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Whole genomic sequence of *Salmonella* Paratyphi B isolated from a hematology patient in the northern region of Brazil

Sequenciamento de genoma completo de *Salmonella* Paratyphi B isolada de paciente hematológico da região norte do Brasil

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ABSTRACT

Salmonella Typhi is a zoonotic pathogen responsible for a life-threatening bloodstream infection that is common in places with limited sanitation and hygiene. This study aimed to characterize the resistance genes and sequence type (ST) of *Salmonella* Paratyphi isolated from a patient with acute myeloid leukemia (AML) undergoing treatment at Fundação HEMOAM, in the north of Brazil. Phenotypic identification and antimicrobial susceptibility testing were performed using the VITEK-2 system. The genomic DNA was sequenced in Illumina® equipment. Resistance genes were identified through the Genome Annotation Service. The microbiological examination of the blood culture identified *S.* Paratyphi B. Regarding resistance genes, aminoglycoside acetyltransferase [aac(6')-Iaa] mutations in the Gly133Glu position of GyrA and Ile249Met of ParC were identified. Multilocus sequence typing detected the ST 313 sequence type. *Salmonella* Paratyphi B isolated from the acute myeloid leukemia patient showed cross-resistance with other antibiotics such as quinolones and tetracyclines, which resulted from mutations in the quinolone resistance-determining region and efflux pump systems.

Keywords: *Salmonella*; Resistance Gene; RamR; Efflux Pump; Antimicrobial Agent

RESUMO

A *Salmonella* Typhi é um patógeno zoonótico, responsável por infecção sanguínea com risco de vida. Comum em locais com saneamento e higiene limitados. Este estudo teve como objetivo caracterizar os genes de resistência e o tipo de sequência de *Salmonella* Paratyphi isolada de um paciente com leucemia mieloide aguda em tratamento na Fundação HEMOAM, no norte do Brasil. A identificação fenotípica e o teste de suscetibilidade antimicrobiana foram realizados utilizando o VITEK-2. Os genes de resistência foram identificados através do Serviço de Anotação do Genoma. O exame microbiológico da hemocultura identificou *S.* Paratyphi B. Em relação aos genes de resistência, foram identificadas mutações da aminoglicosídeo acetiltransferase [aac(6')-Iaa] na posição Gly133Glu do GyrA e Ile249Met do ParC. A tipagem de sequência multilocus detectou o tipo de sequência ST 313. A *Salmonella* Paratyphi B isolada do paciente com leucemia mieloide aguda apresentou resistência cruzada com outros antibióticos, como quinolonas e tetraciclinas, resultantes de mutações na região determinante da resistência às quinolonas e nos sistemas de bomba de efluxo.

Palavras-chave: *Salmonella*; Gene de Resistance; RamR; Bomba de Effluxo; Agente Antimicrobiano

INTRODUCTION

Salmonella ser. Typhi is a zoonotic pathogen that belongs to the Enterobacteriaceae family and is responsible for typhoid fever, a life-threatening bloodstream infection that is common in places with limited sanitation and hygiene, which is transmitted through the ingestion of water or food contaminated with excreta (AKINYEMI et al.2023; VAN PUYVELDE et al. 2019). *Salmonella* enterica is classically divided into non-typhoidal (NTS) or typhoidal *Salmonella* serovars. The category typhoidal is restricted to human such *Salmonella* Typhi and Paratyphi while NTS category include *Salmonella enterica* serovars Enteridis (*S. enteridis*) and Typhimurium (BRANCHU et al., 2018).

In 2019, modelling data from Global Burden of Disease study estimated that 9.2 million typhoid fever cases and 110,000 associated deaths occurred worldwide. The highest estimated incidence occurred in the WHO South-East Asian (306 cases per 100,000 persons), Eastern Mediterranean (187), and African (111) regions. During 2017-2022, seven confirmed outbreaks were identified from ongoing outbreak monitoring activities by CD´s Global Disease Detection Operation Center including the Philippines (2022:14,056 cases), three in Zimbabwe (January-March 2017: 1,312 cases; November 2017-February 2018: 3,187 cases; and August-December 2018:7,134 cases), outbreaks with confirmed antimicrobial- resistant cases in Pakistan (January 2018-December 2019: 14,894 cases) and China (2022: 23 cases) (HANCUH et al. 2023). In Europe, the number of NTS reported was estimated at 690 cases per 100,000 inhabitants (NIKIEMA et al., 2021), while in Brazil, the *Salmonella enterica* is the main isolate pathogen in foodborne outbreaks and *S.* Typhimurium, is the first or second most isolated serovar in the country (SERIBELLI et al., 2020). According data published by Datasus, one death by *Salmonella* occurred in the northern region of Brazil, among the age group of 30 to 39 years old, in the year of 2016 (FURQUIM et al., 2021).

Most cases of salmonellosis are due to the ingestion of contaminated foods, such as poultry products and dairy products (AKINYEMI et al.2023; VAN PUYVELDE et al. 2019; PARK et al., 219; YUSOF et al., 2022; RODRIGUES et al., 2020) with most of them associated with self-limiting gastroenteritis (ASHTON et al., 2017). Individuals with a weakened immune system, adults over 64 years of age, and children under the age of five are considered high-risk groups for contracting invasive disease (MARTINS et al., 2023; ASHTON et al., 2017]. *Salmonella* Typhi and *Salmonella* Paratyphi A, generally cause systemic disease (typhoid or paratyphoid fever) (AKINYEMI et al.2023); in more severe cases, such as sepsis and systemic inflammatory response syndrome, the use of antibiotics is indicated. The spread of this pathogen, combined with resistance to multiple drugs including chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole, has caused great clinical concern (AKINYEMI et al.2023; VAN PUYVELDE et al. 2019; PARK et al., 219; YUSOF et al., 2022; RODRIGUES et al., 2020).

Bacterial resistance is among the top ten global health threats (ERDEM et al., 2022) and, due to the increasing emergence of multidrug-resistant pathogens, along with the need for new effective antibiotics available for treatment, a list of priority pathogens

was published by the WHO, in which Gram-negative bacteria such as *Salmonella* with resistance to fluoroquinolones, is included as PRIORITY 2: HIGH (VAN PUYVELDE et al. 2019; ERDEM et al., 2022).

Different studies have reported the genetic flexibility of *Salmonella* to acquire plasmid, transposon, prophage, and chromosomal gene mutation to develop antibiotic resistance (ARGIMÓN et al., 2022). There are several genes related to antibiotic resistance in *Salmonella* Typhi (*S.* Typhi), such as *catA1* (chloramphenicol resistance), $bla_{\text{TEM-1}}$ (ampicillin resistant), *dhfr7* and *sul1* (cotrimoxazole resistant), which are normally found linked to other resistance genes in class 1 integrons. Macrolide- and carbapenem-resistant *Salmonella* Typhi isolates have also emerged worldwide (PARK et al., 219; YUSOF et al., 2022; RODRIGUES et al., 2020), as has resistance to third generation cephalosporins attributed to the production of ESBLs (extended-spectrum β-lactamases) and mediated by AmpC, KPC or metallo-β-lactamases (MBLs) (ERDEM et al., 2022), whose ESBL and AmpC genes are often located in the plasmid, which carries the *qnr* gene for resistance to fluoroquinolones (PARK et al., 219; YUSOF et al., 2022; RODRIGUES et al., 2020; MARTINS et al., 2023; ERDEM et al., 2022; ARGIMÓN et. Al. 2022). Mutations in *gyrB*, *parC* and *parE*, resulting in the inhibition of antibiotic binding to topoisomerase (ARGIMÓN et al., 2022), have also been reported.

Efflux pump systems also play a significant role in resistance to various antibiotics. *Salmonella* species have five families of efflux pumps, the ABC and MFS superfamilies and the SMR, MATE and RND families, which confer intrinsic resistance to multiple antibiotics. *Salmonella* Typhimurium has five RND efflux systems: AcrAB, AcrAD, AcrEF, MdtABC and MDsABC, with AcrB being the most important as it transports structurally varied antimicrobials and its inactivation confers hypersusceptibility, while unique deletions of other RND efflux pump genes, has little or no effect on susceptibility to most antimicrobials (BUCKNER et al., 2016).

Regarding sequence types (STs), the majority of *Salmonella* Typhimurium strains associated with gastroenteritis in underdeveloped countries belong to STs 19 and 34, with ST19 being the globally circulating lineage since it is also detected in Europe and North America (AKINYEMI et al., 2023; MARTINS et al., 2023). The STs of *Salmonella* Paratyphi detected in Africa are predominantly multidrug resistant (MDR) with resistance to the first three lines of antibiotics, such as ampicillin, chloramphenicol

and trimethoprim-sulfamethoxazole, in addition to the acquisition of extended-spectrum beta-lactamases (ESBLs), conferring resistance to third generation cephalosporins such as ceftriaxone (LYU et al., 2021).

In Brazil, genomic studies on the resistance mechanisms of *Salmonella* are still scarce. Therefore, this study aimed to characterize the resistance genes and sequence type (ST) of *Salmonella* Paratyphi isolated from a patient with acute myeloid leukemia (AML) undergoing treatment at Fundação HEMOAM, in the north of Brazil.

MATERIAL AND METHODS

The study was carried out at the Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM), in the city of Manaus, which is responsible for the diagnosis and treatment of outpatients and inpatients with malignant and benign hematological diseases. HEMOAM is also responsible for the care and control of blood and blood products that are made available to residents of the city of Manaus.

Patient Data

The patient came from the neighboring state of Pará, and the journey to the city of Manaus took place via a regional boat. Upon admission to the service, a daily fever was identified that lasted three weeks, accompanied by diarrheal syndrome that ceased spontaneously, which was followed by intestinal constipation. In the investigation of the hematological condition, a myelogram was performed, and acute myeloid leukemia was diagnosed, with hospitalization to begin a chemotherapy protocol with cytarabine and daunorubicin – Protocol 3+7. During routine admission to the service on March 14th, 2023, blood culture tests were requested, which identified *Salmonella* Paratyphi B. The start of the chemotherapy protocol was delayed due to the need for adequate treatment of typhoid fever, with ciprofloxacin initially being used. Subsequently, with antibiotic escalation, guided by susceptibility testing, piperacillin + tazobactan were prescribed. The patient was discharged on April 12th, 2023, after recovery from the infection. Attention should be paid to the epidemiological history of the Amazon region, in the search for infectious and contagious pathologies that are prevalent in the region.

Cultivation, identification, and susceptibility testing

After positive blood culture (BACT/ALERT FA PLUS, Biomérieux, Brazil), primary seeding was performed with 5% sheep blood and MacConkey agar plates (Himedia-Hexasystems, Mumbai, India), which were then incubated for 24 h at 35.4 °C.

Phenotypic identification and antimicrobial susceptibility testing were performed using the VITEK-2 system (bioMerieux, Brazil); however, the minimum inhibitory concentration (MIC) values for *Salmonella* Paratyphi were defined according to criteria established by the EUCAST manual, 2022 (EUCAST, 2022). The *Staphylococcus aureus* ATCC 25923 strain was used as the quality control for susceptibility testing. Aliquots of *Salmonella* Paratyphi B were stored at -80 °C in a cryovial with brain heart infusion (BHI) broth (Himedia, Hexasystens-Mumbai, India) + 20% glycerol for further molecular testing.

Genomic DNA extraction, whole genome sequencing, MLST, resistance genes, phylogenetic analysis

Total DNA extraction was performed using the PureLink genomic DNA mini-Kit (Invitrogen, CA, USA) according to the manufacturer's instructions and then quantified using Nanodrop 1000 Spectrophotometer (ThermoFisher, USA). The genomic DNA library was prepared with Illumina Microbial Amplicon Prep (iMAP) (https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/microbial-amplicon-prep.html) and sequenced in the MiSeq Illumina Platform, configured for 150 bp paired-end fragments according to standard protocol. Part of the analyses of the genome was performed using online tools available at the Bacterial and Viral Bioinformatics Resource Center (https://www.bv-brc.org) and the Center for Genomic Epidemiology (https://www.genomicepidemiology.org. Multi-locus sequence typing (MLST) was carried out using allele sequence and profile data available at PubMLST.org. The assembly of the whole genome and individual analyses of specific resistance genes extracted from the genome were carried out using software such as Geneious Prime v. 2023. Resistance genes were identified via the Genome Annotation Service (PATRIC) using the k-mer-based antimicrobial resistance (AMR) gene detection method, which utilizes the curated collection of representative variants of the AMR gene sequence.

RESULTS

The microbiological blood culture test carried out during the patient’s hospitalization at HEMOAM made it possible to identify an isolate of *Salmonella* Paratyphi B. The antimicrobial susceptibility test (AST) and the respective MICs are

shown in Table 1, which demonstrates the patient's resistance to the antibiotic's amikacin, gentamicin, ciprofloxacin and tigecycline.

Table 1. ***Salmonella*** **Paratyphi B antimicrobial susceptibility test (AST)**

Antibiotic	MIC (µg/mL)	Interpretation
Ampicillin	≤ 2	S
Ampicillin/sulbactam	≤ 2	S
Piperacillin/tazobactam	≤ 4	S
Cefuroxime	8	S
Cefuroxime axetil	8	S
Cefoxitin	≤ 4	S
Ceftazidime	≤ 1	S
Ceftriaxone	≤ 1	S
Cefepime	≤ 1	S
Ertapenem	≤ 0.5	S
Meropenem	≤ 0.25	S
Imipenem	≤ 0.25	S
Amikacin	≤ 2	R
Gentamicin	≤ 1	R
Ciprofloxacin	≥ 0.25	R
Tigecyclin	1	R
Colistin	≤ 0.5	S

MIC= Minimum inhibitory concentration; R= resistant; S=sensitive

Genome assembly

The *Salmonella* Paratyphi genome sequence presented 95 contigs, an estimated genome length of 4,847,499 bp and an average G+C content of 52.18%. The N50 length is 178,174 bp, and the L50 count is 8. The serotype Typhimurium (05-), with an antigen profile 4: i:1,2 was identified.

Antimicrobial resistance genes

Table 2 describes the resistance genes identified through the k-mer antimicrobial resistance (AMR) gene detection method. The aminoglycoside acetyltransferase [aac(6')-Iaa] was identified, corroborating the phenotypic resistance to the aminoglycoside. The presence of genes related to the production of beta-lactamases was not observed in this study. In relation to quinolones, comparative analyses of *gyrA* and *parC*, extracted from the genome, made it possible to identify mutations in the Gly133Glu position of GyrA and Ile249Met of ParC (Table 3) and the RamR protein

with its 72 mutations is shown in Figure 1. Sequences of the IncFIB and IncFII type plasmids were also identified but not associated with any resistance genes

Table 2. Antimicrobial resistance genes

AMR mechanism	Genes
Antibiotic activation enzyme	KatG
Antibiotic inactivation enzyme	AAC (6')-Ic,f,g,h,j,k,l,r-z
Antibiotic resistance gene cluster, cassette, or operon	MarA, MarB, MarR
Antibiotic target insusceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target protection protein	BcrC
Antibiotic target replacement protein	fabV
Efflux pump conferring antibiotic resistance	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC, AcrZ, EmrAB-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, MexPQ-OpmE, OprM/OprM family, SugE, TolC/OpmH
Gene conferring resistance via absence	gidB
Protein-altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Regulator modulating expression of antibiotic resistance genes	AcrAB-TolC, EmrAB-TolC, H-NS, OxyR

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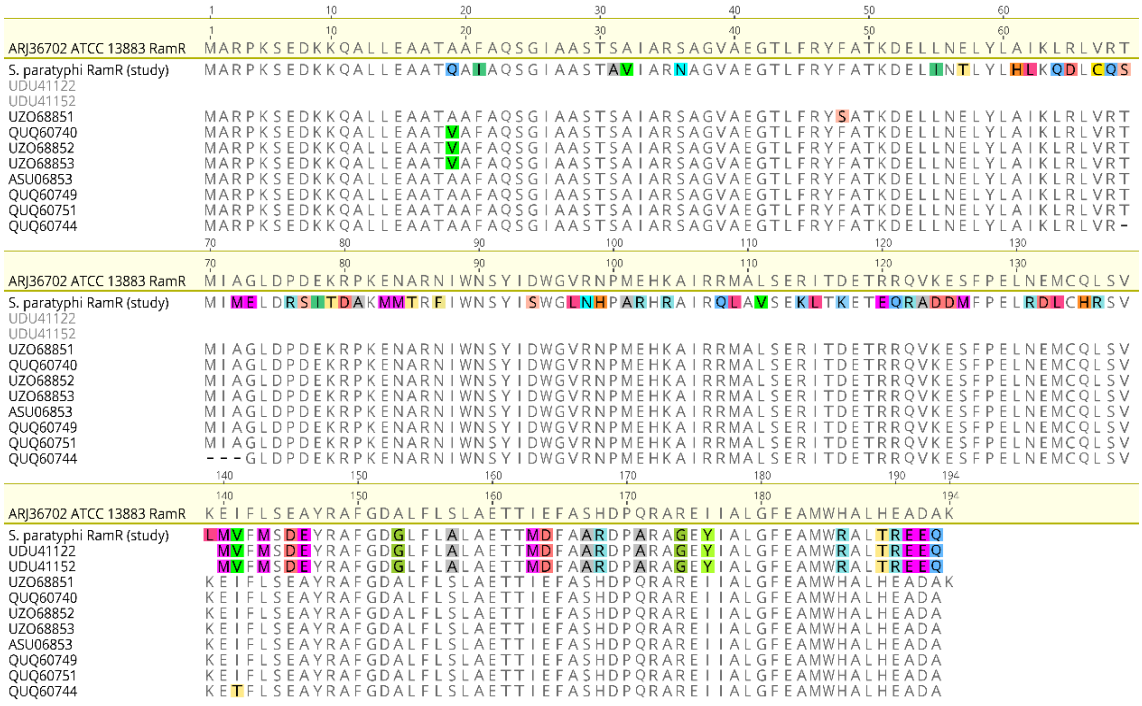
Figure 1. Alignment of *Salmonella* Paratyphi with GenBank ARJ36702 as reference sequence. Colors corresponds the mutations observed in the comparative analysis.



The multi-locus sequence type (ST) analysis detected the ST 313 sequence type, this being the first report of *Salmonella* Paratyphi B ST313, which was isolated from an onco-hematological patient at the HEMOAM Foundation, in the northern region of Brazil.

DISCUSSION

The emergence of multidrug-resistant *Salmonella* isolates has become a cause for global concern. Each year, approximately 11.9 to 20.6 million cases of typhoid fever occur in underdeveloped countries, causing approximately 129,000 to 223,000 deaths LYU et al., 2021). In this study, *Salmonella* Parathyphi B isolated from a patient with hematological disease showed a resistance profile to the class of aminoglycosides and quinolones. In relation to aminoglycosides, this was certainly due to the presence of aminoglycoside acetyltransferase [aac(6')-Iaa]. Similar data were observed in 2020 by Rodrigues et al. when they analyzed the genome of *Salmonella enterica* and identified that the most frequent resistance profile was to aminoglycoside, followed by tetracycline (QIAN et al., 2020). The authors related their findings to the high use of this antimicrobial in livestock farming as a growth promoter in animals (VAN PUYVELDE et al. 2019).

As for quinolones, it was found that, according to the breakpoints currently standardized for *Salmonella* spp. by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022), our isolate showed resistance to ciprofloxacin with a MIC of ≥ 0.25 μg/mL. Bacteria presenting this phenotype are worrying, as they make clinical treatment difficult and can lead to patient death (QIAN et al., 2020). This resistance can be attributed to different factors such as i) chromosomal mutation in the quinolone resistance determinant region (QRDR) in the *gyrA, gyrB, parC* and *parE* genes, ii) overexpression of the AcrAB-TolC efflux system, which decreases the cellular concentration of the drug (ZHANG et al., 2016; ABD EL-AZIZ et al., 2021) and iii) through plasmid (PMQR), encoded by the qnr determinant (*qnrA, qnrB, qnrC, qnrD, qnrS*), *aac (6')-ib-cr, qepA* and *oqxAB* (ABD EL-AZIZ et al., 2021; ALENAZY et al., 2022; BAUCHERON et al., 2014). Furthermore, quinolone resistance in *Salmonella* species is also associated with the resistance-nodulation-division (RND) family of protein transporters in the cytoplasmic membrane. Among them, the most

important is the tripartite efflux pump AcrAB-TolC, which is activated by overexpression of the *acrB* gene, a transporter protein that expels unrelated compounds from bacterial cells and is responsible for the MDR phenotype (VELHNER et al., 2016).

In this study, comparative analyses in the QRDR, in the region of *gyrA* (GyrA) and *parC* (ParC) extracted from the genome, showed no mutations in the usual region of the QRDR, only in position 133 of GyrA (Gly133→Glu). This was detected when compared to *Salmonella* Typhi Ty2 (Table 3) and is in agreement with the study by ACHEAMPONG et al. in 2019. In 2014, GARCIA et al., detected a mutation in *gyrA*, in all their *Salmonella* isolates when using the *Salmonella* Typhimurium LT2 sequence for comparison, which is different to what we found in our study.

The authors also observed that the frequency of this mutation was very low in Asian countries; however, it was high in the Democratic Republic of Congo (GARCIA et al., 2014). Nonetheless, this isolated mutation would not be sufficient to express the resistance phenotype, unless it is combined with other mutations in the QRDR region (QIAN etal.,2020). In ParC, the mutation identified was at position 249 (Ile249→Met). Additional research is necessary to verify whether these unusual mutations are related to the resistance to this group of antibiotics.

The expression of the AcrAB efflux pump is regulated by the *ramR* repressor gene which, in the presence of mutations or deletions, increases the expression of the *ramA* genes that produce the protein RamA, homologous to MarA and SoxS, and *acrAB*. This results in the appearance of the efflux phenotype multidrug resistant and cross-resistance between antibiotics such as tetracyclines, quinolones, beta-lactams and chloramphenicol (ZHANG et al., 2016; ABD EL-AZIZ et al., 2021; ZHENG et al., 2011), due to the broad substrate specificity that the AcrAb-TolC efflux pump can eliminate (ZHANG et al., 2016). In *Salmonella* species, the known efflux pump regulators are i) AcrAB-TolC, which plays an important role in resistance; ii) RamA, which controls a series of genes with diverse physiological functions; iii) MarA which mediates drug resistance, and iv) AcR (ZHENG et al., 2011; FERRARI et al., 2013). However, more studies still need to be carried out to better understand the functions of acrB, MarA, RamA and their regulation in *Salmonella enterica* (VELHNER et al., 2016; FERRARI et al., 2013). In this study, genome analysis showed the presence of

the *AcrAB, MarA, MarB, MarR* and *ramR* genes (ZHANG et al., 2016; ABD EL-AZIZ et al.,2021; BAUCHERON et al., 2014; ZHENG et al., 2011), which may have contributed to the cross-resistance of this isolate.

Regarding resistance to tigecycline, the comparison of the ArcR residues from *S.* Paratyphi in this study, with the sequence from *Salmonella* Typhimurium LT2, showed no difference in the amino acid sequence. Some studies report that, in addition to mutations in efflux proteins, ribosomal protection and enzyme inactivation (*tet* (A), *te*t (M), *tet* (X)), overexpression of the efflux pumps AcrAB-TolC and OqxAB may also be involved in this type of resistance (ABD EL-AZIZ et al.,2021). In our study, the AcrAB-TolC efflux system was identified in *Salmonella*, thus requiring more in-depth genomic studies for better understanding of its action in the resistance mechanism. The Ram-R analysis showed several mutations that need to be better understood and researched in order to verify its relationship with the tigecycline resistance mechanism.

Genetic analysis identified the IncFIB and IncFII plasmid types in our study, but they were not associated with any resistance genes. Similar plasmid findings were reported by ROBERTSON et al., 2023 and LYU et al., 2021, in a genomic characterization study on *Salmonella enterica* strains isolated from meat in China. CHEN et al., in 2016, in a study carried out on multidrug resistant strains of *Salmonella*, identified different types of plasmids such as IncP, HI2, A/C, FIIs, FIA, FIB, I1, IncHI2 IncFIB, showing that the IncHI2 plasmid, in addition to having an important role in the dissemination of genes encoding extended-spectrum beta-lactamases, mainly $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, and PMQR genes *qnrA* and *acc(60)-ib-cr*, was the main lineage in the dissemination of antibiotic resistance (CHEN et al., 2016). In this study, the presence of genes related to the production of beta-lactamases was not observed, which agrees with the results of the susceptibility test, as *Salmonella* Paratyphi B ST313 was resistant to few antibiotics.

The analysis of the MLST alleles of *Salmonella* Paratyphi B in this study made it possible to identify the ST313 sequence type, this being the first report in the northern region of Brazil. A study carried out by SERIBELLI et al., 2021, in samples of *Salmonella* Typhimurium isolated from samples of human diarrheal feces from and food in the city of São Paulo, Brazil, also identified ST313 with a sensitivity profile to antibiotics; however, this ST has been reported in species of *Salmonella* with different

antibiotic resistance profiles. Similar findings were identified by PULFORD et al., 2021, in an evolutionary study on two variant strains of *Salmonella* (ST313 (L1) and ST313 (L2)), with resistance to at least one antimicrobial, associated with blood-borne infectious processes, and involving different African countries such as Kenya and Malawi (East Africa) (RAMACHANDRAN et al.,2015). In the Democratic Republic of Congo, a new II.1 sublineage of *Salmonella* ST313 was identified carrying the extensively resistant IncHI2 plasmid (pSTm-ST313-II.1) (VAN PUYVELDE te al., 2019; MARTINS et al., 2023), which we did not identify in our study. In the United Kingdom, the same ST was detected and isolated from patients with gastroenteritis (ASHTON et al., 2017). Reports of the identification of ST313 in different countries and regions suggest a trend of global distribution for this type of genotype. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JBBLHE000000000. The version described in this paper is version JBBLHE010000000; Bioprojet: PRJNA1071614**.**

CONCLUSION

Our study demonstrated that *Salmonella* Paratyphi B isolated from a patient with AML showed cross-resistance with other antibiotics such as quinolones and tetracyclines, resulting from mutations in the QRDR region and efflux pump systems. Genome studies are important for elucidating the mechanisms of resistance to antimicrobials and provide important epidemiological data for monitoring, therapy, and management of surveillance programs in our region, as well as in proteomics in order to understand how infections can cause significant changes in the proteins of cells.

DECLARATIONS ETHICS APPROVAL

This study was approved by the Human Research Ethics Committee at the Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas – HEMOAM, Manaus, Amazonas state, Brazil (CEP/HEMOAM), under (CAAE Nº 68471223.5.0000.0009).

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