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ORIGINAL RESEARCH

Distribution of ß-Lactamase Genes Among Multidrug-Resistant and Extended-Spectrum ß-Lactamase-Producing Diarrheagenic *Escherichia coli* from Under-Five Children in Ethiopia

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Purpose: *Escherichia coli* strains that produce extended-spectrum ß-lactamase (ESBL) and carbapenemase are among the major threats to global health. The objective of the present study was to determine the distribution of ß-lactamase genes among multidrug-resistant (MDR) and ESBL-producing Diarrheagenic *E. coli* (DEC) pathotypes isolated from under-five children in Ethiopia.

Patients and Methods: A cross-sectional study was conducted in Addis Ababa and Debre Berhan, Ethiopia. It was a health-facility-based study and conducted between December 2020 and August 2021. A total of 476 under-five children participated in the study. DEC pathotypes were detected by conventional Polymerase Chain Reaction (PCR) assay. After evaluating the antimicrobial susceptibility profile of the DEC strains by disk diffusion method, confirmation test was done for ESBL and carbapenemase production. ß-lactamase encoding genes were identified from phenotypically ESBLs and carbapenemase positive DEC strains using PCR assay.

Results: In total, 183 DEC pathotypes were isolated from the 476 under-five children. Seventy-nine (43%, 79/183) MDR-DEC pathotypes were identified. MDR was common among enteroaggregative *E. coli* (EAEC) (58%, 44/76), followed by enterotoxigenic *E. coli* (ETEC) (44%, 17/39)) and enteroinvasive *E. coli* (EIEC) (30%, 7/23). Phenotypically, a total of 30 MDR-DEC pathotypes (16.4%, 30/183) were tested positive for ESBLs. Few ETEC (5.1%, 2/39) and EAEC (2.6%, 2/76) were carbapenemase producers. The predominant β-lactamase genes identified was bla_{TEM} (80%, 24/30) followed by $bla_{\text{CTX-}M}$ (73%, 22/30), bla_{SHV} (60%, 18/30), bla_{NDM} (13%, 4/30), and $bla_{\text{OXA-}48}$ (13%, 4/30). Majority of the ß-lactamase encoding genes were detected in EAEC (50%) and ETEC (20%). Co-existence of different β-lactamase genes was found in the present study.

Conclusion: The bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{NDM}, and bla_{OXA-48}, that are associated with serious and urgent threats globally, were detected in diarrheagenic *E. coli* isolates from under-five children in Ethiopia. This study also revealed the coexistence of the β-lactamase genes.

Keywords: Diarrheagenic *E. coil*, under-five children, ß-lactamase, carbapenemase, ESBL, multidrug resistance, Ethiopia

Introduction

Enterobacterales are Gram-negative bacteria that play a significant role in human disease.[1] Among Enterobacterales, diarrheagenic *Escherichia coli* (DEC) is one of the main etiological agents of diarrheal disease, mainly in children.[2] Six common DEC pathotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC), are associated with diarrhea.[2] According to a systematic review of the global causes of mortality due to diarrheal diseases,

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more than 50% of all diarrheal death in under-five children are caused by EPEC, ETEC, rotavirus and calicivirus.[3] The contribution of DEC to acute diarrhea incidence in children is greater than 30% in developing countries.[4]

The most commonly prescribed antimicrobials for infections caused by Enterobacterales include ß-lactam drugs, aminoglycosides, and fluoroquinolones.[5,6] The ß-lactam drugs include penicillin, cephalosporins, carbapenems, and monobactams. In recent years, antimicrobial resistance (AMR) has become one of the top ten threats to health globally.[7] AMR creates a huge clinical (high morbidity and mortality) and financial (increased healthcare costs) burden globally.[8] Inappropriate use of antimicrobials, poor hygiene and sanitation, and poor infection prevention and control have contributed to AMR occurrence.[9] Transmission of AMR traits between bacterial genera or species is mainly mediated by horizontal gene transfer through mobile genetic elements.[10,11]

Resistance to β-lactams among Enterobacterales occurs through different mechanisms.[6] One of the primary resistance mechanisms to ß-lactam drugs in Enterobacterales is through inactivation of the antimicrobials by ß-lactamase.[6] Currently, β-lactamase-producing Enterobacterales are the most serious and critical threats to the world.[6] Gene mobilization mechanisms mediate the transfer of β-lactamases in Enterobacterales.[12] β-lactamases (penicillinases, extended-spectrum cephalosporinase, carbapenemase, and oxacillinase) hydrolyze β-lactam antimicrobials.[12] Many ß-lactamases are categorized into different classes based on their amino acid sequence, substrate, inhibitor profile, and variation in their active sites (using serine or require divalent zinc ions) for hydrolysis.[12]

Extended-spectrum ß-lactamase (ESBL) and carbapenemase are among the β-lactamases associated with serious and urgent threats globally.[6,12,13] The most common ESBLs are categorized as class A of the Ambler classification and are capable of conferring resistance to penicillins, cephalosporins, extended-spectrum cephalosporins, and monobactams.[12] The common and medically important types of ESBLs include TEM, SHV (sulfhydryl variable), and CTX-M (hydrolytic activity against cefotaxime).[14,15] Currently, there are more than 183 TEM types, with the most common TEM-type found in *E. coli*[6] and more than 178 SHV varieties, mainly in Enterobacterales.[6,14] Carbapenems have been used to treat ESBL-producing bacteria and cephalosporin-resistant infections.[12] Currently, the emergence and spread of carbapenemase-producing *E. coli* strains is of great concern globally.[16] Carbapenemases are β-lactamases that hydrolyze penicillins, cephalosporins, monobactams, and carbapenems.[13] Most commonly carbapenemases are grouped as class A (IMI, SME, and KPC), class B (NDM, VIM, IMP, and SIM) and Class D (OXA ß-lactamase).[13] The KPC, NDM, and OXA-48 types are predominantly isolated from Enterobacterales, including *E. coli* in children.[17–19]

Multidrug-resistant (MDR) is defined as developing acquired resistance to at least one antimicrobial in three or more antimicrobial categories or classes.[20] Currently, there are reports of higher number of MDR Gram negative bacteria including members of Enterobacterales such as *E. coli* strains from clinical specimens.[21–23] MDR *E. coli* strains including DEC pathotypes that produce ESBL and carbapenemase are emerging.[24] High-risk groups are those contaminated with ESBL-producing *E. coli* strains and carbapenemase-producing *E. coli* strains.[12] Understanding the level of the problem in a particular area could help not only for better management of high-risk group patients but also for better infection prevention measures. ESBL-producing Enterobacterales and carbapenemase-producing Enterobacterales are well characterized in different populations, including food handlers,[25] from urine, blood, wounds, and sputum of infected individuals[26–28] in Ethiopia. However, few studies have characterized β-lactamase genes originating from gastrointestinal sites, regardless of their role in AMR occurrence and spread.[10] Moreover, horizontal gene transfer can be enhanced between members of Enterobacterales during inflammatory reactions, like in case of diarrhea.[29] Globally, there are limited data on the molecular epidemiology of ß-lactamase genes in DEC pathotypes of children. ß-lactamase genes are not well characterized in either community-acquired or hospital-acquired diarrhea in children in Ethiopia. Moreover, no study has characterized ß-lactamase genes in MDR and ESBL-producing DEC (ESBL-DEC) pathotypes from under-five children in Ethiopia. This study aims to determine the distribution of β-lactamase genes among MDR and ESBL-DEC pathotypes isolated from under-five children in Ethiopia.

Materials and Methods

Study Setting

A cross-sectional study was conducted in Addis Ababa and Debre Berhan, Ethiopia (Figure 1). It was a health facility-based study and conducted between December 2020 and August 2021. Debre Berhan is located 130 km from Addis

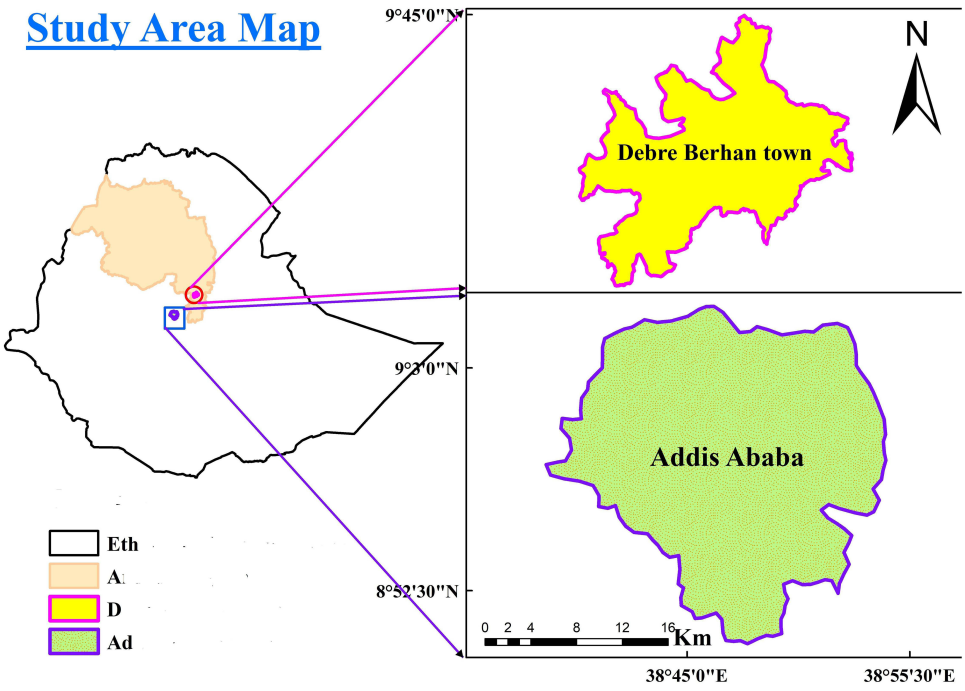

Figure 1 Map of the study area, Addis Ababa and Debre Berhan, Ethiopia.
Note: Eth, Ethiopian Administration boundary; A, Amhara Regional state; D, Debre Berhan Town; Ad, Addis Ababa city.

Ababa (capital city) and found in Amhara administrative region. Recent estimation reported 5.2 million and 95, 000 population for Addis Ababa and Debre Berhan, respectively.[30] A total of five health centers (3 from Addis Ababa and 2 from Debre Berhan) were randomly selected for the study. A total of 391 children with diarrhea and 85 children attending a health facility for causes other than diarrhea were enrolled in the study. All the study participants were under-five children and with no antimicrobial treatment history (for the last three weeks). Standard structured questionnaire (S1 data) was used to collect socio-demographic data, including age, sex, occupation, and family income from parents (or guardians) of the study participants.

Bacterial Isolation and Identification

As described in detail in our previous work,[30] standard bacteriological procedure was followed to isolate and identify the bacterial isolates. Briefly, stool samples collected from study participants in Cary-Blair transport media (Oxoid, UK) were transported (using cold chain) to Microbiology Laboratory. Fecal suspension was prepared and was inoculated onto MacConkey agar (Oxoid Ltd, UK) and incubated at 37 °C for 18–24 hours. A series of biochemical tests were used to identify the bacterial isolates.[30,31] Presumptive *E. coli* isolates were stored at –80 °C in brain heart infusion broth containing 16% (v/v) glycerol until use.

Detection of DEC Pathotypes

Deoxyribonucleic acid (DNA) extraction was done by boiling method, and the concentration and purity of the extracted DNA was assessed by Nanodrop (Thermo Scientific). Each DEC pathotypes were identified based on specific virulence genes using conventional Polymerase Chain Reaction (PCR) assay. The detail procedure for the DNA extraction and PCR-based identification of the DEC pathotypes is described in our previous work.[30]

Antimicrobial Susceptibility Testing

Disk diffusion method was used to assess the antimicrobial resistance profile of the DEC pathotypes.[32] A total of 12 different antimicrobials were evaluated against the DEC isolates.[30] The test procedures and the interpretations were based

on the Clinical and Laboratory Standards Institute (CLSI) guidelines.[32] DEC isolates that were non-susceptible to at least one antimicrobial in three or more class of antimicrobials were defined as MDR bacterial isolates.[20] This specific procedure was also described in our previous work.[30]

Phenotypic Detection of ESBLs and Carbapenemases Production

Isolates resistant to cefotaxime (30 µg) and ceftazidime (30 µg) were tested for ESBL production. Combination disk method and modified carbapenemase inactivation method were used for identifying ESBL and carbapenemase production, respectively.[30,32] Control strains used during the test procedure were *Klebsiella pneumoniae* ATCC 700603 (ESBLs positive), *E. coli* ATCC 25922 (ESBLs negative), *K. pneumoniae* ATCC BAA-1705 (carbapenemase-positive), and *K.* pneumoniae ATCC BAA-1706 (carbapenemase-negative). The detailed procedure is described in our previous work.[30]

PCR Detection of ß-Lactamase Genes

A conventional PCR assay was conducted to identify the ß-lactamase genes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{KPC}, bla_{NDM}, and $bla_{\text{OXA-48}}$) at the Armauer Hansen Research Institute, Addis Ababa, Ethiopia as described previously[33,34] with slight modification through optimization. The PCR assays were set in two separate PCR reactions, reaction 1 and reaction 2. Briefly, the first reaction targeted three ESBL genes ($bla_{CTX-\text{M}}$, bla_{TEM}, and bla_{SHV}), and the second reaction targeted three carbapenemase genes (bla_{KPC}, bla_{NDM}, and bla_{OXA-48}) in a separate tube. The sets of specific primers used to detect ESBL genes ($bla_{CTX-\text{M}}$, bla_{TEM}, and bla_{SHV})[35,36] and carbapenemase genes (bla_{KPC}, bla_{NDM}, and bla_{OXA-48})[17,37] are shown in Table 1. Platinum™ II Hot-Start PCR Master Mix (2X) (Thermo Fisher Scientific) was used as PCR master mix in all the PCR reactions. A final reaction volume of 20 µL, containing 10 µL of the PCR master mix, 1.2 µL of primer (each, 0.2 µM), 1.5 µL test DNA (template), 6.1 µL of nuclease-free water, was used in both PCR reactions. Initial denaturation at 94 °C for 15 min was set for the PCR thermal condition. It was followed by a total of 35 cycles of denaturation (92 °C), annealing (60 °C), and extension (72 °), each for 30s. And it had also a final extension of 5 min (at 72 °C). Agarose gel electrophoresis was used to separate the PCR products. UV transilluminator system (Gel Doc, Bio-Rad) was used to visualize the PCR products following the agarose gel electrophoresis procedure. In the agarose gel electrophoresis, 1.7% (w/v) agarose gel Tris Borate ethylene diamine tetra acetic acid (EDTA) buffer (pH 8.2), and ethidium bromide (10µg/mL) were used. Control strains including *K. pneumoniae* ATCC 700603 (ESBL-positive) and *K. pneumoniae* ATCC BAA-1705 (carbapenemase-positive) were used as quality control in the PCR assay.

Statistical Analysis

SPSS version 20 software program was used for the analysis of the data. Descriptive statistics (including frequency and proportion) were used. Chi-square test, bivariate and multivariate analyses were performed. For the multivariate logistic regression analysis (final model), independent variables were identified with $p < 0.25$. A $p < 0.05$ was considered as statistical significance. The results were interpreted using crude odds ratio (COR) and adjusted odds ratio (AOR).

Table 1 Target ß-Lactamase Encoding Genes for PCR Assay and Primer Profiles

Target genes	5' to 3' Primer (Forward)	5'-3' Primer (Reverse)	Size (bp)	Reference
$bla_{\text{CTX-M}}$	CGCTGTTGTTAGGAAGTGTG	GGCTGGGTGAAGTAAGTGAC	754	[37]
bla_{TEM}	TTTCGTGTCGCCCTTATTCC	ATCGTTGTCAGAAGTAAGTTGG	403	[38]
bla_{SHV}	CGCCTGTGTATTATCTCCCT	CGAGTAGTCCACCAGATCCT	293	[38]
bla_{NDM}	GGTTTGGCGATCTGGTTTTC	CGGAATGGCTCATCACGATC	621	[22]
bla_{KPC}	CGTCTAGTTCTGCTGTCTTG	CTTGTCATCCTTGTTAGGCG	798	[22]
bla_{OXA-48}	TGTTTTTGGTGGCATCGAT	GTAAMRATGCTTGGTTCGC	177	[39]

Abbreviation: bp, base pair.

Results

Characteristics of the Study Population and DEC Pathotypes

Of the total participants, 58% (274/476) were male, and 60% (288/476) of the study participants were in the age range of 25–59 months (Table 2). DEC was detected in 38% (183/476) of study participants. The pathotypes detected in the study were EPEC, ETEC, EIEC, EAEC, STEC, DAEC, and hybrid strains. From the total DEC, 104 (56.8%, 104/183) and 79 (43.2%, 79/183) were found in males and females, respectively. The majority (59.6%, 109/183) of the DEC were detected in children aged 25–59 months, followed by 13–24 months (23.5%, 43/183) and 0–12 months (16.9%, 31/183). Of the total DEC detected, 76 (41.5%), and 107 (58.5%, 107/183) were from Debre Berhan and Addis Ababa, respectively.

Antibiogram of DEC Pathotypes

Two to seven DEC pathotypes were resistant to various antimicrobials (Figure 2). As it can be seen in Figure 2 all antimicrobials (except carbapenems) tested in the present study were associated with four or more DEC pathotypes. DEC isolates were resistant to ampicillin (95%), tetracycline (91%), gentamicin (28%), trimethoprim-sulfamethoxazole (42%), and chloramphenicol (26%). Significant proportions of DEC isolates were resistant to ciprofloxacin (15%), ceftazidime (16%), cefotaxime (16%), cefepime (4%), meropenem, and ertapenem (2%).

Ampicillin and tetracycline resistance were high among DEC strains from children with- and without diarrhea from both study areas, Addis Ababa and Debre Berhan (Table 3). Seventy-nine (43%) of the DEC pathotypes were MDR (Table 4). MDR to three or more antimicrobials was commonly detected in EAEC (58%, 44/76), ETEC (44%, 17/39), and EIEC (30%, 7/23) pathotypes. Rate of MDR among isolates from Addis Ababa and Debre Berhan, and children with- and without diarrhea were not statistically different ($p > 0.05$).

Phenotypic ESBL and Carbapenemase Production

All the ESBL-DEC and carbapenemase-producing DEC strains were MDR. The proportion of ESBL-DEC was 38% (30/79) of total MDR-DEC strains. Carbapenemase-producing DEC pathotypes were 2.2% (4/183). All the three STEC isolates (100%), 19.7% (15/76) EAEC, 15.3% (6/39) ETEC, 10.7% (3/28) EPEC, 8.7% (2/23) EIEC, and 7.7% EPEC/EAEC (1/13, a hybrid strain) were ESBL producers. Except for two ETECs (5.1%) and two EAECs (2.6%), carbapenemase production was not observed in any of the other DEC pathotypes. Sample image of ESBL-producing strains is available in S1 Figure. Phenotypically, 67% (20/30) of the ESBL-DEC strains were from Addis Ababa and the remaining 33% (10/30) were from Debre Berhan. Carbapenemase-producing DEC strains were not detected in Debre Berhan. Aged 25–59 months, mother/guardian being self-employed, and others (farming, doing paid work, and non-regular businesses)

Table 2 Factors Associated with ESBLs Producing DEC Pathotype in Under-Five Children in Addis Ababa and Debre Berhan, Ethiopia

Characteristics		ESBLs		Univariate Analysis		Multivariate Analysis	
		Yes	No	COR (95% CI)	P-value	AOR (95% CI)	P-value
Sex	Male	60 (22%)	214 (78%)	1.00	0.821	1.00	0.852
	Female	46 (23%)	156 (77%)	1.052 (0.680, 1.627)		1.045 (0.657, 1.661)	
Age	0–12 months	27 (34%)	52 (66%)	1.00	0.001	1.00	0.000
	13–24 months	32 (29%)	77 (71%)	1.249 (0.671, 2.326)		1.197 (0.622, 2.302)	
	25–59 months	47 (16%)	241 (84%)	2.926 (1.521, 4.662)		2.827 (1.576, 5.071)	
Study area	Debre Berhan	37 (19%)	162 (81%)	1.00	0.103	1.00	0.209
	Addis Ababa	69 (25%)	208 (75%)	0.688 (0.439, 1.079)		0.726 (0.441, 1.196)	
Case	Diarrheic	88 (23%)	303 (77%)	1.00	0.789	1.00	0.613
	Non-diarrheic	18 (21%)	67 (79%)	0.925 (0.522, 1.639)		1.168 (0.640, 2.134)	
Mothers/guardian occupation	Employed	43 (30%)	101 (70%)	1.00	0.026	1,00	0.039
	Self employed	13 (16%)	68 (84%)	0.584 (0.364, 0.937)		2.159 (1.018, 4.579)	
	Other	50 (20%)	201 (80%)	1.301 (0.666, 2.541)		1.769 (1.07, 2.922)	

Notes: Other includes farming, doing paid work, and non-regular businesses.
Abbreviations: ESBL, Extended spectrum ß-lactamases; COR, Crude odd ratio; AOR, Adjusted odd ratio; CI, Confidence interval.

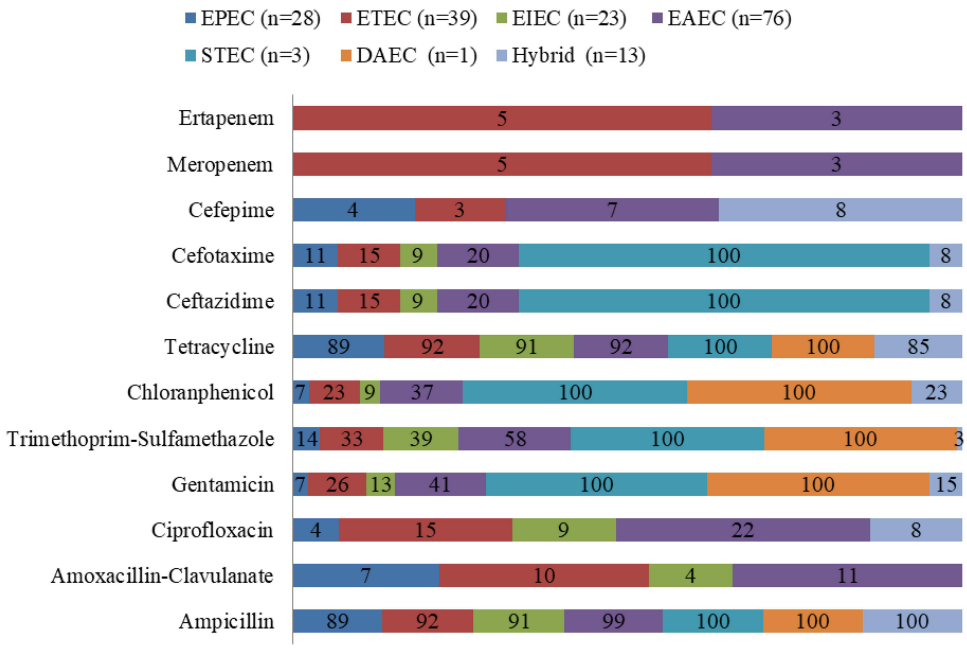

Figure 2 Different antimicrobials (the Y-axis) associated with different resistant DEC pathotypes (the X-axis in percent) isolated from under -five children, Addis Ababa and Debre Berhan, Ethiopia 2020/21.
Abbreviations: EPEC, Enteropathogenic *Escherichia coli*; ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli*; EAEC, Enteroaggregative *Escherichia coli*; STEC, Shiga-toxin producing *Escherichia coli*; DEAE, Diffusely adherent *Escherichia coli*; Hybrid, 5 ETEC/EAEC, 5 ETEC/EPEC, and 3 EPEC/EAEC.

were associated with the acquisition of ESBL-DEC pathotypes. DEC isolated from children aged 25–59 months were more likely (AOR = 2.827, CI = 1.576, 5.071) to be ESBL-producing than those isolated from children aged <12 months. Children whose mothers or guardians were self-employed (AOR = 2.159, CI = 1.018, 4.579) and others (farming, paid work, and small businesses) (AOR = 1.769, CI = 1.07, 2.922) were twice as likely to get ESBL-DEC pathotypes.

Distribution of ß-Lactamase Genes

ß-lactamase genes were detected in all phenotypically ESBL-DEC strains. The five common ß-lactamase genes tested in the present study, *blaCTX-M, blaTEM*, and *blaSHV, bla*$_{NDM}$ and *bla*$_{OXA-48}$, were detected among the ESBL-DEC strains.

Table 3 Antimicrobial Susceptibility Profile of Different Antimicrobials Tested Against DEC Pathotypes Isolated from Diarrheic and Non-Diarrheic Under-Five Children in Addis Ababa and Debre Berhan, Ethiopia

Antimicrobials	Addis Ababa (n=107)		Debre Berhan (n=76)		Diarrheic (n=159)		Non-Diarrheic (n=24)	
	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
Ampicillin	102 (95%)	5 (5%)	72 (95%)	4 (5%)	151 (95%)	8 (5%)	23 (96%)	1(4%)
Amoxicillin-Clavulanate	9 (8%)	98 (92%)	6 (8%)	70 (92%)	14 (9%)	145 (91%)	1(4%)	23 (96%)
Ciprofloxacin	18 (17%)	89 (83%)	9 (12%)	67 (88%)	24 (15%)	135 (85%)	3 (13%)	21 (87%)
Gentamicin	33 (31%)	74 (69%)	19 (25%)	57 (75%)	47 (30%)	112 (70%)	5 (21%)	19 (79%)
Trimethoprim-Sulfamethoxazole	48 (45%)	59 (55%)	30 (39%)	46 (61%)	68 (43%)	91 (57%)	10 (42%)	14 (58%)
Chloramphenicol	29 (27%)	78 (73%)	19 (25%)	57 (75%)	41 (26%)	118 (74%)	7 (29%)	17 (71%)
Tetracycline	94 (88%)	13 (12%)	73 (96%)	3 (4%)	144 (91%)	15 (9%)	23 (96%)	1(4%)
Ceftazidime	20 (19%)	87 (81%)	10 (13%)	66 (87%)	30 (19%)	129 (81%)	0 (0%)	24 (100%)
Cefotaxime	20 (19%)	87 (81%)	10 (13%)	66 (87%)	30 (19%)	129 (81%)	0 (0%)	24 (100%)
Cefepime	7 (7%)	100 (93%)	1 (1%)	75 (99%)	7 (4%)	152 (96%)	1(4%)	23 (96%)
Meropenem	4 (4%)	103 (96%)	0	76 (100%)	4 (3%)	155 (97%)	0 (0%)	24 (100%)
Ertapenem	4 (4%)	103 (96%)	0	76 (100%)	4 (3%)	155 (97%)	0 (0%)	24 (100%)

Abbreviations: R, Resistance; S, Susceptible.

Table 4 Resistance Antibiogram of DEC Pathotypes Isolated from Under-Five Children in Debre Berhan and Addis Ababa, Ethiopia

DEC Path Types	Level of Antimicrobial Resistance, n (%)								
	R0	R1	R2	R3	R4	R5	R6	R7	≥ R8
EPEC (n=28)	2 (7%)	4(15%)	18 (64%)	2 (7%)	2 (7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ETEC (n=39)	2 (5%)	3(8%)	17 (44%)	8 (20%)	3(8%)	2 (5%)	2 (5%)	2 (5%)	0 (0%)
EIEC (n=23)	1 (4%)	3 (13%)	12 (52%)	2 (9%)	2 (9%)	2 (9%)	1 (4%)	0 (0%)	0 (0%)
EAEC (n=76)	1 (1%)	6 (8%)	27 (34%)	18 (23%)	17 (22%)	3 (4%)	3 (4%)	0 (0%)	3 (4%)
Hybrid (n=13)	0 (0%)	3 (23%)	7 (54%)	2 (15%)	1 (8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Others* (n=4)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	1 (25%)	2 (50%)	0 (0%)	0 (0%)
Total (n=183)	6 (3.2%)	19 (25%)	81 (44.3%)	32 (17.5%)	26 (14.2%)	8 (4.4%)	8 (4.4%)	2 (1.1%)	3 (1.6%)

Note: *Others include STEC (n=3) and DAEC (n=1). R0, Susceptible to all antimicrobials tested (or stands for resistance for zero antimicrobial); R1, R2, R3, R4, R5, R6, R8: Resistant to one, two, three, four, five, six, eight and greater than eight antimicrobials, respectively.

Abbreviations: DEC, Diarrheagenic *Escherichia coli*; EPEC=Enteropathogenic *Escherichia coli*; ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli*; EAEC, Enteroaggregative *Escherichia coli*; STEC, Shiga-toxin producing *Escherichia coli*; DEAE, Diffusely adherent *Escherichia coli*; Hybrid strains, five ETEC/EAEC; five ETEC/EPEC; and three EPEC/EAEC.

Table 5 Distribution of ß-Lactamase Genes Among *DEC* Isolates from Under-Five Children in Addis Ababa and Debre Berhan, Ethiopia

DEC Pathotypes	ß-Lactamase Genes (n=30)	ß-Lactamase Gene Variants				
		bla_{CTX-M}	bla_{TEM}	bla_{SHV}	bla_{NDM}	bla_{OXA-48}
EPEC	3 (10%)	0	3	1	0	0
ETEC	6 (20%)	5	5	3	2	2
EIEC	2 (7%)	0	2	2	0	0
EAEC	15 (50%)	13	11	10	2	2
STEC	3 (10%)	3	3	1	0	0
EPEC/EAEC	1 (3%)	1	0	1	0	0
Total	30 (100)	22 (73%)	24 (80%)	18 (60%)	4 (13%)	4 (13%)

Abbreviations: DEC, Diarrheagenic *Escherichia coli*; EPEC, Enteropathogenic *Escherichia coli*; ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli*, EAEC, Enteroaggregative *Escherichia coli*; STEC, Shiga-toxin producing *Escherichia coli*; DEAE, Diffusely adherent *Escherichia coli*.

The predominant ß-lactamase gene was bla_{TEM} (80%, 24/30), followed by bla_{CTX-M} (73%, 22/30), and bla_{SHV} (60%, 18/30) of ESBL-DEC pathotypes (Table 5). All the three common ß-lactamase genes were detected in 33% (10/30) of ESBL-DEC strains in combination (S1 Table). The other ß-lactamase genes detected in the present study were bla_{NDM} (13%, 4/30) and bla_{OXA-48} (13%, 4/30). Fifty percent (15/30) of ß-lactamase genes were detected in EAEC, 20% (6/30) in ETEC, 10% (3/30) in EPEC, 10% (3/30) in STEC, 6.7% (2/30) in EIEC, and 3.3% (1/30) in EPEC/EAEC DEC strains (Table 5). Nearly 67% (20/30) of ß-lactamase genes were detected in DEC pathotypes isolated from Addis Ababa, whereas 33% (10/30) were from DEC pathotypes isolated from Debre Berhan. All the two carbapenemase encoding genes, bla_{NDM} and bla_{OXA-48}, co-existed among 13.3% of ESBL-DEC pathotypes (4/30). In the present study, bla_{KPC} was not detected in any of the tested DEC pathotypes. Sample image of gel electrophoresis for PCR β-lactamase gene products is presented in Figure 3 and is also available in S2 Figure.

Discussion

Diarrhea and pneumonia are among the common childhood diseases in Ethiopia.[38] The diarrheal burden is higher in under-five children[39] and with a significant mortality rate[40] in the country. There is 63.8% of an overall prevalence of antimicrobial use, and most treatments are empiric (96.7%) in Ethiopia.[41] Periodic antibiogram profiling of pathogenic bacteria in such areas could help patient management decisions and outcomes. In a country with high prevalence of diarrheal disease, characterizing the distribution of β-lactamase genes in DEC strains will contribute to inform policies and monitor impact of local and global control strategies.

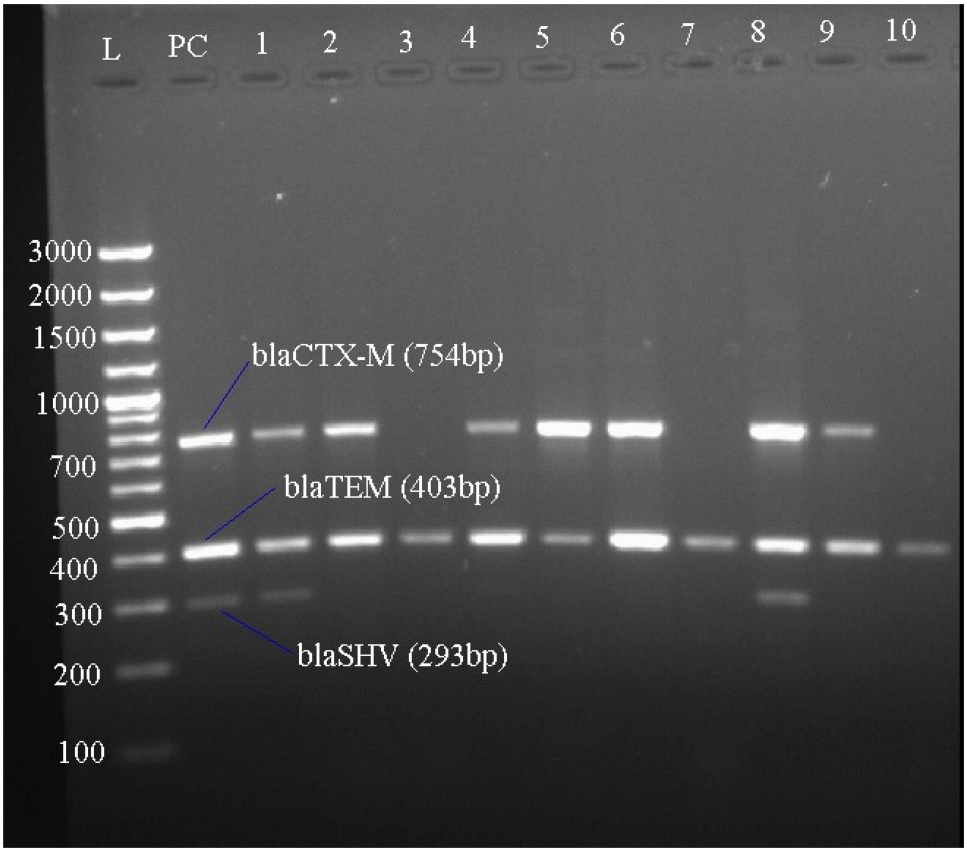

Figure 3 Gel image of the ß-lactamase genes of DEC pathotypes isolated from under -five children, Addis Ababa and Debre Berhan, Ethiopia 2020/21.
Notes: Lane L, 100 kb+ DNA ladder (in bp, base pair); Lane PC, positive control[bla_{CTX-M} (754bp), bla_{SHV}(293bp) and bla_{TEM} (403bp)]; Lanes 1and 8 are positive for bla_{CTX-M}, bla_{TEM} and bla_{SHV}; 2, 4, 5, 6, and 9 positive only for bla_{CTX-M} and bla_{TEM}; and 3, 7, and 10 positive only for bla_{TEM}.

A previous study reported a large proportion of ampicillin (85%) and tetracycline (67%)-resistant enteric pathogens in Ethiopia.[42] In line with this, large proportions of ampicillin-resistant (95%) and tetracycline-resistant (91%) DEC strains were found in the present study. Most antimicrobials (98%) prescribed in Ethiopia are from the national essential medicines list[43] and almost all (except tetracycline) the antimicrobials tested in the present study were among these lists. In the present study, a low resistance rate to Amoxicillin-Clavulanate, third- and fourth-generation cephalosporins, and carbapenems was observed. However, the presence of strains resistant to third- and fourth-generation cephalosporin and carbapenem in the present study could alert health personnel to timely measures. The Ethiopian essential medicine list grouped the 3rd-generation cephalosporin and carbapenems as the watch and reserve group antimicrobials, respectively.[44] Resistance to both groups of antimicrobials could lead to the death of the patient or no treatment options. Thus, protection and prioritization from the misuse of these drugs must be given attention at the right time. The antimicrobial resistance rate did not differ between Addis Ababa and Debre Berhan ($p > 0.05$) for all the antimicrobials tested in this study. This could be due to the inappropriate use of antimicrobials without significant differences between urban and rural communities in Ethiopia.[45]

The human gut serves as a major conduit for the development and environmental spread of MDR organisms.[46] A recent study conducted in Ethiopia[42] and other area[23] reported a higher rate of MDR (≥71%) among Gram-negative bacterial pathogens. The MDR rate in the present study was 43%, which was lower than report from Iran (78.1%)[47] and China (66.7%).[48] This inconsistency may be due to differences in contributing factors, variations in DEC pathotypes, and geographical variations. However, some studies have reported MDR-DEC strains,[49,50] which is in agreement with the present study. A higher rate of MDR was observed in EAEC (58%), ETEC (44%), and EIEC (30%) in this study. Abbasi et al[47] also reported high rates of MDR in EAEC (82%), ETEC (67%) and EIEC from central Iran

(100%). The leading cause of travellers' diarrhea is ETEC followed by EAEC; EAEC can also cause severe chronic diarrhea.[2] EIEC is also associated with shigellosis.[2] Antimicrobial treatment is required when infections associated with EIEC, ETEC, and EAEC are severe or prolonged.[2] However, the present findings showed that EIEC, ETEC, and EAEC developed resistance to the most commonly prescribed antimicrobials, which could be a challenge in treating resistant strains in the study area.

Globally, ESBL- and carbapenemase-producing Enterobacterales are considered as a serious threat to health.[46] Prevalence of ESBL (17.1–67.3%) - and Carbapenemase-producing Enterobacterales (2.4–7.7%) were reported in Ethiopia.[25,28,51] However, there have been no reports of ESBL-DEC and carbapenemase-producing DEC strains in Ethiopia. The present study identified 16.4% ESBL- and 2.2% carbapenemase-producing DEC strains. The presence of ESBL-DEC strains in the gut may result not only in high dissemination of the resistant trait to non-resistant bacterial strains[10] but will also be a problem in patient management. Bacterial infections with ESBL-producing Gram-negative bacteria are associated with high mortality rates in Ethiopia.[52] Intervention is needed for timely control of the spread of resistant strains.

ESBL-DEC strains differed between children with and without diarrhea in the present study. This difference could be due to the small sample size of non-diarrheic children participated in the study compared with diarrheic children, which is a limitation of the present study. Inflammatory reaction occurred in the gut (e.g. diarrhea) that could mediate gene transfer among bacterial strains could explain the higher ESBL-DEC strains in diarrheic children.[29] Mandal et al reported the prevalence of ESBLs in ETEC (18.32%), EPEC (10.9%), EAEC (6.8%), and EIEC (1.57%) among diarrheic children in India.[53] In the present study, the prevalence of ESBLs in the ETEC, EPEC, and EIEC groups was consistent with Mandal et al report.[53] Among the DEC pathotypes, EAEC (19.7%) and ETEC (15.3%) showed a high rate of ESBLs compared to other pathotypes ($p < 0.001$) in the present study. This high rate could be associated with their high prevalence in the study area compared with other pathotypes. Since EAEC is a commonly emerging pathogen, and ETEC is most prevalent in low-income countries,[2] the presence of resistant strains of both pathogens requires attention. Regular assessment of antimicrobial resistance profile may help to treat infections caused by EAEC and ETEC in a given area.

The association of socio-demographic factors with phenotypic ESBL-producing bacterial strains varies among different studies.[51,54,55] Lower educational level (mothers) and drinking tap water (children) were associated with ESBL-producing bacterial strains in previous study done in Ethiopia.[51] According to the present findings, children at the age range of 25 −59 months were less likely to develop ESBL-DEC than those aged <1 year, which is consistent with another report.[55] However, those with age greater than or equal to one year was found significantly associated with ESBL-producing Enterobacterales in other study.[54] This discrepancy may be due to other factors included in the analysis. Additionally, children whose mothers/guardians were self-employed or engaged in other occupations (farming, paid work, and non-regular businesses) were more likely to be positive for ESBL-DEC. Exposure of farmers to animals and workers in non-regular settings (high workload or low income) may contribute to the acquisition of ESBL-DEC compared to employed mothers or caretakers who have less exposure.

Currently, *E. coli* strains carrying ß-lactamase-encoding genes including $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM} are distributed in human, animal, food and the environment.[56] These common ß-lactamase-encoding genes have also been reported from *E. coli* isolates in sepsis patients in Ethiopia.[27,28] In agreement with these reports, the present study also found β-lactamase-encoding genes ($bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM}) in DEC pathotypes. The β-lactamase-encoding genes detected in the present study were bla_{TEM} (80%), $bla_{CTX\text{-}M}$ (73%), and bla_{SHV} (60%). In India, 86.1% $bla_{\text{CTX-M}}$, 68% bla_{SHV}, and 52% bla_{TEM} were detected in children with diarrhea[53] which is in-line with the present study. The findings from other studies conducted in Iran[47] and Ghana[57] are also in line with those of the present study. However, Monira et al[58] reported 39% $bla_{\text{CTX-M}}$, 26% bla_{TEM}, and 12% bla_{SHV} in children from Bangladesh. The higher prevalence of β-lactamase genes in the present study may be due to difference in risk factors contributing to emergence of such genes. This study revealed the presence of DEC strains carrying ß-lactamase-encoding genes that circulate in the community and could be a threat to effective patient management.

In the present study, the predominant β-lactamase was bla_{TEM} which is contrary to other reports in Ethiopia from Enterobacterales in patients with sepsis[27,28] where $bla_{\text{CTX-M}}$ is more prevalent. $bla_{\text{CTX-M}}$ has also been reported as the predominant ß-lactamase in extraintestinal invasive *E. coli* in Ethiopia.[59] This discrepancy may be due to differences in

the types of bacterial strains and cases involved in the analysis. In a study conducted in Iran among under-five children[60] and in Ghana on diarrheic patients,[47] bla_{TEM} was found to be the predominant β-lactamase gene. EAEC was the predominant MDR strain with a higher prevalence of bla_{TEM} in the present study. The DEC pathotypes may have contributed to the epidemiology of common β-lactamase genes. Except TEM-1 and TEM-2 variants, all bla_{TEM} are ESBLs and show activity against cefotaxime.[14] It could contribute not only to the patient management problem but also to the spread of resistant traits to other strains that could result in no drugs for use.[44]

In the present study, bla_{TEM} was detected in 83% (5/6) ETEC, 73% (13/15) EAEC, 67% (2/3) STEC, in all EPEC (3/3), EIEC (2/2), and EPEC/EAECs (1/1). This finding is in agreement with a study conducted in Iran[47] in which bla_{TEM} was detected in all EAEC (9/9), EPEC (5/5), ETEC (2/2), and EIEC (1/1) isolates. bla_{CTX-M} from EPEC (0/3) and EIEC (0/2), and bla_{TEM} from EPEC/EAEC (0/1) were not detected in the present study. bla_{SHV} was detected from ETEC (2/6) and EIEC (2/2) in the present study, in contrast to the report by Abbasi et al,[47] whereas bla_{SHV} was not detected from EIEC and ETEC. This discrepancy may be due to variations in the acquisition of antimicrobial resistance determinants at different selection pressures.[61]

In Norway, data from 2007 to 2014 show an increasing number of carbapenemase-producing Enterobacterales cases yearly.[62] Recent data showed an increased detection of carbapenemase-producing Gram negative bacteria from clinical isolates in Ethiopia.[63,64] A systematic review and meta-analysis showed 5.4% pooled prevalence of carbapenemase-producing Enterobacterales, with high prevalence in Central Ethiopia.[65] Recent data reports bla_{KPC}, bla_{NDM}, and bla_{OXA-48} from clinical isolates of Enterobacterales in Ethiopia.[66,67] Carbapenemase encoding genes, bla_{NDM}, and bla_{OXA-48} are becoming the dominant carbapenemase variants in *E. coli* strains.[62] In another study, carbapenemase genes, including bla_{NDM-1}, were identified in the DEC strains.[68] Carbapenemase-producing DEC, bla_{OXA-48} (13%), and bla_{NDM} (13%) were found in the present study. Carbapenemase bla_{OXA-48} reported in different studies included 31% in Egypt,[69] 57% in Burkina Faso,[70] 29% in Kenya,[71] and 33% in Uganda.[18] The higher prevalence in these studies compared to the present study may be due to the source of the samples and geographical differences. In studies conducted in China[48] and Egypt,[69] bla_{NDM} has been detected in DEC isolated from children. Both bla_{OXA-48} and bla_{NDM} hydrolyze penicillin and carbapenem, while bla_{NDM} hydrolyzes cephalosporins and extended-spectrum cephalosporins.[12] The presence of carbapenemase-producing strains in the present study could indicate problems that lead to a lack of drugs in the reserve antimicrobial group for use.[44]

In the present study, 33% of the DEC pathotypes contained all three common ß-lactamase encoding genes (bla_{CTX-M}, bla_{SHV}, and bla_{TEM}) in combination (co-occurrence), which as reported in Nigeria.[72] Co-occurrence of carbapenemase genes was also identified in this study. The carbapenemase encoding genes, bla_{NDM-1} and bla_{OXA-48} were isolated from clinical isolates of *Klebsiella pneumonia* (48%) in Egypt.[17] The co-occurrence of β-lactamase genes in a single strain showed the incidence of MDR-DEC strains, rate of dissemination of resistant determinants, and emergence of resistant strains in the study area. Mobile genetic elements such as plasmid and integrons[11,73] could play vital roles in the spread of the resistance traits and the occurrence of MDR. Ssekatawa et al[18] reported 14.8% bla_{KPC} in DEC and extra-intestinal pathogenic *E. coli* in Uganda. Dembele et al also reported[70] low prevalence of bla_{KPC}[64] in EAEC and aEPEC. In the present study, bla_{KPC} was not found in DEC strains isolated from under-five children. Prolonged hospital stay, invasive devices, lack of immune-competency, history of antimicrobial therapy could contribute to the acquisition of KPC-producing bacteria.[74] The presence of these risk factors or exposure to them will determine the epidemiology of KPC-related infections,[74] and thereby dissemination or spread of bla_{KPC} among bacterial strains. In a study done by Han et al, the prevalence of bla_{KPC} among Enterobacterales varied among strains and it was higher in *K. pneumoniae* (64.5%) but lower in *E. coli* (2.7%).[19] Thus, the lack of bla_{KPC} in the present study may be due to differences in bacterial strains, types of cases, and risk factor exposures that contribute to selection pressure.

Conclusions

DEC with a multidrug resistance profile and ß-lactamase-encoding genes (bla_{CTX-M}, bla_{SHV}, and bla_{TEM}, bla_{NDM-1} and bla_{OXA-48}) that are associated with serious and urgent global threats were found in under-five children in Ethiopia. The coexistence of β-lactamase genes suggests the severity of this problem. The presence of MDR ß-lactamase, including carbapenemase-producing DEC, in the gut of under-five children could reveal the presence of risk for emergence of MDR bacterial strains in the area. Therefore, there is a need for the timely control of the dissemination of such resistant strains. Antibiogram profile-based drug prescriptions and health facility-based surveillance could help manage and control ESBL- and carbapenemase-producing DEC-related infections.

Abbreviations

AMR, Antimicrobial resistance; ATCC, American Type Culture Collection; CLSI, Clinical and Laboratory Standards Institute guidelines; DAEC, diffusely adherent *E. coli*; DEC, Diarrheagenic *E. coli*; DNA, Deoxyribonucleic acid; EAEC, Enteroaggregative *E. coli*; EIEC, Enteroinvasive *E. coli*; EPEC, Enteropathogenic *E. coli*; ESBL-DEC, ESBL-producing DEC; ESBLs, extended-spectrum ß-lactamase; MDR, Multidrug-resistant; PCR, polymerase-chain reaction; STEC, Shiga toxin-producing *E. coli*.

Data Sharing Statement

All the information is presented in the main manuscript, and there are no remaining data or materials.

Ethical Approval and Informed Consent

The study was approved at institutional and national level, by Institutional Review Board of College of Health Science, Addis Ababa University (PN_025/20/DMIP), and Ethiopian National Research Review Committee (Ref.No_RED/1.14/9428/21), respectively. Informed consent (verbal and written) was obtained from parents (or guardians). The study was conducted in accordance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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