ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *Shigella* spp. ISOLATED FROM PATIENTS SUSPECTED OF ACUTE GASTROENTERITIS

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ABSTRACT

Shigellosis, an intestinal infection caused by *Shigella* species, is manifested by bloody diarrhea. Due to the surge in multidrug-resistant (MDR) *Shigella* species, the control of shigellosis has been a big challenge. This study aims to determine the prevalence and assess the antibiotic susceptibility pattern of *Shigella* species. During our study period of five months from April 2014 to August 2014 at Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu, a total of 653 stool samples were collected from the patients suspected of acute gastroenteritis. The standard microbiological procedure was followed for the isolation and identification of *Shigella* species. Assessment of antibiotic susceptibility pattern of the *Shigella* species was done by Kirby-Bauer disk diffusion method following CLSI guidelines. The study found 25(3.82%) cases were *Shigella* positive. Among them, 18(72%) were *S. flexneri*, 6(24%) were *S. dysenteriae*, and 1(4%) was *S. sonnei*. The patients in the age group 16-45 years were highly susceptible to infection as the higher proportion 16(64%) of *Shigella* species were isolated from this age group (p> 0.05). *Shigella* species were found to be highly susceptible to Cefotaxime (100%), a third-generation cephalosporin. Nalidixic acid, on the other hand, was the least effective antibiotic as 20(80%) of the *Shigella* isolates were resistant, followed by Ampicillin 18(72%), Cotrimoxazole 13(52%), and Ciprofloxacin 9(36%). A higher proportion of [10(40%)] of our study isolates were MDR. Our results show that Nalidixic acid, Ampicillin, Cotrimoxazole, Ciprofloxacin, and Ofloxacin cannot be used as empirical therapy for the treatment of *Shigella* infection as *Shigella* species were highly resistant to these antibiotics. So, for the MDR *Shigella* infection, we suggest third-generation cephalosporin as an option.

Keywords: Antibiotic susceptibility, empirical therapy, MDR, *Shigella* species

INTRODUCTION

Shigella, a food-borne pathogen, is responsible for acute invasive gastroenteritis and traveler's diarrhea (von Seidlein *et al.*, 2006). It is a major global health problem as it is a prime cause of diarrhea, with 212,000 deaths every year worldwide (Arnold, 2021). It is a major cause of mortality and morbidity in children under the age of five in developing nations (Kotloff *et al.*, 2018). It is manifested by bloody diarrhea and is caused by *Shigella flexneri*, *S. sonnei*, *S. boydii*, or *S. dysenteriae* (Heymann, 2008). *S. flexneri* is the major cause of diarrhea in the least developed and developing countries, whereas *S. sonnei* is the major species isolated in the developed nations (Kotloff *et al.*, 2018; Bardsley *et al.*, 2020). The organism is usually transmitted by the feco-oral route when contaminated food or water is ingested. The definitive diagnosis of *Shigella* infection is done by isolating the organism from the stool and serotyping the isolate (WHO, 2005).

The severity of shigellosis and potential complications can be avoided by the proper use of antimicrobials (Lima *et al.*, 1995); however, antibiotic resistance to *Shigella* species and serotypes has emerged throughout the world (Taneja *et al.*, 2012). Previously effective first-line drugs such as Sulphonamide-trimethoprim, Ampicillin, and Nalidixic acid are almost ineffective against the prevalent *Shigella* strains (Kotloff *et al.*, 2018; Puzari *et al.*, 2018). With the recent rise in fluoroquinolone-resistant strains, the choice of drugs for effective antimicrobial therapy has vastly reduced (Kotloff *et al.*, 2018; Puzari *et al.*, 2018). As an alternative antimicrobial therapy, WHO has recommended three antibiotics, namely Pivmecillinam, Ceftriaxone, and Azithromycin (WHO, 2005). However, resistance to these WHO-recommended drugs has been observed as suggested by the findings of various studies.

In Nepal, *S. flexneri* is the predominant species, followed by *S. dysenteriae* (Wilson *et al.*, 2006; Khan *et al.*, 2013). Studies conducted in Nepal have found that most patients who are vulnerable to infection and develop infection are under the age of ten (Bhattacharya *et al.*, 2005; Khan *et al.*, 2013). Nepal has experienced *Shigella*

species resistance to multiple drugs over the past two decades. There are a few reasons for the emergence of antibiotic-resistant strains in Nepal, such as easy access to antibiotics in the pharmacies without a doctor's prescription, poor antibiotic policies, including poor laboratory diagnosis and antibiotic susceptibility tests (Dhital *et al.*, 2017). Therefore, this study aims to provide more insight into the prevalence and the antibiotic susceptibility pattern of *Shigella* species isolated in a tertiary care hospital in Kathmandu.

MATERIALS AND METHODS

This cross-sectional study was done in Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu, over five months from April to August 2014.

Patients and sample collection
A total of 653 stool samples were collected from the patients complaining of acute gastrointestinal disorder, i.e., patients with fever, fatigue, frequent defecation of painful stools containing blood and mucus defined by physicians as a probable case of bacillary dysentery. Therefore, the stool samples containing blood and mucus from patients with no antibiotics taken within three days were included as study samples, whereas those with firm consistency without blood and mucus were excluded from the study. One gram of stool samples was collected in clean screw-capped plastic containers. The samples were labeled with the patient's name, age, sex, and address. If there was a possible delay in the processing of the sample, it was stored in the refrigerator at 2-8 ºC. The stool samples were further processed in the microbiology laboratory following standard operating procedures (McCartney *et al.*, 1989).

Bacteriological analysis
A loopful of stool sample from each container was then inoculated in the Xylose lysine deoxycholate agar (XLD) and Mac-Conkey agar (MAC) plates and incubated at 37ºC for 24 hours for the isolation of *Shigella* species. Simultaneously, enrichment was done in Selenite-F broth and incubated at 37ºC for 24 hours. Subculture was then done in XLD agar and MAC and further incubated at 37ºC for 24 hours. The colony morphology resembling *Shigella* species was identified microscopically and biochemically. The non-lactose fermenting (NLF) colonies from both XLD and MAC were identified by the IMViC test, Urease test, Nitrate reduction test, and Triple sugar iron (TSI) test.

Serological identification
Differentiation of *Shigella* species was done by slide agglutination test using polyvalent antisera (Denka Seiken Co. Ltd. Japan). On the clean glass slide, milky suspension of the organism was made with saline. *Shigella* polyvalent antiserum was added starting from A to D polyvalent. The mixture was tilted to and fro for 30-60 seconds. While viewing under a good light against a dark background, distinct clumping within 60 seconds was a positive result. The suspension alone, on the other hand, as control was tested to exclude auto-agglutination. The appearance of agglutination (distinct clumping) by Group A polyvalent, Group B polyvalent, Group C polyvalent, and Group D polyvalent in kit were identified as *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* respectively.

Antibiotic susceptibility test
The antibiotic susceptibility test was done by the Kirby-Bauer disk diffusion method in Muller-Hinton Agar (MA) following 2014 CLSI guidelines (CLSI, 2014). The antibiotic susceptibility pattern of six antibiotics, namely Ampicillin (Amp, 10 μg), Cefotaxime (Ctx, 30 μg), Cotrimoxazole (TMP-SMX, 25 μg), Ciprofloxacin (Cip, 5 μg), Ofloxacin (Ofx, 15 μg), and Nalidixic acid (Nal, 30 μg) (Hi Media Laboratory Ltd. Mumbai, India) was assessed. The zone of inhibition of each antibiotic was measured, and they were interpreted as sensitive, intermediate sensitive, and resistant (CLSI, 2014). For the quality control purpose, strict aseptic conditions and techniques were followed according to the National Committee for Clinical Laboratory Standard (NCCLS) recommendation. Positive and negative control was always used during each testing with the standard organism. For the antibiotic susceptibility test, standard organism ATCC 25922 *Escherichia coli* was used for the quality control.

Statistical analysis
Data from the laboratory findings was analyzed using Microsoft Excel 365. The t-test was performed to compare the statistical significance. The relationship with a p-value greater than 0.05 was indicated as insignificant.

Ethics clearance
The written informed consent was taken prior to the study from the patients involved in the study. Our study was accepted by the Institutional Review Committee (IRC) at Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu.

RESULTS
Out of a total of 653 shigellosis suspected cases, 25(3.82%) were confirmed as positive cases (Fig. 1, Table 1). Among 25 positive shigellosis cases, *S. flexneri*, *S. dysenteriae*, and *S. sonnei* were found to be 18(72%), 6(24%), and 1(4%), respectively (Fig. 1, Table 2). Shigellosis positive female patients were found to be comparatively higher than male patients (female-14(56%), male- 11(44%)) (Table 1). The highest number of *Shigella* species were recovered from the age group 16-45 years 16(64%) followed by age group >45 years 7(28%), whereas 2(8%) of the *Shigella* species was isolated from the age group <16 years (Table 2). However, there was no significant association between age and the number of species.

The antibiotic susceptibility pattern of *Shigella* species suggests that Cefotaxime, the third-generation cephalosporin, was found to be the most effective (100% sensitive) against all the isolated *Shigella* species (Table 3). Among the tested antibiotics, Nalidixic acid was the least effective as 20(80%) of *Shigella* species were resistant to it (Table 3). The second least effective antibiotic was

Ampicillin, as 18(72%) of *Shigella* species were resistant to it (Table 3). This was followed by Cotrimoxazole 13(52%) and Ciprofloxacin 9(36%), respectively. Similarly, out of a total of 25 isolates, 10 isolates were found to be MDR, representing 40% of the total isolates (Fig. 2).
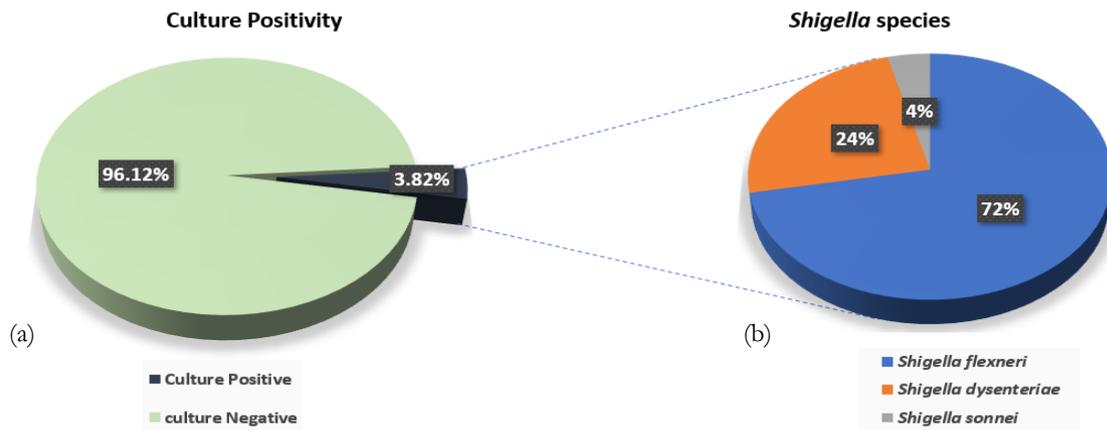

Figure 1. (a) The pie-chart represents the fraction of culture-positive isolates, (b) the pie-chart represents the proportion of *Shigella* species within the culture-positive isolates.

Table 1. Gender wise distribution of *Shigella* positive and negative patients

Sex	Culture		Total
	Positive (%)	Negative (%)	
Male	11(44)	287(45.7)	298(45.6)
Female	14(56)	341(54.3)	355(54.4)
Total	25(3.82)	628(96.12)	653(100)

*The figures in the parentheses indicate the percentage

Table 2. Number of *Shigella* species isolated in different age groups

Age groups (years)	*Shigella* species			Total (%)
	S. flexneri	*S. dysenteriae*	*S. sonnei*	
<16	2	0	0	2 (8)
16-45	10	5	1	16 (64)
>45	6	1	0	7 (28)
Total (%)	18 (72)	6 (24)	1 (4)	25 (100)

*The figures in the parentheses indicate the percentage

Table 3. The antibiotic susceptibility pattern of *S. flexneri*, *S. dysenteriae*, and *S. sonnei*

Antibiotics	*S. flexneri* (n=18)			*S. dysenteriae* (n=6)			*S. sonnei* (n=1)			Resistance total (%)
	S	I	R	S	I	R	S	I	R	
Amp	1(5.6)	3(16.6)	14(77.8)	1(16.7)	2(33.3)	3(50)	0(0)	0(0)	1(100)	18(72)
TMP/SMX	4(22.2)	4(22.2)	10(55.6)	3(50)	0(0)	3(50)	1(100)	0(0)	0(0)	13(52)
Cip	4(22.2)	6(33.3)	8(44.5)	5(83.3)	1(16.7)	0(0)	0(0)	0(0)	1(100)	9(36)
Ofx	6(33.3)	7(38.9)	5(27.8)	4(66.7)	2(33.3)	0(0)	0(0)	0(0)	1(100)	6(24)
Nal	1(5.5)	2(11.1)	15(83.4)	1(16.6)	1(16.6)	4(66.8)	0(0)	0(0)	1(100)	20(80)
Ctx	18(100)	0(0)	0(0)	6(100)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)

*The figures in the parentheses indicate the percentage; S-Sensitive, I-Intermediate Sensitive, R-Resistant
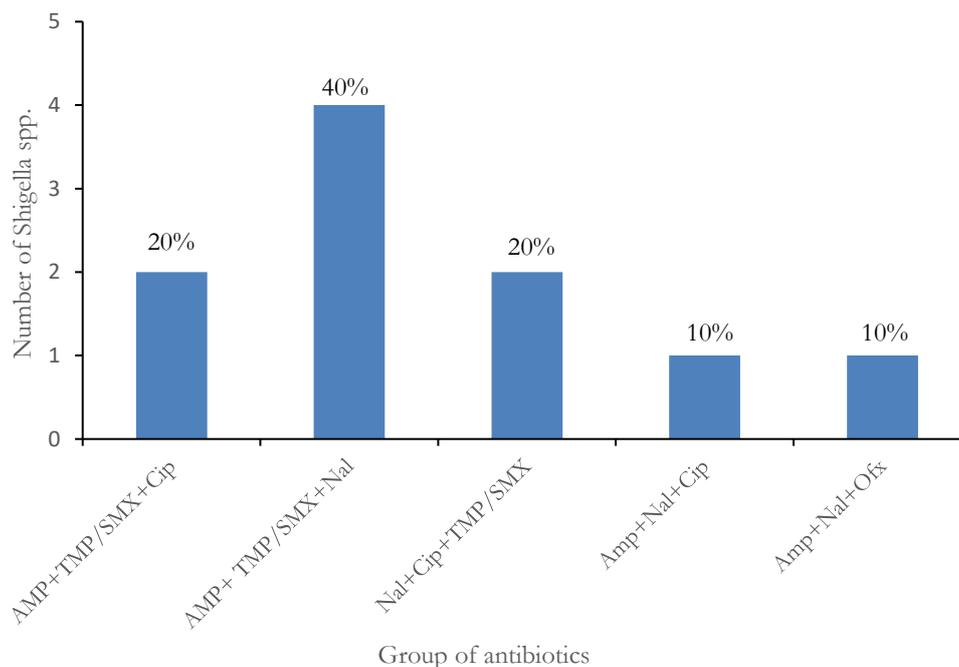
Figure 2. Bar-graph showing multi-drug resistance (MDR) pattern of *Shigella* species. (Amp-Ampicillin, TMP/SMX-Cotrimoxazole, Cip- Ciprofloxacin, Nal- Nalidixic acid, Ofx-Ofloxacin).

DISCUSSION

This study was conducted from April to August 2014 among the patients suspected of acute gastroenteritis visiting Sukraraj Tropical and Infectious Disease Hospital, Teku. Out of 653 studied cases, 25(3.82%) cases were *Shigella* positive. This isolation rate is comparable to a study done in Tribhuvan University Teaching Hospital and Kanti Hospital (2.1% culture positive) (Dhital *et al.*, 2017). In contrast to our findings, two different studies conducted in Nepal reported a relatively higher prevalence rate (13.61% and 10.8%) (Wilson *et al.*, 2006; Khan *et al.*, 2014). The prevalence of food and water-borne infections like Shigellosis increases in the rainy season as the infection rate fluctuates according to the seasons. Our study shows that the patients in the age group 16-45 years were more susceptible to *Shigella* infection (16(64%)). However, there was no significant difference in the infection rate among all the age groups. In contrast to our study, the infection rate was higher in the age group <10 years in most studies (Kansakar *et al.*, 2006; Wilson *et al.*, 2006; Khan *et al.*, 2014; Mengistu *et al.*, 2014). This discrepancy is because the children usually visit the children's hospitals in Kathmandu rather than the tertiary care hospital where this study was carried out. This is also reflected in our study, as participants below the age of 10 were lower than other age groups.

S. flexneri [18(72%)] was found to be the predominant *Shigella* species in our study, followed by *S. dysenteriae* [6(28%)]. This finding is congruent to hospital-based studies in Nepal (Wilson *et al.*, 2006; Khan *et al.*, 2014), whereas incongruent to a study done in eastern Nepal where the predominant isolate was *S. dysenteriae* (73.6%) followed by *S. flexneri* (23%) and *S. boydii* (4%) (Bhattacharya *et al.*, 2005). In the studies in Nepal from 1995 to 2002, the prevalence of *S. dysenteriae* was higher than the *S. flexneri*, while in the studies done from 2003 to 2005, *S. flexneri* was found to be the predominant species (Kansakar *et al.*, 2006). The recent studies conducted in India (Puzari *et al.*, 2018), Nepal (Maharjan *et al.*, 2017; Thapa *et al.*, 2017), Iran (Nikfar *et al.*, 2017), China (Chang *et al.*, 2016), and Latin America (Sati *et al.*, 2019) show that *S. flexneri* is the predominant species. On the other hand, nations such as England (Bardsley *et al.*, 2020), Australia (O'Sullivan *et al.*, 2002; Ingle *et al.*, 2020), and the United States (Kozyreva *et al.*, 2016) showed a higher predominance of *S. sonnei*. These findings support the claim that *S. flexneri* infection is associated with developing and underdeveloped countries, and *S. sonnei* infection is associated with developed countries (Kotloff *et al.*, 2018; Bardsley *et al.*, 2020). However, a rapid rise in the *S. sonnei* cases has been observed in developing countries in recent years (Anderson *et al.*, 2016). This could be possibly associated with the climatic and environmental changes and the innate changing nature of *Shigella* serogroups and serotypes from time to time.

All the antibiotics used in our study were the routine antibiotics used in the diagnostic microbiology laboratory in Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu. Our study showed the increased resistance to Nalidixic acid (80%), Ampicillin (72%), Cotrimoxazole (52%), and Ciprofloxacin (36%) corresponding to various studies conducted in different parts of the world (Srinivasa *et al.*, 2009; Bhattacharya *et al.*, 2012; Gharibi *et al.*, 2012). Although further confirmation is needed, our study suggests that Nalidixic acid, Cotrimoxazole, Ampicillin, and Ciprofloxacin can be used no longer for the empirical treatment of shigellosis. Similarly, Nalidixic acid is a drug of choice in developing countries, and Ciprofloxacin in developed countries (Gharibi *et al.*, 2012). The increased resistance (80% resistance) of Nalidixic acid was observed in the present study and other studies conducted in Nepal (Bhattacharya *et al.*, 2005; Khan *et al.*, 2014; Dhital *et al.*, 2017). This increased resistance to Nalidixic acid in Nepal can be attributed to the over-the-counter availability of drugs, self-prescription by patients, and poor antibiotic policies. In the early 1990s, *Shigella* isolates were susceptible to most prescribed antibiotics such as Gentamicin, Norfloxacin, and Nalidixic acid (Jesudason, 2002; Dutta *et al.*, 2003; Rahbar *et al.*, 2007). However, during the late 1990s, *Shigella* isolates were resistant to most of these antibiotics but susceptible to Ciprofloxacin (Sack *et al.*, 1997). A study conducted in Bangladesh has shown the rising trend of Ciprofloxacin resistance, i.e., from 0% in 2004 to 48% in 2010 (Azmi *et al.*, 2014). A similar study of the *Shigella* isolates between 2010 and 2015 revealed South-Asia as a reservoir for the global spread of Ciprofloxacin resistant *S. sonnei* (Chung The *et al.*, 2016). Similarly, a study conducted from the *Shigella* isolates over the six years from 2011 to 2016 in Iran also suggested the higher resistance (76%) to Ciprofloxacin (Mamishi *et al.*, 2019). These statistics show that the Ciprofloxacin-resistant *Shigella* species has increased considerably over time. Although WHO has recommended Ciprofloxacin as a drug of choice to treat shigellosis (WHO, 2005), our result suggests that Ciprofloxacin cannot be trusted blindly as the effective drug against shigellosis.

According to our findings, cefotaxime, a third-generation cephalosporin, was the most sensitive (100% of *Shigella* isolates were susceptible to it). This result is congruent to the studies performed by Dhital *et al.* (2017), where 93% of *Shigella* isolates were susceptible to Ceftriaxone, and by Kansakar *et al.* (2006), where 100% of *Shigella* isolates were susceptible. In similar settings, Maharjan *et al.* (2017) found 97% of *Shigella* species, and Thapa *et al.* (2017) found 71.4% of *Shigella* species susceptible to Ceftriaxone. Several studies across the world have also shown the increased resistance of *Shigella* species to third-generation cephalosporins (Radice *et al.*, 2001; Vinh *et al.*, 2009; Mandal *et al.*, 2010). More recent research also consistently supports the fact that third-

generation cephalosporins cannot be relied on treating Ciprofloxacin resistant *Shigella* species due to surge in third-generation cephalosporin-resistant *Shigella* species (Hosseini Nave *et al.*, 2016; Nüesch-Inderbinen *et al.*, 2016; Madhavan *et al.* 2018; Abbasi *et al.*, 2019; Bian *et al.*, 2019). Therefore, although our study has shown the high susceptibility of *Shigella* species to a third-generation cephalosporin (Cefotaxime), we still suggest using third-generation cephalosporin with caution. Our study was conducted seven years ago, and recent studies across the world have shown increased resistance of *Shigella* species to third-generation cephalosporins. So, for further studies, we recommend carrying out the study in similar settings with few other third-generation cephalosporins included before concluding with certainty that third-generation cephalosporins are the effective options for treating shigellosis.

Forty percent (40%) of the Shigella-positive isolates were MDR. This figure corresponds to a study conducted in the hospital settings in Pokhara (74.7%) and Kathmandu (86%) (Wilson *et al.*, 2006; Dhital *et al.*, 2017). MDR isolates in our study were resistant to Nalidixic acid, Ciprofloxacin, Cotrimoxazole, and Ampicillin, and Ofloxacin. The emergence of MDR *Shigella* isolates is of great concern in South Asian countries, including Nepal (Rahman *et al.*, 2004). The high resistance level in *Shigella* can be attributed to the transfer of resistant determinants such as plasmids, integrons and transposons (Barrantes & Achí, 2016). Due to the rise in resistance to third-generation cephalosporins (Abbasi *et al.*, 2019), a recent study has suggested using Azithromycin and Piperacillin tazobactam as an alternative (Madhavan *et al.*, 2018). However, resistance to Azithromycin has also been reported (Nüesch-Inderbinen *et al.*, 2016). Therefore, we can see that the MDR *Shigella* species is increasing continuously. Continuous monitoring of the MDR strains and an effective antibiotic prescription policy in Nepal could help mitigate increased drug resistance seen in *Shigella* species.

CONCLUSIONS
Our study found the predominant *Shigella* serovar to be *Shigella flexneri*. *Shigella* species were highly resistant to commonly prescribed antibiotics in Nepal, such as Nalidixic acid, Ampicillin, Cotrimoxazole, and Ciprofloxacin, whereas susceptible to a third-generation cephalosporin (Cefotaxime). We found a higher prevalence of MDR *Shigella*, and we suggest using third-generation cephalosporins to treat MDR *Shigella* infection.

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AUTHOR CONTRIBUTIONS
BB contributed to the study design, laboratory work, and manuscript writing. DN performed data analysis and manuscript writing. JA and SB contributed to the conceptualization and study design.

CONFLICT OF INTEREST
The authors declare no competing interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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