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Sickle cell disease children's gut colonization by extended-spectrum β-lactamase (ESBL)-producing *Enterobacterales*: an antibiotic prophylaxis effect?

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1.4 Keyword

Sickle cell disease; antibiotic prophylaxis; gut microbiota; *Enterobacterales*; multidrug resistance and extended-spectrum β-lactamase (ESBL)-producing *Enterobacterales*.

2. Abstract

Introduction: Sickle cell disease (SCD) children have a high susceptibility to pneumococcal infection. For this reason, they are routinely immunized with pneumococcal vaccines and use antibiotic prophylaxis (AP).

Hypothesis/Gap Statement: Yet, little is known about SCD children's gut microbiota. If antibiotic-resistant *Enterobacterales* may colonize people on AP, we hypothesized that SCD children on AP are colonized by resistant enterobacteria species.

Objective: To evaluate the effect of continuous AP on *Enterobacterales* gut colonization from children with SCD.

Methodology: We analysed 30 faecal swabs from SCD children on AP and 21 swabs from children without the same condition. *Enterobacterales* was isolated on MacConkey agar plates and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (bioMérieux, Marcy l'Etoile, France). We performed the antibiogram by Vitek 2 system (bioMérieux, Marcy l'Etoile, France), and the resistance genes were identified by multiplex PCR.

Results: We found four different species with resistance to one or more different antibiotic types in the AP-SCD children's group: *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Citrobacter farmeri*. Colonization by resistant *E. coli* was associated with AP (prevalence ratio 2.69, 95% confidence interval [CI], 1.98–3.67, $P<0.001$). Strains producing extended-spectrum β-lactamases (ESBL) were identified only in SCD children, *E. coli*, 4/30 (13%), and *K. pneumoniae*, 2/30 (7%). The ESBL-producing *Enterobacterales* were associated with penicillin G benzathine use (95 % CI, 22.91–86.71, $P<0.001$). CTX-M-1 was the most prevalent among ESBL-producers (3/6, 50%), followed by CTX-M-9 (2/6, 33%), and CTX-M-2 (1/6, 17%).

Conclusion: Resistant enterobacteria colonize SCD children on AP, and this therapy raises the chance of ESBL-producing *Enterobacterales* colonization. Future studies should focus on prophylactic vaccines as exclusive therapy against pneumococcal infections.

3. Introduction

Children with sickle cell disease (SCD) are at high risk of *Streptococcus pneumoniae* infection [1], but death by invasive pneumococcal is now rare [2, 3] due to prophylactic measures by vaccination and penicillin use [4–6]. However, *Acinetobacter* spp. and *Enterobacterales* such as *Salmonella* spp., *Escherichia coli*, and *Klebsiella pneumoniae* have already been identified as important bacterial agents associated with SCD children invasive infections [2, 7, 8].

In the last decade, researchers have focused attention on the human gut microbiota. However, in SCD children, the microbiota profile has not been yet elucidated, especially related to enterobacteria. *Enterobacterales* species, bacteria of the intestinal microbiota, are often

associated with extraintestinal infections, especially in immunosuppressed individuals. The most common observed infections are abscesses, pneumonia, meningitis, bloodstream (sepsis), surgical site, and urinary tract infections (UTI) [9]. Antibiotic resistance has become a global threat to public health due to its overuse or inappropriate prescription [10, 11]. The emergence and dissemination of multidrug-resistance *Enterobacterales* are one of the causes of this threat [11]. At present, β-lactam resistance is a major concern worldwide [12].

Antibiotic prophylaxis (AP) may lead to gut colonization by antibiotic-resistant bacteria [13, 14], and for this reason, we hypothesize that SCD children on AP are colonized by resistant enterobacteria. In this study, we evaluate the effect of continuous AP on *Enterobacterales* species gut microbiota colonization from SCD children. Also, we investigate the antimicrobial susceptibility profile and the resistance genes associated with β-lactamase production.

4. Methods

Study design

We conducted a sample convenience cross-sectional observational study. Fifty-one children participated in the study. In the SCD group, 30 participants (ages 2–12 years) had the followed hemoglobin (Hb) genotypes: HbSS (sickle cell anemia; 16/30), HbSC (hemoglobin SC disease; 13/30), and HbSβ-thalassemia (sickle/β-thalassemia; 1/30), all on AP. The next group was composed of 21 children (ages 2–17 years) who were SCD group relatives, genotyped as HbAA (normal hemoglobin; 8/21), HbAS (sickle cell trait; 8/21), and, unfortunately, five children (5/21) did not have their genotype information on their medical records. This group lives in the same SCD children's group household in close contact, all

without AP. The calculated sample size corresponded to approximately 10% of the annual incidence of SCD in Bahia, Brazil.

Inclusion and non-inclusion criteria

In the AP-SCD children's group, children under 2 years of age who discontinued AP in the last year, showing risk factors associated with gut microbiota alteration, such as inflammatory bowel disease, colon cancer, irritable bowel syndrome, gastric ulcers, non-alcoholic fatty liver disease, obesity, metabolic syndromes, asthma, atopy, and hypertension; and who did not sign the adult consent, or a child's assent form to participate in the research, were not included.

Similarly, in the group without AP use, children under 2 years of age, who used antibiotics in the last 3 months, and do not reside in the same home as the AP-SCD children, with the presence of the above-mentioned risk factors associated with gut microbiota alteration, and who did not agree with the research, were not included.

Sample collection

Stool samples were obtained at home by participants in a sterile stool sample container that we previously provided. Swabs were directly immersed in the fresh faecal samples and were transported in Stuart medium, at room temperature, to the Clinical and Research Microbiology Laboratory at the Federal University of Bahia's Faculty of Pharmacy. The average time between faecal swab collection and processing was around 24 h.

***Enterobacterales* isolation, identification, and antimicrobial susceptibility determination**

All swabs were streaked onto MacConkey agar plates, which were incubated for 24 h at 36±1 °C. For each plate, two to five different colony morphologies were transferred to a Triple Sugar Iron agar slant to screen *Enterobacterales*. The isolates were kept at −80 °C in a Brain Heart Infusion broth medium supplemented with 10% (v/v) glycerol for further analysis.

The isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (bioMérieux, Marcy l'Etoile, France). The antibiotic susceptibility profile was determined by Vitek 2 system (bioMérieux, Marcy l'Etoile, France), testing the following antibiotics: amikacin, ampicillin/sulbactam, cefepime, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, ertapenem, gentamicin, imipenem, meropenem, and piperacillin/tazobactam. Antibiotic susceptibility was interpreted using the Clinical and Laboratory Standards Institute (document M100, 2019) [15] and the Brazilian Health Regulatory Agency (ANVISA) (technical note 01/2013) [16] guidelines. Multidrug resistance was defined as resistance to three or more antimicrobial categories [17]. When the participants were colonized with more than one bacteria of the same species, we distinguished the different strains by evaluating the antibiotic susceptibility profile.

DNA extraction and antibiotic resistance gene identification

The DNA extraction and multiplex PCR were performed according to the protocol developed by Dallenne et al. [18], using primer sequences for the bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1-like}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-9}}$ genes.

Statistical analysis

We performed descriptive data analysis, by using medians and proportions (i.e. frequencies). We did Pearson's chi-square test association analysis by inserting data in the contingency tables to obtain prevalence ratios. Also, we used a chi-squared proportions test comparison. The significant association was reached when $P<0.05$ to a confidence interval (CI) of 95%. The study data was analysed on GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA) and at the online tool medcalc.net. Graphical representations were also performed on GraphPad Prism v. 6.01 software.

5. Results

Characteristics of the study population

From the 51 participants investigated in this study, over half of SCD children (16/30, 53%) had HbSS, 13/30 (43%) had HbSC, and 1/30 (3%) HbSβ-thalassemia. The genotypes of the group without AP were 8/21 (38%) HbAA, 8/21 (38%) HbAS, and a minority of 5/21 (24%) was not determined. Eighty percent (24/30) of SCD children were using oral penicillin V, and a minority (6/30, 20%), intramuscular penicillin G benzathine (Table 1).

Table 1. Distribution and characteristics of children with sickle cell disease on antibiotic prophylaxis, and children without antibiotic prophylaxis according to age, sex, genotype, and antibiotic used

Characteristics	On AP (n=30)	Without AP (n=21)
Age*		
	4 (2-12)	8 (2-17)
Sex		
Female	17 (57%)	12 (57%)
Male	13 (43%)	9 (43%)
Genotype		
HbSS	16 (53%)	-
HbSC	13 (43%)	-
HbSβ-thalassemia	1 (3%)	-
HbAA	-	8 (38%)
HbAS	-	8 (38%)
Unknown	-	5 (24%)
Antibiotic prophylaxis		
Oral penicillin V†	24 (80%)	-
Intramuscular penicillin G benzathine‡	6 (20%)	-

*Age expressed in years and median (minimum to maximum).
†<3 years, 125 mg twice daily; >3 years, 250 mg twice daily.
‡600,000 Units once montly.
AP, Antibiotic prophylaxis; HbAA, normal hemoglobin; HbAS, sickle cell trait; HbSC, hemoglobin SC disease; HbSS, sickle cell anemia; HbSβ-thalassemia, sickle/β-thalassemia.

Enterobacterales species diversity

We found seven *Enterobacterales* species in the AP-SCD children's group and nine in the group without the evaluated condition. Five common species were found in both groups. The *E. coli* and *K. pneumoniae* species were the most frequent in the groups (51/86 [59%] and 25/51 [49%] for *E. coli*, and 22/86 [26%] and 12/51 [24%] for *K. pneumoniae*, on AP-SCD and non-AP groups, respectively), followed by *Enterobacter cloacae* (7/86 [8%] and 6/51 [12%], on AP-SCD and non-AP groups, respectively) (Table 2). It is worth mentioning that some participants were colonized with more than one *E. coli, K. pneumoniae, E. cloacae*, and *Klebsiella aerogenes* strains. There were no significant differences between bacterial isolates and bacterial diversity when evaluating the AP-SCD and non-AP children's groups (95% CI,

−1.22–23.30, *P*=0.08), although the group without AP showed higher *Enterobacterales* diversity in a smaller number of isolates.

Table 2. Frequencies and diversity of *Enterobacterales* isolated from the gut microbiota of children with sickle cell disease on antibiotic prophylaxis, and the group without antibiotic prophylaxis

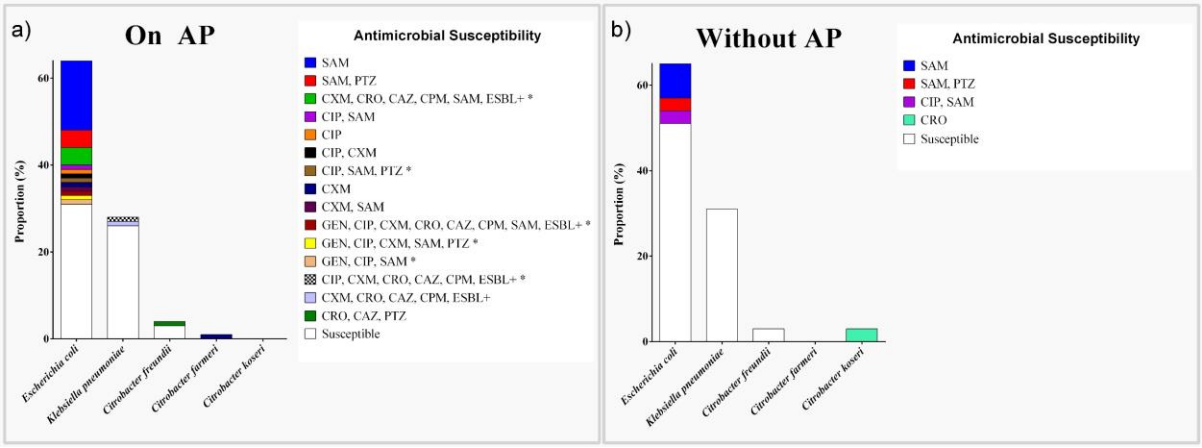
Isolated *Enterobacterales*	**On AP (n=30)**	**Without AP (n=21)**	***P***
	Frequency (%)	**Frequency (%)**	
Escherichia coli	51 (59%)	25 (49%)	
Klebsiella pneumoniae	22 (26%)	12 (24%)	
Enterobacter cloacae	7 (8%)	6 (12%)	
Citrobacter freundii	3 (3%)	1 (2%)	
Klebsiella aerogenes	1 (1%)	3 (6%)	
Citrobacter farmeri	1 (1%)	-	
Citrobacter werkmanii	1 (1%)	-	
Citrobacter koseri	-	1 (2%)	
Escherichia fergusonii	-	1 (2%)	
Klebsiella oxytoca	-	1 (2%)	
Kluyvera ascorbata	-	1 (2%)	
Total	**86 (100%)**	**51 (100%)**	**0.08***
Different species	**7 (8%)**	**9 (18%)**	

*No statistical significance at *P*<0.05.
AP, Antibiotic prophylaxis.

Antimicrobial susceptibility profiles of *Enterobacterales* isolates

Five species of resistant *Enterobacterales* were identified: *E. coli, K. pneumoniae, Citrobacter freundii, Citrobacter farmeri,* and *Citrobacter koseri*. Among them were observed a high rate of antibiotic resistance in *E. coli* isolates (27/51, 53%) in the AP-SCD children's group (Fig. 1). All bacterial isolates were susceptible to amikacin and carbapenems.

Fig. 1. Antimicrobial susceptibility profile. *E. coli, K. pneumoniae, C. freundii, C. farmeri,* and *C. koseri* isolates on (a) sickle cell disease children on antibiotic prophylaxis and (b) children without antibiotic prophylaxis. * Indicates a multidrug-resistant profile. ESBL+ denotes ESBL-producing isolates. AP, antibiotic prophylaxis; CIP, ciprofloxacin; CXM, cefuroxime; CRO, ceftriaxone; CAZ, ceftazidime; CPM, cefepime; ESBL, extended-spectrum β-lactamase; GEN, gentamicin; PTZ, piperacillin/tazobactam; SAM, ampicillin/sulbactam.



Carriage prevalence of antibiotic-resistant *Enterobacterales*

Sixty-seven percent of SCD children on AP (20/30) were colonized by resistant *E. coli*, 7/30 (23%) by *E. coli* multidrug-resistant, and 4/30 (13%) by ESBL-producing *E. coli*. On the other hand, 4/21 (19%) of children without AP were colonized by resistant *E. coli*. By the way, two of these children were living in close contact with the AP-SCD patients colonized by *E. coli* with the same resistant pattern. No multidrug-resistant or ESBL-producing *Enterobacterales* were found in the children without AP (Table 3).

Table 3. Frequency of children with or without isolates of antibiotic-resistant *Enterobacterales* and extended-spectrum β-lactamase producers

Presence of antibiotic resistance	On AP (n=30)	Without AP (n=21)
Escherichia coli		
Presence	20 (67%)	4 (19%)
Multidrug-resistant	7 (23%)	-
ESBL +	4 (13%)	-
Klebsiella pneumoniae		
Presence	2 (7%)	-
Multidrug-resistant	1 (3%)	-
ESBL +	2 (7%)	-
Citrobacter freundii		
Presence	1 (3%)	-
Multidrug-resistant	-	-
ESBL +	-	-
Citrobacter farmeri		-
Presence	1 (3%)	-
Multidrug-resistant	-	-
ESBL +	-	-
Citrobacter koseri	-	
Presence	-	1 (5%)
Multidrug-resistant	-	-
ESBL +	-	-

+ Positive.
AP, Antibiotic prophylaxis; ESBL, extended-spectrum β-lactamase.

When antibiotic-resistant and non-resistant *E. coli* were compared, concerning antibiotic use or not in both groups, we found a significant association between AP and *E. coli* resistance (95% CI, 1.98–3.67, $P<0.001$). The prevalence ratio of resistant *E. coli* was 2.69 times higher in the AP-SCD children's group compared to the group without the evaluated condition.

Two SCD children (2/30, 7%) on AP were colonized by resistant *K. pneumoniae*. Although this colonization was by ESBL-producing strains, only one *K. pneumoniae* showed multidrug resistance. On the other hand, the children's group without AP was not colonized by resistant

K. pneumoniae. Two SCD children on AP were colonized by resistant *Citrobacter* (1/30 *C. freundii* and 1/30 *C. farmeri*). One child (1/21) without AP was colonized by resistant *C. koseri* (Table 3); however, we do not find resistant enterobacteria in the AP-SCD child who was living in close contact with this non-AP child.

The prevalence of ESBL-producing *Enterobacterales* was higher among SCD children who used penicillin G benzathine, 4/6 (67%) children were colonized. On the other hand, the prevalence of ESBL-producing *Enterobacterales* among children who used penicillin V was 1/24 (4%). To evaluate the association between penicillin G benzathine use and ESBL-producing *Enterobacterales* colonization, a proportion's comparison test was used. The evaluated association was significant (95% CI, 22.91-86.71, $P<0.001$).

Molecular profiles of β-lactamases

The molecular analysis of β-lactamase producing isolates demonstrated that AP-SCD children were colonized by enterobacteria with the bla_{TEM} (13/30, 43%), $bla_{\text{CTX-M-9}}$ (2/30, 7%), $bla_{\text{CTX-M-1}}$ (1/30, 3%), $bla_{\text{CTX-M-1}}$ and bla_{TEM} (1/30, 3%), $bla_{\text{CTX-M-2}}$ and bla_{TEM} (1/30, 3%), and $bla_{\text{CTX-M-1}}$, $bla_{\text{SHV,}}$ and bla_{TEM} (1/30, 3%) genes. Five SCD children (5/30, 17%) were colonized by enterobacteria resistant to β-lactam; however, genes related to this resistance were not between the investigated genes. On the other hand, just the bla_{TEM} gene (4/21, 19%) was found in the children's group without AP, and one child (1/21, 5%) was colonized by β-lactamase-producing *Enterobacterales* whose gene was not identified. No resistant bacteria showed the $bla_{\text{OXA-1-like}}$ resistance gene in the investigated groups (Table 4).

Table 4. β-lactamases associated with resistance in *E. coli*, *K. pneumoniae*, *C. freundii*, *C. farmeri*, and *C. koseri*

Microorganism/β-Lactamases found	On AP (n=30)	Without AP (n=21)
Escherichia coli		
TEM	13 (43%)	4 (19%)
CTX-M-9	2 (7%)	-
CTX-M-1, TEM	1 (3%)	-
CTX-M-2, TEM	1 (3%)	-
Other unidentified	3 (10%)	-
Klebsiella pneumoniae		-
CTX-M-1	1 (3%)	-
CTX-M-1, SHV, TEM	1 (3%)	-
Citrobacter freundii		-
Other unidentified	1 (3%)	-
Citrobacter farmeri		-
Other unidentified	1 (3%)	-
Citrobacter koseri	-	
Other unidentified	-	1 (5%)

SHV, SHV variants including SHV-1; TEM, TEM variants including TEM-1 and TEM-2; CTX-M-1, Variants of CTX-M group 1 including CTX-M-1, CTX-M-3, and CTX-M-15; CTX-M-2, Variants of CTX-M group 2 including CTX-M-2; CTX-M-9, Variants of CTX-M group 9 including CTX-M-9 and CTX-M-14.
AP, Antibiotic prophylaxis.

6. Discussion

Children with SCD have a high susceptibility to pneumococcal infection, especially in those aged <5 years [1, 19]. For this reason, adherence to penicillin AP and vaccine regimens is recommended [4–6], which is a safe treatment against *S. pneumoniae*, especially since penicillin-resistant *S. pneumoniae* serotypes are not selected [20].

Despite the discrepancy in children's ages between the groups in this study, the gut microbiota stabilizes and resembles the adult gut microbiota around the age of 2 years old

[21], which justified the comparative study between the groups. Also, the elevated prevalence of oral penicillin V use between AP-SCD children (80%) is in line with the literature [22].

In contradiction with the literature, we found no significant difference between species diversity. Several studies demonstrated the association of antibiotic use with reduced gut microbiota diversity [23, 24]. We believe that the sample size could be the reason for non-significance, given that our findings demonstrate a tendency.

In the current study, 67% (20/30) of SCD children on AP were colonized by antibiotic-resistant *E. coli*, and 13% (4/30) of those children were colonized by this ESBL-producing bacteria. Therefore, these children are at high risk of severe infection by resistant *E. coli*, especially UTI. *E. coli* is the pathogen most associated with this infection, including in children [25]. Besides, it is believed that infective strains of *E. coli* from the gut microbiota contaminate and cause UTI [26].

The prevalence of resistant *E. coli* was 2.69 times higher among SCD children on AP compared to the group without the evaluated condition. Some studies have already shown that antibiotic use may lead to the emergence of new mutants or growth of existing antimicrobial-resistant gut microbiota populations, promoting the proliferation of Gram-negatives with these characteristics due to selective pressure [13, 14, 27, 28].

It was found that two SCD children on AP (2/30, 7%) were colonized by ESBL-producing *K. pneumoniae*. Although *K. pneumoniae* presents a high resistance rate, even higher than *E. coli* [29], this pattern is observed in hospitals, but *K. pneumoniae* is also a pathogen causing

community-acquired infections [30, 31]. However, based on this prevalence, this is a target for surveillance. The association between gastrointestinal colonizing *K. pneumoniae* with subsequent infections, particularly for pneumonia and UTI, has been demonstrated [32]. Besides, this bacteria is the second most common *Enterobacterales* in UTI in children [25, 33].

According to the multidrug-resistant classification criteria described by Magiorakos et al. [17], microorganisms resistant to at least three antibiotic categories, 8/30 SCD children on AP were colonized by multidrug-resistant *Enterobacterales*. This finding is worrying due to the relationship of these microorganisms with community-acquired infections [34–36], which can cause severe infections in these children due to difficult-to-treat resistances.

We found an association between ESBL-producing *Enterobacterales* and penicillin G benzathine use. We considered ESBL producer's prevalence high, 4/6 (67%) cases in the penicillin G group. Given that this finding is based on a limited number of children that used this antibiotic (n=6), this result should be treated with caution. However, we suggest the attention on AP with this antibiotic in SCD children, and further research is warranted to confirm this association.

In the molecular analysis of β-lactam resistant isolates, we found a high prevalence of children colonized by TEM-enzyme producers *Enterobacterales*, especially *E. coli*. This is consistent with the literature since this enzyme is the most commonly encountered β-lactamase in Gram-negative bacteria. Besides, TEM-1 is responsible for approximately 90% of ampicillin resistance in *E. coli* [37]. Although β-lactamase inhibitors typically inhibit the

TEM-enzyme, several studies demonstrated variants of this enzyme as responsible for resistance to β-lactam/β-lactamase inhibitor combinations, including ampicillin/sulbactam and piperacillin/tazobactam, which justifies our results [38, 39].

Regarding the CTX-M-group enzymes on ESBL-producers, CTX-M-1 was the most prevalent in isolates and found in three SCD children (3/30, 10%), followed by CTX-M-9 (2/30, 7%) and CTX-M-2 (1/30, 3%). In a study that determined CTX-M-producer's prevalence of faecal isolates in asymptomatic people in Thailand, the CTX-M-9 group (61%) was the most prevalent, followed by CTX-M-1 (39%) [40]. In another study in Brazil with bloodstream infection isolates, CTX-M-1 was the most predominant [41]. In South American countries, CTX-M-2 is the most detected [42], with CTX-M-2 most prevalent in Brazil followed by CTX-M-1, particularly in *K. pneumoniae* and *E. coli* [43]. SCD children colonized by CTX-M-type ESBL-producing *Enterobacterales* raise the chance of severe infection by these bacteria, given the ability of this enzyme to hydrolyse broad-spectrum cephalosporins [44].

Although SCD children in the present study colonized by resistant *Enterobacterales* were in a stable state, the chance of translocating resistant *E. coli* strains to different extraintestinal sites is raised, mainly due to it being the most frequent microorganism in translocations, especially in postoperative processes. This event also occurs in spontaneous conditions [45, 46]. Also, faecal contamination in wounds, surfaces, medical devices, and hands contributes to the subsequent spread of these microorganisms [47], and these pathogens are released into the environment through faecal contamination in soils and water, mainly in regions of poor or

non-existent basic sanitation. Therefore, these SCD children are a source of community transmission of resistant enterobacteria.

The limitations of this study were its cross-sectional design, which made it impossible to gauge causality among the studied phenomena, and the sample size, which limited the analysis's statistical power. We had difficulty finding children without SCD and AP living in the same house as AP-SCD children. Also, in the group without AP, five children did not have their genotype information in their medical records. As the association of penicillin G benzathine use with the presence of ESBL-producing *Enterobacterales* was based on a limited number of SCD children cases who used this antibiotic, this association needs to be well evaluated for a better understanding.

In summary, we observed that SCD children on AP from our casuistic have a 2.69 times higher chance to have resistant *E. coli* gut colonization. We also found that penicillin G benzathine use appeared to be associated with ESBL-producing *Enterobacterales*, and the CTX-M-1 group enzyme was the most prevalent among the ESBL-producing (3/6, 50%), followed by CTX-M-9 (2/6, 33%), and CTX-M-2 (1/6, 17%) groups. Further studies on AP in SCD children are urgently needed to prevent the spread and risk of resistant-*Enterobacterales* infections. Based on these results, we suggest that alternative therapies regarding prophylactic antibiotic use in SCD children should be evaluated. Future studies should consider the use of prophylactic vaccines in SCD children as an exclusive therapy against pneumococcal infections.

7. Author statements

7.1 Authors and contributors

Adriano de Souza Santos Monteiro: conceptualization; formal analysis; investigation; data curation; writing (original draft preparation).

Eduardo Gomes de Oliveira: conceptualization; investigation; data curation.

Djanilson Barbosa dos Santos: conceptualization; methodology.

Soraia Machado Cordeiro: methodology; resources; writing (review and editing); supervision.

Ricardo David Couto: conceptualization; methodology; formal analysis; resources; writing (review and editing).

Fábio David Couto: conceptualization; methodology; resources; writing (review and editing); supervision; project administration; funding.

7.2 Conflicts of interest

The authors declare that there are no conflicts of interest.

7.3 Funding information

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7.4 Ethical approval

All legal guardians and participants gave their informed consent after being informed about the aims, study risks, and benefits. General information about the participants was collected through interviews. The study was conducted only after the ethics committee approval, authorization numbers 77667917.0.0000.0056 (Federal University of Reconcavo of Bahia)

and 77667917.0.3001.8035 (Faculty of Pharmacy, Federal University of Bahia), according to the Brazilian bioethics resolutions.

7.5 Acknowledgements

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