

Molecular Profiling of Class I Integron Gene in MDR *Salmonella typhi* Isolates

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Abstract

Typhoid fever is a paramount reason for horribleness that more mortal sin “around the sum ages aggregations clinched alongside iraq it initiated by salmonella typhi. *Salmonella typhi* is diagnosed serologically by the Widal test and confirmed by vitek and using polymerase chain reaction (PCR) based amplification of DNA from the bacterial samples of typhoid fever patients. The present study was designed to detect class I integron gene encoding antimicrobial of *S. typhi* using appropriate primers by PCR. These isolates of this study were collected from postgraduate laboratories (Prepared samples in vitro prepared diagnostics), they were a previous collected from carried out in Al Najaf provenance, throughout those period from July 2018 on March 2019 including 231 cases from blood, stool samples collected from patients suffering from typhoid fever were attended to Al-Sader Medical City and Al-Hakim General Hospital in Al-Najaf province. Biochemically tests and monovalent antisera gave 117 (50.64%) positive result *S. typhi* isolates and confirmed by Vitek system and PCR which showed positive result 59 (50.42%). Fifty nine isolates of *S. typhi*, were collected from patients with typhoid fever that distributed to 40/59 (34 %) from blood , 19/59 (15.1%) stool. Molecular detection revealed that most isolates of *S. typhi* were positive results to (*intI*) gene 43/59 isolate (the specific primer (*intI*) gene for *S. typhi* bacteria was designed in this study by using bioinformatics programs with NCBI website). According to the different diagnostic above, Vitek and PCR method were more sensitivity technique for *S. typhi* detection among typhoid patients. The results of virulence factors of *S.typhi* isolates were negative results for gelatinase, hemolysin, protease and capsulated. Multidrug resistance (MDR) of *S. typhi* isolates were represented by 18 antibiotics resistance to class and sub class of antibiotic. All *S. typhi* isolates appeared high resistance 100% to Aztreonam (AZM₁₅), Nitrofurantion (F), Amoxicillin/clavulanicacid (AMC₃₀), (PY₂₅), Clarithromycin (CLR), Cefoxitin (FOX₃₀), Penicillin(P₁₀), Cefotaxime (CTX₃₀), ampicillin (AMP), Meropenem (MEM), Tetracycline(TE₃₀). Also resistance of isolates that revealed 91% to Impinem (IP), 88% Ampicillin (AM₁₀), 85%Amoxillin (AX), 81% Gentamicin (CN₁₀), 80% Chloramphenicol (C₃₀), 74% Cefpirome (CPR) and 68% Carbenicillin (CB).

Keyword: *Salmonella typhi*, Typhoid fever, PCR, integron class I, *intI* gene, Virulence Factors

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INTRODUCTION

Typhoid fever is an acute illness, potentially fatal systemic caused by *Salmonella typhi* and *Salmonella paratyphi*, pathogen only specific to humans. It has been shown that typhoid occurs in many developing countries, which it still remains an important cause of mortality and morbidity¹. Typhoid fever is still a major health concern in the developing world as it is found endemically throughout Asia Africa, particularly in South Asia, East Asia and South America. In a broader sense, typhoid fever is categorized under enteric fever which is described as a systemic illness described by abdominal pain, fever and other symptoms as vomiting, nausea, anorexia and headache². When enteric fever is caused by *Salmonella typhi*, it is known as typhoid fever and when it is due to *Salmonella Paratyphi* A, B, or C, it is called paratyphoid fever.

Salmonella typhi bacterium are an obligate parasite with no other known reservoir outside of humans. A 100,000 organisms of *Salmonella typhi* make up an infectious dose, and the disease is typically spread through feces and urine of infected people in contaminated food and water². Typhoid is transmitted by the fecal-oral route, in most cases an asymptomatic carrier of *S.typhi*, or an individual who has recently recovered from the infection, continues to excrete large number of organisms in the stool and contaminates food or water, either through direct food handling, through transfer of bacteria by flies and other insects, or by contamination of potable water³. Approximately 10% of patients recovering from typhoid fever excrete *S.typhi* in the stool for three months, and in the 2-3% became permanent carriers⁴.

Salmonella typhi contains an endotoxin typical of gram negative organisms, as well as the Vi antigen which is thought to increase virulence. Many of the *S.typhi* virulence factors, such as toxin genes invasion and adhesion are clustered in certain areas of the chromosome known as *Salmonella* pathogenicity islands⁵. Virulence factors present on the surface of *Salmonella typhi* such as lipopolysaccharides, capsule and fimbriae can affect the virulence of the bacterium, and also are key targets of the host immune system⁶. Endotoxin play an important role in the pathogenesis of *Salmonella typhi* infection

during the bacteremia stages of typhoid fever that produced as a result of the acting endotoxin directly and indirectly through the release of endogenous pyrogens from leukocytes⁷.

Most *Salmonella* strains carry a large number of plasmids that contains virulence genes that are essential to trigger systemic disease, their involvement in the enteric stage of the infection is unknown⁸. Antimicrobials is important for the control and treatment of *Salmonella* spp. However, antimicrobial- and multidrug-resistant *Salmonella* strains have emerged, leading to treatment failure⁹. Many Strains of *Salmonella typhi* resistant to all three first-line drugs were in existence, with the development of quinolone resistance, third-generation cephalosporin's were used for treatment, but sporadic reports of resistance to them also followed^{10,11}.

Multidrug resistance is an increasing threat to global health. There are > 14 million cases of typhoid fever every year and > 135,000 deaths, the disease is primarily controlled by antimicrobial treatment, but this is becoming increasingly difficult due to MDR¹¹. Multidrug-resistant typhoid fever is defined as typhoid fever caused by *Salmonella typhi* are resistant to all the three first-line recommended drugs for treatment, including cotrimoxazole ampicillin and chloramphenicol¹². ESBL-producing *Salmonella* isolates resistant to several antibiotic agents, third-generation cephalosporin's referred to extended spectrum cephalosporin's were effective against ampicillin hydrolyzing β -lactamases and gained widespread use in clinical use. They include ceftriaxone, ceftazidime, cefotaxime and others. Treatment of *Salmonella typhi* with third-generation cephalosporin, azithromycin and fluoroquinolones drugs are the main treatment¹³.

Integron are genetic elements that contain the genetic determinant for the components of asite-specific recombination system that recognizes and captures mobile gene cassettes. An integron is a gene capture system found on plasmids, on chromosome and in transposon, platforms that incorporate exogenous open reading frames by site-specific recombination and convert them to functional genes by ensuring their correct expression¹⁴. Integron can be divided into two major group : resistance integrons and super-integrons Although not independently mobile,

integrons are widespread versatile DNA elements and can be divided into two distinct subsets: the mobile integrons and the chromosomal integron¹⁵.

Integrons are classified based on sequence similarity, there are at least five classes of integrons with class I integrons being the most studied and characterized¹⁶. Class 1 integrons are the most common and widely distributed among Gram-negative bacteria and are associated with functional and nonfunctional transposons^{17,18}. Class 1 integron is composed of the (3[′]CS) and including 2 (ORFs) open reading frames. QacE1 gene, it confers resist to qac quaternary ammonium compound; and Soul gene is confer resist to sulphonamides. There are responsible for the mobility of transposons and also found in *Salmonella typhi*¹⁹. PCR is rapid and sensitive assay used to detect microbial pathogens in clinical samples. PCR method had diagnostics value for detection of *Salmonella* among all the diagnostic tests²⁰⁻²². The present study carried out to achieve the following objects: 1- Isolation and identification of *Salmonella typhi* collected from patients with typhoid fever from blood and stool by Vetik and PCR diagnosis . 2- Determination of the virulence factors gelatin and protease among clinical isolates. 3- determination of MDR of the antimicrobial susceptibility in *S.typhi* isolates. 4- Detection of the Class I integron (*intl*) gene in *S.typhi* isolates.

MATERIAL AND METHODS

Samples collection

These isolates of this study were collected from postgraduate laboratories (Prepared samples in vitro prepared diagnostics), they were a previous collected from 231 samples were involved 122 stool samples and 109 blood samples. Those period from July 2018 on March 2019 were attended to Al-Hakim General Hospital and Al-Sader Medical City Hospital in Al-Najaf province. It collected from patients were suffering from typhoid fever and swabbed onto Xylose-Lysine-Desoxycholate Agar (XLD) and MacConkey agar media were incubated overnight.

Identification of *Salmonella typhi* isolates

- **Microscopic Properties:** Gram's stain was used to examine the isolated bacteria for studying the microscopic properties as initial identification of *S.typhi*.²³

- **Cultural Characteristics:** Morphological characteristics of *Salmonella typhi* were recorded on the specific media for primary identification²³.
- **Biochemical Tests:** 1-2 colony of *S.typhi* isolates were tested for oxidase, Simmone Citrate, catalase, Kligler Iron agar and Indole tests.
- **VITEK@2 GN ID Card:** *Salmonella typhi* isolates were confirmed with the automated VITEK@2 compact system by using GN ID cards. It was carried out on each bacterial isolate to complete the final identification. The GN ID card is based on established biochemical (64 reaction) methods and newly developed substrates, measuring various metabolic activities (BioMerieux Company/ <http://www.bioMérieux.com>).

C - Molecular Profiling of Class I Integron *intl* gene in *Salmonella typhi* isolates

- **Bioinformatics of *intl* gene:** Bio-statistical service technology and Bioinformatics has advance of diagnostics and medical in (NGS) and ecological research. Bioinformatics specialized in Microbiology and set new standards for high level services of data analysis including high expertise in NGS^{18,24}. In this study is using Bioinformatics program to designing the specific primer of Class I *intl* gene in MDR *Salmonella typhi*.
- **Genomics Bacterial DNA Extraction:** DNA (Promega /USA) Extraction of Bacteria DNA is (59) of *Salmonella typhi* bacterium. The wizard chromosomal DNA purification kit is designed for extraction of *Salmonella* DNA.
- **Polymerase Chain Reaction:** PCR has been amplifying specific primer of Class I integron *intl* gene with genomic DNA of all isolates *S.typhi*. DNA purification kit was designed for extraction of *S.typhi* DNA. Gel-electrophoresis was used for detection of DNA by UV transilluminator²⁵. The PCR assay was detected the (*intl*) gene. These specific primer synthesized by Alpha DNA company, Canada. the DNA marker and PCR product were resolved (1.5%) agarose gel by electrophoresis.
- **Multidrug Resistance Tests in *Salmonella typhi* isolates:** Antimicrobial susceptibility tests assay, as described by CLSI²⁶. It was used

antibiotics disks assay and Muller Hinton Agar medium in current study to the susceptibility of 18 antibiotics commonly including as Aztreonam(AZM₁₅), Nitrofurantion(F), Amoxicillin/clavulanicacid (AMC₃₀), (PY₂₅), Clarithromycin(CLR), Cefoxitin(FOX₃₀), Penicillin(P₁₀), Cefotaxime(CTX₃₀), ampicillin(AMP), Meropenem(MEM), Tetracycline(TE₃₀), Impinem(IP), Ampicillin(AM₁₀), Amoxillin(AX), Gentamicin (CN₁₀), Chloramphenicol(C₃₀), Cefpirome(CPR) and Carbenicillin(CB)²⁶.

Virulence factors of *Salmonella typhi*

- **Detection of Hemolysin:** This agar medium was inoculated with *S.typhi* isolates, and incubated for 24/48 hr, at 37°C. Positive results that show the clear zone around the bacterial colony appears which is referred to β -hemolytic and α -hemolytic or green zone referred to but negative results weren't that show²⁷.
- **Detection of Protease Enzyme:** *S.typhi* isolates were inoculated the Skim agar medium, it was incubated for 24-48hr, at 37°C. the positive result it was observed when clear zone was appeared around the colony while the negative results weren't that show²⁸.
- **Detection of gelatin production:** The specific media for detection gelatinase liquefaction was inoculated with a single colony of overnight culture from nutrient medium and incubated at 37°C, for 24 hr of incubation period by placing the tube in refrigerator for (30)min. A positive result was indicates if the media was liquid which refer to ability of

bacteria to produced gelatinase and negative results was solidify because of no ability to produced it²⁹.

Statistical Analysis

In the current study using the program of SPSS computing (version 24) for the data analysis.

RESULTS

Identification of *Salmonella typhi* Isolates

During the current study period were collected 231 clinical samples from (122) blood samples and, (109) stool samples from patients with typhoid disease. Identification of *Salmonella typhi* isolates have been depending on initial identification the morphological, biochemical tests, and microscopically. Microscopically identification of *S.typhi* was appeared gram negative bacilli singly or in pairs, peritrichous flagellated, motile, non-spore forming bacteria, and non-capsulated. While the Morphological of *S.typhi* isolates were used the specific media such as XLD agar and MacConkey agar, appeared on culture medium once appeared the typical characteristics at 37°C after 18-24h, on blood agar *S.typhi* colonies appeared smooth, rounded, convex, and nonhemolytic and grey white color. But on MacConkey agar were appeared pale yellow (non-lactose ferment), 1-3mm in diameter and after 18-24 hour at 37°C. as well as, good growth of *S.typhi* colonies on XLD agar were appeared gray color with black center colonies because of it was ability to produce the H₂S, as showed in [Fig. 1].

Biochemically tests results of *S.typhi* isolates were revealed on TSI, Sugars, Oxidase, Indole, Ureases and simmone citrate test. In



Fig. 1. *Salmonella typhi* colonies on XLD agar

Table 1. Biochemical tests of *Salmonella typhi* isolates

No.	Biochemical tests	<i>Salmonella typhi</i> results
1.	TSI test	AKL/ACID with H ₂ S
2.	Glucose, mannitol, L-arabinose sorbitol	+ Sugars fermented
3.	Oxidase test	-
4.	Indole test	-
5.	Ureases test	-
6.	simmone citrate slant test	-
7.	Motility	Motile +
8.	Spore	-
9.	H ₂ S Production	+

TSI slants test, the slant and butt turned into AKL/ACID red and yellow colour respectively indicating to non-fermentation of glucose on slant and production of acid with H₂S in the bottom. Glucose, mannitol, L-arabinose and sorbitol sugars were fermented by all isolates. Other tests of *S.typhi* isolates were appeared negative result for oxidase test, indole production, urease production and citrate utilization as showed in Table [Table.1].

VITEK@2GNID Cards System results of *S.typhi* isolates, the identification were included many biochemical tests. The results revealed *S.typhi* were revealed with cards ID the ranging excellent of isolates (percentage% from 95 to 99%). The 59 *Salmonella typhi* colonies on MacConkey agar.

Detection virulence factors of *Salmonella typhi*

The ability of (59) *S. typhi* to produce many virulence factors. All isolates of *S.typhi* have been produce the non-hemolytic on blood medium and non-capsulated. Likewise *S.typhi* isolates have no ability to produce of Gelatinase on specific gelatin media as well as it has non-produced of Proteinase on Skim agar medium.

Multi-drug Resistance of *Salmonella typhi* isolates

Among 59 *Salmonella typhi* isolates, they were susceptible to describe this study

by susceptibility of 18 antibiotics, including as high resistance of *Salmonella typhi* isolates appeared high resistance 100% to Aztreonam (AZM₁₅), Nitrofurantion (F), Amoxicillin/clavulanicacid (AMC₃₀), (PY₂₅), Clarithromycin (CLR), Cefoxitin (FOX₃₀), Penecillin (P₁₀), Cefotaxime (CTX₃₀), ampicillin (AMP), Meropenem (MEM), Tetracycline(TE₃₀). Also resistance of isolates that revealed 91% to Impinem (IP), 88% Ampicillin (AM₁₀), 85%Amoxillin (AX), 81% Gentamicin (CN₁₀), 80% Chloramphenicol (C₃₀), 74% Cefpirome (CPR) and 68% Carbenicillin (CB).as mentioned in [Fig. 2]. They have been need an overall increment in MDR typhoid created by *S.typhi* on different antimicrobial operators. A high variables would responsible, those development MDR isolates encountered with urban decay because of deindustrialization, government lodgin and engineering imagined.

Molecular Characteristics *Salmonella typhi* isolates

Characteristics of Class I Integron *intl* Gene of *S.typhi* isolates

The present investigation indicates that the vicinity about a integron might have been altogether connected with 43(73 %) MDR isolates. Those distinguished genes encodes

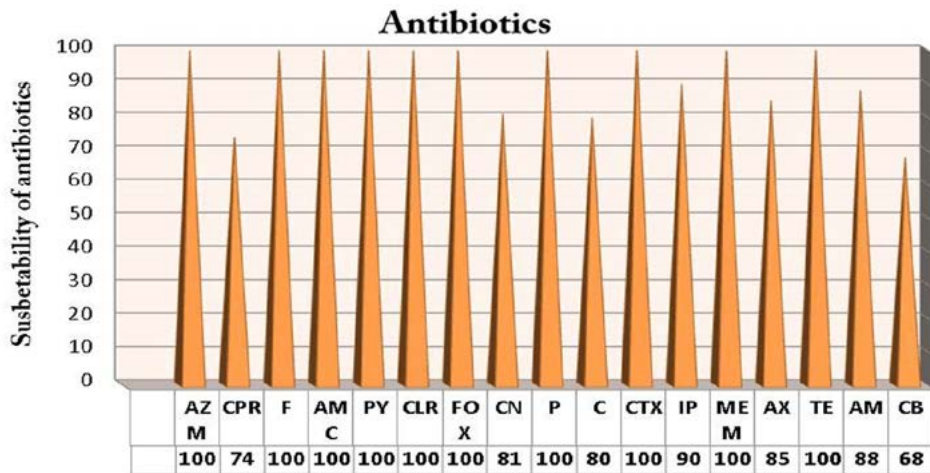


Fig. 2. Multi-drug Resistance of *Salmonella typhi* isolates for many antibiotics

Table 2. The Sequence and concentration of intl primers

No	Primers	Primers sequences 3- 5	Product size	Temperature Annealing	References
1.	<i>intl- F</i> <i>intl- R</i>	ACCCCGTAATGGCTAACTT GCAGTTGCAAACCTCACTG	613 bp	60°C	Primer designed in this study

(integrase) from claiming class 1 integron. Specific primer designed in this study within NCBI web/Bioinformatics program. PCR was used to detect the *(intl)* gene of *Salmonella typhi*. The results of *Salmonella typhi* diagnosis by PCR technique that 43/59(73%) isolates visualized in [Table 2] and [Fig. 3].

DISCUSSION

The current study identified of *Salmonella typhi* isolates were depended on, microscopically, the morphology, biochemical tests and initial identification. *Salmonella typhi* colonies on XLD agar were appeared gray color with black center colonies. Results from previous studies suggested that iNTS disease and Typhoid fever were major causes of invasive bacterial febrile illness in the sampled locations, most commonly affecting child in both high and low population density³⁰. Typhoid fever infection was caused by *S.typhi* and was transmitted through the oral route by the contaminated poultry, meat,, milk and eggs. Disease is most common in young child and elderly with peak incidence in summer and fall rainy season³¹.

Biochemically tests results of *Salmonella typhi* were observed negative result to simmons citrate, indole production, urease oxidase and H₂S-production and TSI was ALK/ACID for all isolates. It was founded *S. typhi* isolates couldn't produce Urease enzyme. All biochemically result were agreed with³²⁻³⁴.

All results of virulence factors of *Salmonella typhi* showed that all isolates didn't produced genelatinase, hemolytics and proteinase enzymes. Thus, entero-chelin secretion in vivo

could be a vital step in the stages of the infection cycle in which intestine – population, penetrate, the mucous layer covered the intestinal, translocate out of intestinal lumen through the epithelial cells, and finally step spread to organs and they may survive³⁵.

MDR results in this study by susceptibility of (18) antibiotics, including as high resistance of *Salmonella typhi* isolates 100% to Aztreonam(AZM₁₅), Nitrofurantion(F), Amoxicillin/clavulanicacid (AMC₃₀), (PY₂₅), Clarithromycin (CLR), Cefoxitin(FOX₃₀), Penecillin (P₁₀), Cefotaxime (CTX₃₀), ampicillin (AMP), Meropenem (MEM), Tetracycline (TE₃₀). Antibiotics resistance in *S.typhi* is a threat to human public health³⁶. In previously studies, MDR*Salmonella* bacteria were frequently observed among pigs,chickens and ducks. In addition, MDR *Salmonella* was serotype dependent³⁷. As well as *Salmonella enteritidis* and *Salmonella typhimurium* were strongly association with MDR antibiotics phenotypes. MDR isolates could transfer to humans via animal or animal production and a great risk to public health³⁸.

While, these isolates gave high resistance to other antimicrobial such as 91% to Impinem(IP), 88% Ampicillin(AM₁₀), 85%Amoxillin(AX), 81% Gentamicin (CN₁₀), 80% Chloramphenicol(C₃₀), 74% Cefpirome(CPR) and 68% Carbenicillin(CB).as mentioned in [Fig.2]. Other studies mentioned that the 41 *Salmonella typhi* screened for antibiotics susceptibility, 25 (61.0%) were resistant to five or more antibiotics. Also, 84% of bacteria were isolated from patients who didn't respond to initial treatments. The high percentage of MDR*Salmonella typhi* revealed was worrying

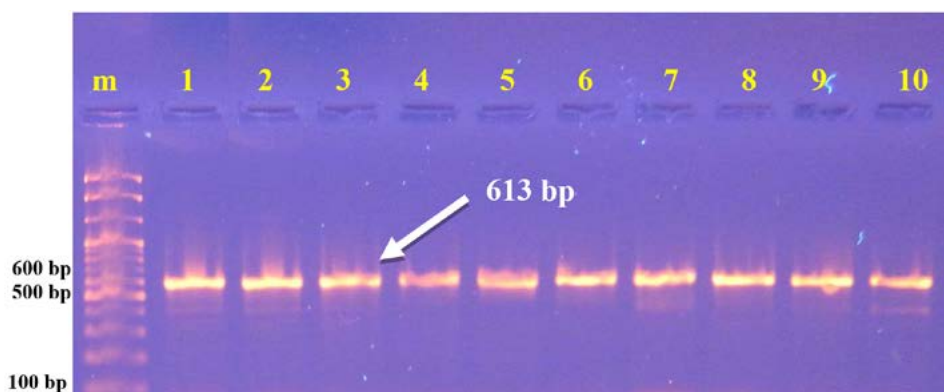


Fig. 3. The Agarose Gel Electrophoresis (1.5%) of PCR products of *intl* gene (613 bp) of *Salmonella typhi* for (45) min at (100) volt.

and quite revealing³⁹. MDR *Salmonella typhi* in Nigeria, Lagos. Periodic review of antimicrobial used in hospitals was essential as the efficacies of cotrimoxazole chloramphenicol and ampicillin were now doubtful. ofloxacin and Ciprofloxacin were effective antibiotics for treatment of typhoid infection, but with current trends of drug in Nigeria, resistance was likely to development. Therefore, provide an early warning signal for the prudent use of fluoroquinolone DRUGS to preserve their usefulness³⁹.

Our results of molecular profiling of Class I Integron gene in *S.typhi* isolates, the present investigation indicates that the vicinity about a integron might have been altogether connected with 43/59 (73%) MDR isolates. Those distinguished genes encodes (integrase) from claiming class 1 integron. Integron were universal Around medication of Gram-negative clinical segregates. In addition, A percentage combinations for gene cassettes appear to be should need a nearby distribution, inasmuch as others appear will have a broad appropriation. Class 1 integron need aid spotted every now and again on plasmids that could make exchanged by conjugation. Those integron-carrying segregates would generally multidrug resistant, while those integron-lacking detaches would additional defenseless with drugs, Anyhow Likewise over other cases, not the sum safety determinant were spotted on Integrons⁴⁰.

CONCLUSION

The conclusion of this study included into 1- *Salmonella typhi* isolates have been a big role in causing typhoid disease among clinical isolates. 2- Virulence factors of *Salmonella typhi* isolates have no ability to produce the gelatinase, proteinase and non-hemolytic among clinical isolates. 3- Genotypic detection by PCR revealed the presence of Class1 integron gene that have are relationships with MDR. 4- the current study revealed high resistance of *S.typhi* isolates have a reach to resistance 100% to Aztreonam (AZM₁₅), Nitrofurantion (F), Amoxicillin/clavulanicacid (AMC₃₀), (PY₂₅), Clarithromycin (CLR), Cefoxitin(FOX₃₀), Penicillin(P₁₀), Cefotaxime (CTX₃₀), ampicillin (AMP), Meropenem (MEM), Tetracycline (TE₃₀).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY

All data were analyzed during this work are included in the manuscript.

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