# Acute diarrhea associated with *Salmonella enterica* in Belo Horizonte-MG: prevalence and characterization of isolates

## *Diarreia aguda associada a* Salmonella enterica *em Belo Horizonte-MG: prevalência e caracterização das amostras isoladas*

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## ABSTRACT

**Introduction:** Acute infectious diarrhea is still regarded as a public health problem associated with a wide range of etiologic agents, from which *Salmonella enterica* is particularly worth mentioning inasmuch as it is a major cause of inflammatory diarrhea in both developed and developing countries. **Objective:** To assess the distribution of *S. enterica* among children with acute diarrhea in Belo Horizonte and to characterize bacterium isolates. **Material and methods:** The study group comprised a total of 157 children from low socioeconomic background. Stool samples were collected for leukocyte analysis and Salmonella bacterial culture. The isolates were serotyped and evaluated as to antimicrobial susceptibility profile, extended-spectrum β-lactamases (ESBL) production, and presence of virulence markers (*invA*, *iroB*, and *spvC*). **Results:** A total of 5/3.2% children were infected by *S. enterica*, 3/60% by *S. enterica* Typhimurium, 1/20% by *S. enterica* Enteritidis and 1/20% *S. enterica subsp. enterica* serotype 8.20:z4,z23:-. Fecal leucocytes were detected in two out of five fecal specimens positive for *S. enterica*. Isolates from three children were resistant to nalidixic acid, nalidixic acid + chloramphenicol, and nalidixic acid + chloramphenicol + ampicillin. ESBL production was not detected. All samples presented *invA* and *iroB* genes. *spvC* marker was observed in isolates from two children infected by *S.* Typhimurium and *S.* Enteritidis. **Conclusion:** The results demonstrate that *S. enterica* infection is uncommon among children from our region. Furthermore, they indicate the need for periodic monitoring of bacterial antimicrobial susceptibility profile in order to establish suitable antimicrobial therapy when required.


**Key words:** *Salmonella enterica*; acute diarrhea; salmonellosis; antimicrobial susceptibility; epidemiology.

## INTRODUCTION

Acute infectious diarrhea still poses a great challenge for public health authorities worldwide. It is estimated the annual occurrence of approximately 1.5 to 2 billion cases of the disease and 1.5 to 2 million deaths among children aged up to five years[9, 42]. Most episodes of acute diarrhea are considered to be mild and are ultimately solved with neither medical care nor a specific diagnosis. However, the etiologic diagnosis of diarrheal disease is of utmost importance to the establishment of effective prevention and eradication programs[23].

A wide range of diarrheagenic agents have already been identified and nontyphoidal salmonellae is among them. *Salmonella enterica* comprises a large division of hydrogen sulfide-producing Gram negative bacteria hugely diverse from the antigenic perspective [27]. The species is regarded as a major agent of acute inflammatory diarrhea both in industrialized as well as developing countries. Approximately 94 million cases of *Salmonella* enteritis occur worldwide leading to around 150,000 deaths[22, 31, 32] annually.

We addressed the distribution of *S. enterica*-associated diarrhea in a pediatric population of Belo Horizonte, the association between the disease and clinical and epidemiological parameters, and the characterization of bacterium isolates as to virulence markers and antimicrobial susceptibility profile.

## MATERIAL AND METHODS

This protocol was approved by the Ethics Committee of Universidade Federal de Minas Gerais (UFMG). Written informed consent was obtained from the parents or guardians of all children included in this investigation.

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This study comprised a total of 157 children (73 girls and 84 boys, age range 1-48 months, mean age 11.7 months, median 8.0 months) from low socioeconomic background (monthly income below U$300.00), who searched for assistance at Hospital Infantil João Paulo II, Belo Horizonte-MG, from March 2004 to March 2005. All of them presented acute diarrhea and no patient had history of hospitalization or antimicrobial therapy in the last 15 days prior to specimen collection. Clinical and epidemiological data were obtained through questionnaires filled in by the children's guardians.

Stool samples were individually transferred to two sterile leak proof wide mouth screw cap vials, one of them containing a transport medium of equal parts of glycerol and 0.033 M phosphate buffer, maintained in an ice bath, and processed within one hour.

For leukocytes search, fecal smears were stained with May-Grünwald-Giemsa and examined under bright field microscopy at 400× and 1,000×.

The specimens transported in buffered glycerol were streaked onto MacConkey Agar (Difco, Sparks, MD, USA) and SS Agar (Difco). They were also inoculated into Tetrathionate Broth (Acumedia, Baltimore, MD, USA) and, following incubation for about 18 h at 35ºC, subcultured on SS Agar. All agar cultures were incubated for up to 24 h at 35ºC. Afterwards, around five lactose-negative colonies from MacConkey Agar and five lactose-negative and five $H_2S$-positive colonies from each SS Agar plate were picked and inoculated into Triple Sugar Iron Agar (Acumedia), Escola Paulista de Medicina (EPM)[36] and Citrate medium (Biobrás, Montes Claros-MG, Brazil). Whenever possible, morphologically different colonies were selected.

Following identification, *Salmonella* isolates were antigenically characterized by using somatic antisera directed against A, B, C1, C2, D, and E serogroups (Probac, São Paulo-SP, Brazil), according to manufacturer's instructions. Bacterium suspensions were boiled for 10 min before performing agglutination test when necessary. Subsequently, one *S. enterica* isolate obtained from each child was sent to Instituto Oswaldo Cruz (IOC) (Rio de Janeiro-RJ, Brazil) and serotyped by use of polyvalent and monovalent antisera against somatic and flagelar *S. enterica* antigens[19].

Antimicrobial susceptibility profile of *S. enterica* strains were determined by disk diffusion according to Clinical and Laboratory Standards Institute (CLSI) guidelines[6]. Ampicillin (AMP), ceftriaxone (CRO), chloramphenicol (CLO), ciprofloxacin (CIP), nalidixic acid (NAL), and trimethoprim/sulfamethoxazole (SUT) (Cecon, São Paulo-SP, Brazil) were tested. Screening for extended-spectrum β-lactamases (ESBL) was performed by using ceftriaxone, ceftazidime, aztreonam, and cefotaxime (Cecon)[6].

When strains isolated from the same patient showed divergent susceptibility profiles determined by disk diffusion technique, minimum inhibitory concentration was evaluated by agar dilution technique[2]. This was the case exclusively for chloramphenicol. Drug concentrations from 4 to 128 µg/ml were employed. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were included as controls for antimicrobial susceptibility testing.

In order to search for virulence determinants, *S. enterica* isolates were cultivated overnight on Tryptic Soy Agar (Difco) plates at 35ºC, bacterial cells were suspended in 500 µl sterile distilled water and centrifuged for 15 min at 6,000 g. Total deoxyribonucleic acid (DNA) was isolated by a phenol-chloroform method[12] and employed in amplification reactions targeting *invA*[5] and *iroB*[3]. Additionally, plasmidial DNA extracted by a protocol proposed by Birnboim & Doly[4] was used for detecting *spvC*[5].

About 20 ng of bacterial DNA was used as template for amplification reactions. Details of the protocols are described in **Table 1**. *S. enterica* Enteritidis (ATCC 13076), *S. enterica* Typhimurium (ATCC 14028), *Shigellla flexneri* (ATCC 12022), *Shigella sonnei* (ATCC 25931), *Shigella dysenteriae* (ATCC 13313), enteropathogenic *E. coli* (INCQS 00184), enteroinvasive *E. coli* (ATCC 43893), enterohemorrhagic *E. coli* (ATCC 43895), *E. coli* (ATCC 25922) and *Aeromonas hydrophila* IOC/*Food and Drug Administration* [FDA] 110-36) were used to validate the polymerase chain reaction (PCR) protocols.

Amplicons were resolved in 8% polyacrylamide gels, stained with ethidium bromide, and visualized under UV light. Standard of 100 bp (Life Technologies, Gaithersburg, MD, USA) was used as molecular size marker. Positive, negative, and negative internal controls (sterile water) were included in each batch of reaction.

The results generated were analyzed by using the $\chi^2$ test with Yates' correction or Fisher's exact test. The level of significance was set at 0.05.

## RESULTS

*S. enterica* was isolated from fecal specimens obtained from five out of 157 (3.2%) children. A total of 53 strains (4 to 22 isolates/child, mean 10.6, median 8.0) of *S. enterica* were isolated. Data regarding serotyping of *S. enterica* strains are shown in **Table 2**.

**TABLE 1 – PCR conditions employed for the detection of virulence markers of *Salmonella enterica***

| Gene | [primer] (µM) | [MgCl2] (mM) | [Taq] (U) | Annealing temperature | Reference |
|---|---|---|---|---|---|
| *invA* | 0.25 | 1.5 | 0.5 | 56ºC | Chiu & Ou, 1996 |
| *iroB* | 0.125 | 1.5 | 0.1 | 57ºC | Bäumler *et al.*, 1997 |
| *spvC* | 0.25 | 1.5 | 0.5 | 56ºC | Chiu & Ou, 1996 |

*PCR: polymerase chain reaction.*

**TABLE 2 –** Serotyping of 53 *Salmonella enterica* isolates obtained from five out of 157 children with acute diarrhea

| Child | Number of isolates | Serogroup | Serotype |
|---|---|---|---|
| 1 | 4 | B | *S. enterica* Typhimurium |
| 2 | 7 | B and C2 | *S. enterica* subsp. *enterica* serotype 8,20:z4,z23:- |
| 3 | 22 | A and B | *S. enterica* Typhimurium |
| 4 | 12 | A and D | *S. enterica* Enteritidis |
| 5 | 8 | B | *S. enterica* Typhimurium |

Fecal leucocytes were detected in two out of five *S. enterica*-positive fecal specimens.

There was no statistically significant association between infection by *S. enterica* and any epidemiological (seasonal bacterium distribution, gender, and age of the child) or clinical (volume and consistency of stools, frequency of bowel movements, presence of blood, mucus, and pus in stools, fever, vomit, and dehydration) parameters. Four out of five *S. enterica*-infected children were aged up to 18 months. In regard to clinical data, all *S. enterica*-positive patients reported unaltered fecal volume and watery stools and four of them had no blood or pus in feces and presented fever.

*S. enterica* strains obtained from two children (one infected by *S.* Typhimurium and one by *S. enterica* 8,20:z4,z23:-) were susceptible to all antimicrobial drugs tested. Isolates from one child (*S.* Enteritidis) were resistant exclusively to nalidixic acid. Isolates obtained from the other two children (*S.* Typhimurium) exhibited the following patterns: $NAL^{R}CLO^{R}$ and $NAL^{R}CLO^{R}AMP^{R}$. When susceptibility to chloramphenicol was evaluated by disk diffusion method, these strains showed divergent (resistant and intermediate) profiles. Data generated by agar dilution technique confirmed that all isolates were resistant to the drug minimum inhibitory concentration (MIC) ≥ 32 µg/ml. ESBL production was not detected.

All *S. enterica*-positive children were infected by strains harboring *invA* and *iroB*. *spvC* was observed in isolates recovered from two fecal specimens, one positive for *S.* Typhimurium and one for *S.* Enteritidis. There was no diversity among strains obtained from the same child concerning the presence of the three virulence markers surveyed.

## DISCUSSION

*Salmonella* is considered one of the three most relevant agents of inflammatory diarrhea worldwide, affecting mainly those individuals living in areas lacking safe food and water supplies, basic medical care, and adequate nutrition and hygiene practices[23, 31]. Despite its relevance, few reports on the prevalence of *S. enterica*-associated diarrhea are available for Brazil.

Our data demonstrate a low prevalence (3.2%) of *S. enterica* among children with diarrhea in our region. Similar finding was recently reported for the Northeast region of our country. Nunes and coworkers[29] detected *S. enterica* in 2% of the study group exclusively among children with diarrhea.

Among more than 2.500 *S. enterica* serovars, only a few of them seem to be commonly associated with diarrheal disease. Geographical and temporal variations have been observed, but worldwide *S.* Enteritidis (65%) and *S.* Typhimurium (12%) have been considered the most common serotypes[7, 10, 11, 13, 14, 17]. In agreement with these reports, four out of five isolates were serotyped as *S.* Typhimurium (three) and *S.* Enteritidis (one). For Latin America the dominance of *S.* Enteritidis (31%) has been reported[13].

Patients with salmonellosis usually develop nausea, vomiting, abdominal cramping, diarrhea, and fever. Additionally, the presence of red and white blood cells in feces is common due to the inflammatory nature of the disease[11, 23]. In this study we did not detect statistically significant association between *S. enterica*-associated diarrhea and any of the clinical and epidemiological parameters evaluated. This could be due to the low prevalence of the organism, which may compromise statistical analysis. Fecal leukocytes were detected in 40% of *S. enterica*-positive fecal specimens, results similarly described by Huicho and coworkers[18].

In most cases a supportive treatment with no antibiotics is advised for patients presenting uncomplicated salmonellosis. The disease is generally self-limiting and antimicrobial therapy apparently does not alter the clinical course and increases the carrier rate. Notwithstanding, prescription of antimicrobial drugs is necessary in some specific situations[15, 25, 26, 28, 30, 33].

As observed in several other infections, the high frequency of multidrug resistance *Salmonella* strains has made the selection of antibiotics a medical challenge[8, 16, 23, 34, 38]. Chloramphenicol, ampicillin, and trimethoprim-sulfametoxazole could be employed for treating patients with salmonellosis. However, due to increasing resistance to these drugs, fluroquinolones and third generation cephalosporins have now been frequently selected[20, 24, 40, 41].

The high resistance rate to nalidixic acid corroborates data reported by other researchers[29, 39] and should be considered a predictable result since the drug is frequently used in Brazil for treating patients diagnosed with inflammatory diarrhea. Only one out of five *S. enterica*-infected children harbored ampicillin-resistant strains, an unexpected result considering that this antimicrobial has also been largely employed in our country. Nunes and coworkers[29] recently described a 100% resistant rate to the drug. Admittedly, overall drug resistance rates described by these authors were higher than those

found in this investigation. The lack of resistance to trimethoprim-sulfametoxazole is also surprising considering the drug is frequently chosen for treating patients with diarrhea.

The detection of the virulence markers *invA* and *iroB* in all *S. enterica* strains confirms the identification of the organism at the species level and is in accordance with previously reported data. As to *spvC*, only isolates recovered from two children harbored the gene. *spvC* is located in the plasmid and it is not found in all bacterium isolates. *spvC*-positive *S. enterica* strains tend to be associated with more severe cases of the disease. In this investigation *spvC*-positive strains were serotyped as *S.* Typhimurium and *S.* Enteritidis, which are known to harbor the gene[1, 3, 5, 21, 35].

## CONCLUSION

Our data demonstrate that *S. enterica* infection is uncommon among children from our region and stress the need for monitoring the emergence of resistant strains periodically in order to guide the establishment of empirical antimicrobial therapy when required. Despite the fact that infectious diarrhea is a frequent condition, mainly in resource-poor regions, the etiology of the disease is mostly unknown. *S. enterica* is one of the most prevalent diarrheagenic agents worldwide commonly associated with severe cases of the disease. Considering the existence of geographic and temporal variations, periodic comprehensive surveys are needed to allow the understanding of the prevalence of different enteropathogenic bacteria. Evaluation of antimicrobial susceptibility profile is also of major relevance to the establishment of specific antimicrobial therapy whenever required.

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## RESUMO

***Introdução:*** *A diarreia infecciosa aguda é considerada um problema de saúde pública associado a uma ampla gama de agentes etiológicos, entre os quais destaca-se* Salmonella enterica*, causa importante de diarreia inflamatória em países desenvolvidos e em desenvolvimento.* ***Objetivos****: Avaliar a distribuição de* S. enterica *em crianças com diarreia aguda em Belo Horizonte e caracterizar as amostras isoladas.* ***Material e método:*** *O grupo de estudo consistiu de 157 crianças de nível socioeconômico baixo. Espécimes fecais foram empregados para pesquisa de leucóc itos e cultivo de* Salmonella*. As amostras isoladas foram sorotipadas e submetidas à avaliação do perfil de suscetibilidade a antimicrobianos, da produção de betalactamases de amplo espectro (ESBL) e da presença de marcadores de virulência (*invA, iroB e spvC*).* ***Resultados:*** *Cinco/3,2% crianças apresentaram-se infectadas por* S. enterica*; três/60%, por* S. enterica *Typhimurium; uma/20%, por* S. enterica *Enteritidis; e uma/20%, por* S. enterica *subsp.* enterica *sorotipo 8,20:z4,z23:-. Leucócitos fecais foram detectados em dois dos cinco espécimes positivos para* S. enterica*. As amostras isoladas de três crianças apresentaram resistência a ácido nalidíxico, ácido nalidíxico + cloranfenicol e ácido nalidíxico + cloranfenicol + ampicilina. Nenhuma amostra produziu ESBL. Todas as amostras albergavam os genes* invA *e* iroB*. O marcador* spvC *foi observado em amostras isoladas de duas crianças infectadas por* S. *Typhimurium e* S. *Enteritidis.* ***Conclusão:*** *Os resultados demonstram que diarreia associada a* S. enterica *é raramente observada entre crianças da nossa região e indicam a necessidade de avaliação periódica do perfil de suscetibilidade a antimicrobianos da bactéria para orientar o estabelecimento de antibioticoterapia, quando indicada.*

***Unitermos:*** Salmonella enterica*; diarreia aguda;* salmonelose*; suscetibilidade a antimicrobianos; epidemiologia.*

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