using Kirby-Bauer disc diffusion method.

Results: Enteroaggregative *E.coli* (12%), Enteropathogenic E.coli (5%), Enterotoxigenic *E.coli* (3%) and mixed infections (3%) respectively. From the DEC children sample isolates, antimicrobial agents showed 84.6% sensitivity towards Meropenem followed by Gentamicin (80.8%) and then Nalidixic acid (73.1%). High resistance was recorded among the Tetracycline and Sulfamethoxazole antibiotics. Of the 26 DEC children sample isolates, 24(92%) exhibited multidrug resistance patterns: 46% showed resistance to four different antibiotics, 38% to five different antibiotics and 4% to six different antibiotics. Animal samples showed 100% susceptibility to most antibiotics, with only two isolates showing multidrug resistance to five antibiotics and one showing resistance to four and three antibiotics, respectively. The multidrug index value for the DEC pathotypes isolated in children was 0.5, indicating a high risk of DEC transmission.

Conclusion: Multidrug resistant bacteria reported in food animals and children populations suggested the possibility of transmission of multidrug resistance bacteria between children and food animals. Other sources of transmission also need to be investigated.

Background

The increasing number of bacterial infections represent the global health crisis regarding infectious diseases [1]. Animals are always proposed as pathogenic bacterial reservoirs and may play a critical role in transmission to humans [2]. The interaction between animals and humans allows both populations to act as reservoirs from which antimicrobial-resistant bacterial infections can be bidirectionally transmitted [3]. The role of animals in the emergence and transmission of antimicrobial resistance to the human population is yet to be fully understood.

Improper use of antimicrobial agents by humans are considered major drivers of antimicrobial resistance [4]. Escherichia coli (E.coli) is a bacteria that normally lives in the intestines of both healthy people and animals. The prevalence of antibiotic resistance among E. coli isolates from patients with acute diarrhoea in Egypt was 68.2%, 57.2%, and 24.2% for ampicillin, trimethoprim-sulfamethoxazole, and ampicillin respectively [5]. Studies conducted in Asia have reported the highest resistance to the penicillin class of antibiotic with amoxicillin recording 80.9% followed by ampicillin at 73.5% [6]. Escherichia coli also represents a major reservoir of AMR genes that may be responsible for treatment failures in both human and animals. An increasing number of AMR genes has been identified in E. coli many of which are located in mobile genetic elements and hence can be transmitted between animals, humans and the natural world [7].

AMR represents a global public health problem, for which the patterns among bacteria isolated from children with close contact to food animals is not widely documented. Hence, this study a clear understanding of the antibiotic profiles of both children below five years and food animals in Kisumu County. The data generated from this study would establish epidemiological surveillance systems that would promote collaboration directed on the wellbeing of children below five years and animals.

Materials and methods

Study area: This study was carried out in Kisumu County from August 2022 to February 2023. Kisumu has an annual rainfall of 276.22 mm and temperature of 23.93°C. It sits at an elevation of 1,131 m (3,711 ft). Kisumu is 200 miles northwest of Nairobi and it is located at the shores of lake Victoria.
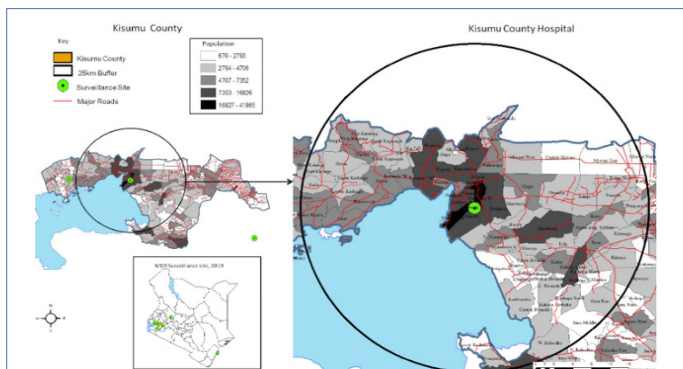

Figure 1: Map of Kisumu county (Source: Govt. of Kenya website).

Study population: All children below five years with diarrhea who attended Kisumu County hospital and had a food animal kept at their homes were included in the study.

Study design: This was a cross sectional study conducted in Kisumu County Hospital and this was chosen because of availability of clinical cases (children with diarrhea) and willingness of the childrens' parents to consent.

Sample size determination: Prevalence based methodology was used to estimate burden of diarrhea disease in Kisumu County among children below five years. Kisumu County reports diarrhea prevalence of 18% higher than the neighboring areas [8]. The P-value of less than 0.05 were considered statistically significant with a detection rate at 18% for children below 5 years with diarrhea [9].

The sample size was determined using the Fishers method.

Sample size $N_0 = Z^2pq/d^2$

Where: n = desired minimal sample size (when population is greater than 10,000)

z = standard normal deviate which is 1.96 at 95% confidence level.

p = estimated prevalence of diarrheal disease among under five children in Kisumu (18%).

d = degree of accuracy (0.05)

q = 1-p

Hence, using a confidence of 95% that corresponds to the standard normal deviate of 1.96, the proportion in the target population estimated at 18% and the degree of accuracy required set at 0.05, the sample size is:

$$= \frac{1.96^2 * 0.18 * 0.82}{0.05^2}$$

=227

10% of 227+23=250 (The 10% was to cover for lost samples during processing) = 250

Therefore a total of 250 samples were collected from both children and animals residing in Kisumu County, Kenya.

Sample collection: The sample collection procedure was done by a certified medical laboratory technician having the stool laboratory request from the pediatrician for the diarrheal child below five years who visited Kisumu County Hospital. The stool samples for children below two years who were unable to give stool at the moment were collected by researchers from the diapers, the stool samples were well labelled and aseptically transferred into a Cary Blair tube and transported to the Kenya Medical Research Laboratory in an ice box containing ice packs for bacterial isolation.

Isolation and identification of diarrheagenic E. coli: Isolation of E.coli was done on MacConkey agar, each of the 250 correctly collected fecal sample was streaked onto the MacConkey plate using a sterile wire loop in a biosafety cabinet, following incubation at 37°C for 18-24 hours. Pink colonies (lactose fermenters) and pale colonies (non-lactose fermenters) were observed. Three to five well separated lactose fermenting colonies were sub-cultured in a nutrient agar taken as pure E.coli culture. Each culture was then subjected to standard biochemical testing for identification of suspected E.coli. The pure E. coli isolates were suspended in tryptone soy broth supplemented with 20% glycerol and stored at -80°C awaiting subsequent analysis.

DNA isolation: The first four colonies of E.coli from overnight growth on MacConkey (Oxoid, Basingstoke, UK) were suspend-

ed in 200 μl of nuclease-free water and mixed thoroughly using a vortex. This was followed by boiling the bacterial suspension at 95° C for 20 minutes. The rich DNA supernatant was harvested after centrifugation at 12,000 ×g for 10 minutes and stored at -20°C[10].

Multiplex polymerase chain reaction: Characterization of the three selected E.coli pathotypes; EAEC, EPEC, and ETEC, was carried out using a multiplex PCR. The 25 μl PCR reaction mixture contained 12.5 μl of 2X DreamTaq Green PCR Master Mix (Thermo Scientific, Waltham, MA, USA), 0.5 μl (30 μM) each of forward and reverse primers, 6.5 μl of nuclease-free water and 5 μl of DNA template. The PCR reaction was performed on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation of 95°C for 5 min, followed by 25 cycles of amplification (94°C for 1 min, 58°C for 1:30 min and 72°C for 1:30 min) and a final extension step at 72°C for 10. Positive and negative controls were ATCC 25922 strain and nuclease-free water, respectively.

Antimicrobial susceptibility testing: The antibiotic susceptibility profiles of confirmed Diarrheagenic strains were determined against eleven antibiotic (Table 1). Using the disk diffusion method on Muller Hinton Agar (Oxoid, Basingstoke, Hampshire England) as described by [11]. The E. coli ATCC 25922 was used for quality control. The diameters of the zones of inhibition were measured using a Vernier caliper and classified as sensitive, Intermediate and resistant according the clinical and laboratory standard institute guidelines (CLSI, 2022). Multidrug resistant Index (MDRI) of individuals isolates was calculated by dividing the number of antimicrobial agents to which the isolates were resistant by the total number of drugs to which isolates were tested against. Values lower than 0.2 were considered low risk while values higher than 0.2 were considered high risk [12].

Table 1: Antimicrobial discs used for susceptibility testing of diarrheagenic E. coli Isolates.

Antibiotic class	Antimicrobial agent (Potency)
Aminoglycosides	Gentamycin (10 μg)
Carbapenems	Meropenem (10 μg)
Penicillin	Ampicillin (10 μg), Amoxillin (10 μg)
Beta lactam combination	Amoxicillin
Cephems	Ceftriaxone (30 μg)
Phenicols	Chloramphenicol (30 μg)
Sulphonamides	Trimethoprim- sulfamethoxazole (25 μg)
Quinolones	Nalidixic acid (30 μg)
Tetracycline	Tetracycline (30 μg)
Macrolides	Azithromycin (15 μg)
Fluoroquinolones	Ciprofloxacin (5 μg)

Data analysis: All data from the laboratory tests were coded, filtered, and recorded in Microsoft Excel spreadsheet 2007 (Microsoft Corporation) before being analyzed with Stata version 15.1. All data from diarrheic children of under the age of five were subject to contingency table analysis, and Pearson's Chi-square test was used to determine the statistical significance of the relationships and/or associations between the dependent and independent variables from the data. To examine the relationship between each predictor variable and the result variable, a logistic regression analysis was used. Throughout the investigation, a significance level of p<0.05 at 95% confidence intervals was employed.

Results

Antimicrobial sensitivity of diarrheagenic Escherichia coli isolated from children aged below five years: The antimicrobial sensitivity patterns of 26 diarrheagenic E. coli showed highest sensitivity patterns to various antibiotics; Of these 22(84.6%) diarrheagenic E. coli positive isolates were sensitive to Meropenem, 21(80.8%) of the diarrheagenic E. coli were sensitive to Gentamicin, 19(73.1%) diarrheagenic E. coli positive isolates showed sensitivity towards Nalidixic acid and 18(69.2%) of the diarrheagenic E. coli positive isolates were sensitive to Chloramphenicol Figure 2.
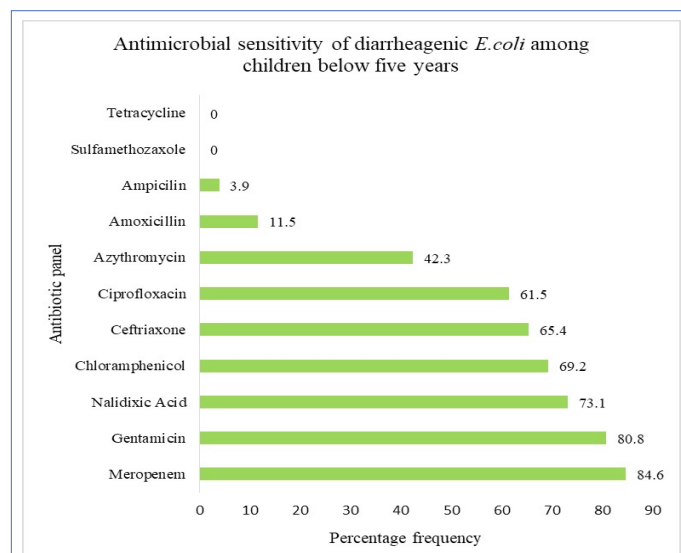

Figure 2: Overall antibiotic sensitivity of diarrheagenic E. coli among children below five years with contact to food animals.
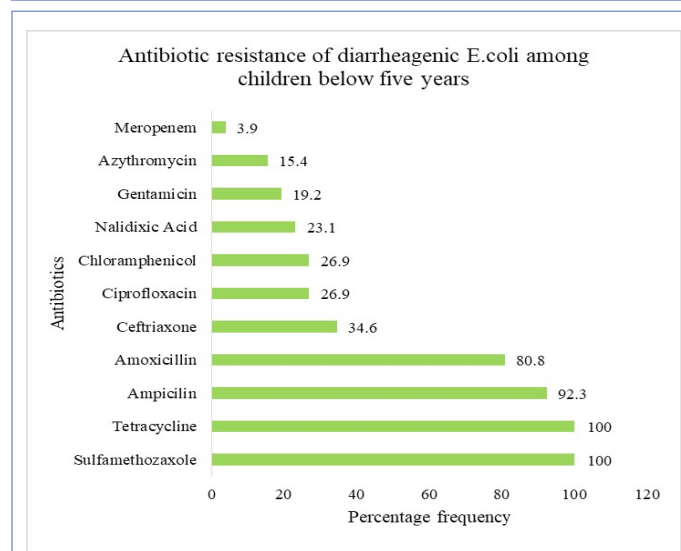

Figure 3: Overall antibiotic resistance of diarrheagenic E.coli among children below five years.

Antimicrobial resistance patterns of diarrheagenic Escherichia coli isolated from children aged below five years: The antimicrobial resistance of 26 diarrheagenic *E. coli* isolates showed 100% resistance to tetracycline and Sulfamethoxazole-Trimethoprim. Of the positive diarrheagenic *E. coli* isolates, 24(92.3%) showed resistance towards ampicillin and 21(80.8%) showed resistance to Amoxicillin (Figure 3).

Antimicrobial resistance patterns of diarrheagenic Escherichia coli from food animals: Of the six food animal samples

that were positive for various diarrheagenic E.coli strains 3/6 isolates showed resistance lower than 20% towards the three drugs Amoxicillin, Azithromycin and Ampicillin. They showed 100% sensitivity among the other eight antibiotics tested (Figure 4).

Multidrug resistance among DEC isolates from children: Of the 26 DEC isolates 24 showed multidrug resistance to more than three different drugs (Table 2).

Figure 4: Overall antibiotic resistance of diarrheagenic E. coli across food animals.

Table 2: Multiple drug-resistant DEC isolates among children.

No of antibiotic-resistant	No of isolates (24)	Percentage
3	3	12%
4	11	46%
5	9	38%
6	1	4%

All the Enteroaggregative E. coli strains in diarrheal children were resistant to tetracycline and Sulfamethoxazole –trimethoprim while 85.7% and 78.6% were resistant to Ampicillin and Amoxicillin respectively. The Enterotoxigenic and Enteropathogenic E. coli strains in diarrheal children showed (100%) resistance to Sulfamethoxazole-trimethoprim, tetracycline and ampicillin while resistance to Amoxicillin was 66.7% and 80% respectively. While the mixed infection (EAEC/ETEC and EAEC/EPEC) samples showed high level of resistance to Sulfamethoxazole –trimethoprim, Tetracycline, Ampicillin and Amoxylin (100%) (Table 3).
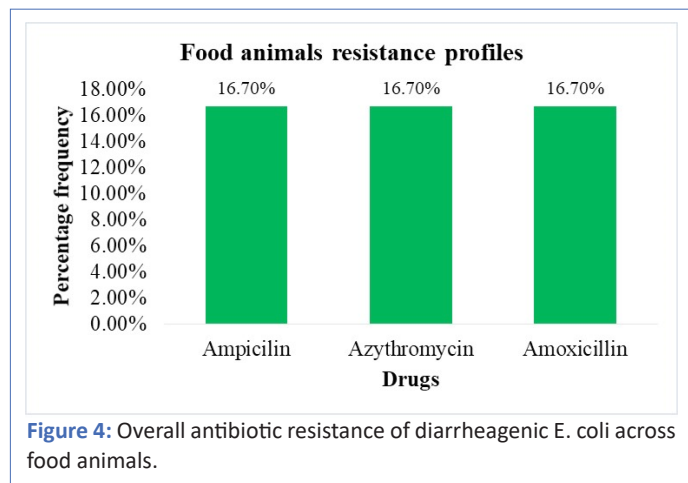
Table 3: Antimicrobial resistance percentage of diarrheagenic Escherichia coli strains/virulence genes.

Drugs	EAEC			EPEC			ETEC			MIXED INFECTIONS			P-value (95%CI)
	Resistance	Intermediate	Sensitivity	Resistance	Intermediate	Sensitivity	Resistance	Intermediate	Sensitivity	Resistance	Intermediate	Sensitivity	
Chloramphenicol	21.4%	0	78.6%	33.3%	33.3%	33.3%	20%	0	80%	50%	0	50%	0.134
Sulfamethoxazole	100%	0	0	100%	0	0	100%	0	0	100%	0	0	-
Tetracycline	100%	0	0	100%	0	0	100%	0	0	100%	0	0	-
Ciprofloxacin	14.3%	14.3%	71.4%	66.7%	33.3%	0	20%	0	80%	50%	0	50%	0.219
Nalidixic Acid	21.4%	0	78.6%	33.3%	0	66.7%	40%	20%	40%	0	0	100%	0.31
Ampicillin	85.7%	7.1%	7.1%	100%	0	0	100%	0	0	100%	0	0	0.932
Gentamicin	7.1%	0	92.9%	33.3%	0	66.7%	20%	0	80%	50%	0	50%	0.247
Azithromycin	14.3%	42.9%	42.9%	33.3%	66.7%	0	20%	20%	60%	0	50%	50%	0.665
Meropenem	0	14.3%	85.7%	33.3%	33.3%	33.3%	0	0	100%	0	0	100%	0.082
Amoxicillin	78.6%	7.1%	14.3%	66.7%	0	33.3%	80%	20%	0	100%	0	0	0.655
Ceftriaxone	21.4%	0	78.6%	66.7%	0	33.3%	40%	0	60%	50%	0	50%	0.404

Discussion

The present study has revealed sensitivity to Meropean, Gentamicin and Nalidixic acid above 70% among children below five years residing in Kisumu County. However there was resistance to Tetracycline, Sulfamethoxazole-trimethoprim and Amoxicillin above 90%. Isolates from animal samples showed sensitivity to most antibiotics and reported low levels of resistance below 20% towards Ampicillin, Amoxicillin and Azithromycin. Our analysis has shown that poor sanitation and environment contamination could link AMR transmission between animals and humans and could have contributed to increase in disease burden and demand of antibiotics among the children below five years.

The present study reports DEC pathotypes resistant to Tetracycline and Sulfamethoxazole (100% 26/26) and (92.3% 24/26), Ampicillin (80.8% 21/26), Amoxicillin (34.6% 9/26) and Ceftriaxone (26.9% 7/26). The resistance reported could be problematic in the management of bacteria-caused diarrhea. DEC pathotypes were also found resistant to these commonly prescribed drugs in other studies, including Ethiopia [13] and Nigeria [14]. Our findings also tend to concur with a study by Verma et al. in India that have reported high resistance to Trimethoprim 96% and tetracycline and ampicillin 97.3% [15]. This concurs with studies performed in Nairobi Kenya [16]. However, compared to other studies, prevalence to tetracycline and ampicillin was lower 61.4%, 55.2% [17,18]. This perhaps

means Sulfamethoxazole trimethoprim, tetracycline, ampicillin and amoxicillin need to be reviewed in the treatment of diarrhea in this population. DEC resistance was significantly higher among children below five years compared to domestic food animals that reported less than 20% resistant to only four antibiotics. This finding could be explained by the fact that gut inflammation like inflammatory diarrhea can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae [19]. In Kenya, antibiotic therapy is recommended for treatment of dysentery and salmonellosis for children below five years, while oral rehydration solutions and zinc sulphate are recommended for the management of DEC-related diarrhea (MOH 2016). However, low diagnostic capacities in low-middle income countries promote empiric treatment of childhood diarrhoea with antibiotics, which likely contributes to the emergence of resistant strains [20]. This study reports on low prevalence of resistance towards Gentamicin and ciprofloxacin to EAEC, EPEC and ETEC isolates which contrast certain studies in Burkhina faso [21]. The environment foremost likely permits comprehensive disease progression that lead to the clinical manifestation of resistant genes. Numerous reports advocate the global all-inclusive One Health approach to ameliorate the emergence and dissemination of antimicrobial resistance [22]. Efforts towards antibiotic management are best complemented with water, sanitation and hygiene interventions to reduce childhood diarrhea-related deaths.

This study has reported sensitivity to Merepenem 84.6%, Gentamicin 80.8%, Nalidixic acid 73.1%, Chloramphenicol 69.2%, Ceftriaxone 65.4 and Ciprofloxacin 61.5%. The findings towards merepenem sensitivity may be due to limited access as it is not prescribed at the outpatient clinics in Kenya. The susceptibility findings contradict studies conducted in India that reported high prevalence of antibiotics resistant of E.coli among a community in the rural settings, the high prevalence was mostly detected among the children [23]. This findings highlights the knowledge of the antibiogram profiles of pathogens could help understand the treatment of choice [18].

Analysis of food animal samples showed sensitivity towards most of the antibiotic that were used for treatment of DEC. This reveals the low usage of antibiotics in the food animals feeds and water in the farms by farmers for the purpose of growth promotion that creates favourable conditions for selection and spread of resistant bacteria within animals. These findings contrasts those from a study performed in south Africa that reported resistance towards similar antibiotics to food animals. However, reported resistance below 20% to Ampicillin, Azithromycin and Amoxicillin, the low resistance cannot not be ignored since it reveals that animal products pose a public health risk [11].

The growing Multidrug-Resistance (MDR) have been witnessed and broadly spreading amongst gram-negative bacteria [24]. This study reports the MDR rates of DEC isolates (70%) Sulfamethoxazole/Trimethoprim, ampicilin, Amoxicillin and tetracycline were the greatest contributors of MDR; SXT 29(100 %), Amp 28(92.3%) and tet 29(100 %) while Ceftriaxone and Nalidixic Acid contributed only 10% to MDR respectively.This means that diarrhea case management among children below five years perhaps remains with a narrow choice of treatment. This finding concurs to studies conducted in Qatar and Iran (31). However, the present finding of Multidrug-Resistant (MDR) phenotypes was lower than those reported by [27]. The current multidrug resistance is probably due to over-the-counter antibiotics that are widely available and used inappropriately

by humans and on animal production farms and this bacteria are known to accumulate genes responsible for coding antimicrobial resistance mechanisms [28]. Most MDR index reported greater than 0.2 suggesting high risk of pathogenic transmission in the environment [29]. The high percentage of MDR isolates revealed by this study among the children samples suggests that these organisms were exposed to high antibiotic use in the environment. Hence, rise in MDR index exacerbate the diarrhea case management resulting to limited treatment options.

Environment contaminated with animals infected dug contaminates animals teats and udder and this is passed to the raw milk hence facilitates transmission of antimicrobial resistant bacteria to children. Additionally,the variable of ''children eating soil'' during crawling as part of development exposes them to increased AMR load in the environment. Lack of personal hygienic practices such as washing of hands after use of the public toilets is associated with increased ampicillin resistant in children and increased tetracycline resistant E.coli in adults. Lastly, Sulfa drugs used as antibiotics are used as malaria treatment options in endemic areas, thus this practice correlates to load of Sulfamethoxazole resistant E.coli in children and it is revealed as a contributing factor to resistant [30].

Our study had few limitations. Isolates analysed from this study were collected from sick children who visited the health facility and they represent a non-randomized subset of the population. Consequently, our results may not generalize the entire children population. Lastly, given that DEC strains isolates are secondary cause of diarrhea among children we were not able to conclusively assess the severity of the pathogens in the population. Hence, longitudinal cohort studies that include a randomized population of children may be suited to address the limitation.

Conclusion

High prevalence of multidrug resistance was reported among children compared to food animals suggesting possibility of transmission of multidrug resistance bacteria between children and food animals. Therefore, Global and National Action plan need to re-direct its effort to reduce the burden of antimicrobial resistance in-order to attain optimal health in children, animals and environment.

Declarations

Ethical approval: This study was approved by Jaramogi Oginga Odinga Ethical Review Board REF: ISERC/JOOTRH/600/22. Confidentiality was observed throughout the study period.

Competing interest: Authors have no competing interest.

Consent to participate: Informed consent was obtained from all participants involved in the study.

Authors' contributions: R.Y Conceptualization and writing – original draft, R.Y, E.K & C.O Methodology, data curation formal analysis. G.M, J.G & G.K Supervision and writing-review and editing.

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