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ORIGINAL ARTICLE

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Cryptosporidiosis is predominantly an urban, anthroponotic infectious disease among Zambian children

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Background: *Cryptosporidium* species are leading causes of diarrhoea in children and immunocompromised individuals. This study aimed to characterise *Cryptosporidium* species from children in rural and urban settings of Zambia.

Methods: Stool samples collected from 490 children aged <5 y with diarrhoea were assessed for *Cryptosporidium* oocysts microscopically. A structured questionnaire was used to collect demographic and socioeconomic characteristics. Positive samples were subjected to PCR and *gp60* sequence analysis.

Results: The overall prevalence was 10% (50/490, 95% CI 7.8 to 13.2) with a peak in March, the late rainy season. Children who came from households where boiling water was not practised (OR=2.5, 95% CI 1.29 to 5.17; p=0.007) or who had experienced recurrent episodes of diarrhoea (OR=9.31, 95% CI 3.02 to 28.73; p=0.001) were more likely to have *Cryptosporidium* infection. Genotyping of 16 positive samples (14 from urban and 2 from rural sources) revealed *Cryptosporidium hominis* (14/16) and *Cryptosporidium parvum* (2/16). The *Cryptosporidium hominis* subtypes identified were Ia, Ib and Ie with subtype families IeAIIG3 (1), IbA9G3R2 (2), IaA31R3 (3), IbA9G3 (5), IaA27R3 (1), IaA30R3 (1) and Ia (1). Subtypes IbA9G3 and Ia were identified in children from a rural area. *Cryptosporidium parvum* subtypes were IIcA5G3R2 (1) and IIcA5G3a (1).

Conclusions: All isolates successfully genotyped were *C. hominis* or anthroponotic *C. parvum*, suggesting that anthroponotic transmission dominates in Lusaka and the surrounding countryside.

Keywords: children, *Cryptosporidium*, *gp60* gene, risk factors, subtypes, Zambia

Introduction

Since the first description of *Cryptosporidium muris* in 1907 by Tyzzer,[1] the *Cryptosporidium* genus (now including over 45 species) has emerged as a major human pathogen,[2] gaining more attention as an opportunistic infection in HIV-infected individuals.[3] Multiple studies now attest to the importance of *Cryptosporidium parvum* and *Cryptosporidium hominis* as causes of childhood diarrhoea across the world.[4] More recently, their importance as contributors to childhood malnutrition, even when there is no diarrhoea, has been established. Efforts at developing new therapies have so far not borne fruit and the only licenced treatment to date, nitazoxanide, is ineffective in individuals with HIV infection.[5]

Transmission of *Cryptosporidium* occurs through the faecal-oral route, either indirectly by accidental ingestion of contaminated water or food, or by direct contact with infected individuals, animals or fomites.[6,7] In immunocompetent individuals, cryptosporidiosis typically results in transient (up to 2–3 wk) self-limiting illness characterised by watery diarrhoea, abdominal pain and, less frequently, nausea, vomiting, fever and weight

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loss.[8–12] *Cryptosporidium parvum* and *C. hominis* are responsible for 90% of human infections, especially in resource-poor settings.[13] *Cryptosporidium parvum* consists of IIa-IIt subtypes, of which IIb, IIc and IIe have been detected in humans and their transmission appears to be anthroponotic, but IIa and IId are transmitted through zoonotic routes. *Cryptosporidium hominis* consists of subtypes Ia-In.[13,14]

Previous work in Zambia using modified Ziehl-Neelsen microscopy has shown a prevalence of 18–26%, associated with risk factors such as inadequate sanitation and hygiene,[15] as well as malnutrition.[16] A more recent study in Zambia identified *C. hominis* (59%), *C. parvum* (38%), *Cryptosporidium felis* (1%) and *Cryptosporidium meleagridis* (1%) species using PCR.[17] There is also evidence that zoonotic transmission (*C. parvum*) occurs in and around cattle farms.[18] Here, we report a direct comparison of the prevalence of cryptosporidiosis in young children with diarrhoea in rural and urban areas, alongside the genotype of isolates.

Methods

Study sites

The study was conducted at four locations during August 2018–March 2019. All children with diarrhoea (defined as ≥3 loose stools in the preceding 24 h) presenting to the study sites over this period were eligible for inclusion. The urban recruitment site was the University Teaching Hospital (UTH) in Lusaka, the capital city of Zambia. Chongwe District Hospital (CDH), Ngwerere Rural Health Centre (RHC) and Luimba Rural Health Centre (RHC) were chosen to represent rural areas. UTH caters for a catchment population of approximately 2 million people. It also receives tertiary referral cases from other parts of the country. UTH Children's Hospital receives paediatric emergencies, including diarrhoea cases, from all socioeconomic groups, including residents of the peri-urban densely populated areas that have poor sanitation, erratic and inadequate water supply and poorly constructed houses. Diarrhoeal diseases are very common. People who seek medical attention at UTH have different occupational backgrounds, including civil servants and professional groups. Municipal piped water is filtered and chlorinated and pumped from the Kafue river and large urban boreholes, but only residents of low-density housing have indoor taps and high-density residents have to use communal stand pipes and kiosks. CDH serves the main settlement of Chongwe town, to the east of Lusaka, which has a population of approximately 50000 inhabitants. Chongwe District also covers the areas of Ngwerere, Chalimbana and Luimba. Agriculture is the main economic activity in these rural areas with crop production, horticultural production and livestock production. Rural settlements rely for drinking water mainly on rivers, boreholes and shallow wells.

Sociodemographic data and specimen collection

A structured questionnaire was used to collect sociodemographic characteristics such as age, gender, type of residence density (low, medium and high density), maternal and paternal education level attained (primary, secondary or tertiary), keeping of animals and water treatment, period of sample collection (month of the year) and type of area (urban, rural). Rural areas were defined as areas dominated by the natural environment and agricultural activity, whilst an urban area was defined as an area of higher population density and dense social and economic organisation in a built-up environment. The questionnaire was pretested before it was administered to respondents and entries were checked by a single author (BB). A single stool specimen was obtained from each participant in a sterile leak-proof, disinfectant-free container and transported within 3 h of collection to the UTH parasitology laboratory.

Formol ether concentration technique

The formol ether concentration technique was performed on stool samples.[19] The supernatant was drained and a smear was prepared from the sediment. The smear was stained with the modified Ziehl-Neelsen stain, counterstained with malachite green and microscopically examined to detect oocysts.[20,21] *Cryptosporidium* positive samples were stored at −20°C for an average of 7 mo until DNA extraction could be performed for molecular characterisation.

DNA extraction

DNA was extracted using the ZR faecal DNA mini kit (Zymo Research, CA, USA), according to the manufacturer's instructions and stored again at −20°C until molecular analysis.

Genotyping

Samples that were positive on modified Ziehl-Neelsen stain were genotyped by amplification of the *gp60* gene using a nested PCR. The PCR was conducted in a 10-μl reaction volume using 0.4 μL of each primer, 1.5 μl of template, 0.8 μl deoxynucleoside triphosphate, 10X PCR buffer, 0.1 μl of Ex-Tag DNA polymerase and 5.8 μl of nuclease-free water. In the primary PCR reaction, the following primers were used: AL3531: 5′-ATAGTCTCCGCTGTATTC -3′ (forward) and AL3535: 5′-GGAAGGAACGATGTATCT-3′ (reverse). In the secondary reaction, 1 μl of the primary PCR products was amplified using the following primers: AL3532: 5′-TCCGCTGTATTCTCAGCC-3′ (forward) and AL3534: 5′-GCAGAACCAGCATC-3′ (reverse), as described by Alves et al.[22] The thermal cycling conditions for the first and the second reactions were as follows: denaturation at 98°C for 5 min followed by 35 cycles of 98°C for 40 s (denaturation), 58°C for 1 min (annealing) and 72°C for 1 min (extension), then a final extension at 72°C for 5 min. Electrophoresis of the PCR products was performed on 1% LE agarose gel containing ethidium bromide. A 100-bp ladder was used as a marker and all gels were visualised using Bench top 300 Transilluminator (BioDoc Imaging System, CA, USA). The expected products were 850 bp.

DNA sequencing

The PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The products were then analysed on a 3500 Genetic Analyser using the Bigdye

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Table 1. Sample collection, August 2018–March 2019

Month	Urban	Crypto positive	Rural	Crypto positive
August	24	3	27	0
September	29	0	27	0
October	41	0	35	1
November	38	3	29	1
December	36	1	17	0
January	50	6	13	0
February	47	12	12	0
March	58	23	7	0
Total	323	48	167	2

terminator sequencer kit (Applied Biosystems, CA, USA). The sequences were compared with the published sequences available in GenBank using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and alignment was performed using ATGC software (GentXY version 12.0). Phylogenetic analysis was performed in MEGAX.[23] The neighbour-joining method with the Kimura two-parameter evolutionary model was used to determine the phylogeny.[24–26] All positions containing gaps and missing data were eliminated. Sequences with short sequences were removed and were not included in the phylogenetic analysis. The sequences have been uploaded to GenBank (accession numbers MZ351216-MZ351230).

Data analysis

Data were analysed using STATA version 15.1 (StataCorp, College Station, TX, USA). χ^2 test or Fisher's exact test were used to understand the association between independent variables with *Cryptosporidium* infection as appropriate. Multiple logistic regression was used to determine the predictors of *Cryptosporidium* species infection. For all analysis, $p<0.05$ was considered statistically significant.

Results

The study was conducted at four locations during August 2018–March 2019. Of the 490 collected samples, the total number of positive samples was 50 (10%, 95% CI 8 to 13%).

Child characteristics

Stool samples were collected from 323 children aged <5 y at UTH; 100 samples were collected at Ngwerere RHC, 33 from CDH and 34 from Luimba RHC representing rural areas (Table 1). In total, there were more males than females (188 vs 135 for urban, 89 vs 78 for rural). Children from urban areas were younger, more likely to have reported previous episodes of diarrhoea, more likely to come from high density housing and less likely to come from households that treat their drinking water (Table 2). Their parents were more likely to be in formal sector employment and had higher educational attainment. Although not directly asked about in the questionnaire, child care centres are extremely unusual in Zambia, even for parents who are in formal employment, so this is unlikely to have contributed significantly to transmission.

Table 2. Demographic characteristics of children with diarrhoea in Lusaka and rural areas

Variable	Urban	Rural	p
N	323	167	
Age (mo)	16 (11–24)	24 (14–38)	<0.001
Gender			
Male	188 (58)	89 (53)	0.299
Female	135 (42)	78 (47)	
Residence (density)			
Low	67 (21)	6 (3)	<0.001
Medium	90 (28)	140 (84)	
High	166 (51)	21 (13)	
Father's education			
Primary	27 (8)	40 (24)	<0.001
Secondary	160 (50)	105 (63)	
Tertiary	136 (42)	22 (13)	
Mother's education			
Primary	74 (23)	73 (44)	<0.001
Secondary	153 (47)	82 (49)	
Tertiary	96 (30)	11 (7)	
Father employed			
No	109 (34)	83 (50)	<0.001
Yes	214 (66)	84 (50)	
Mother employed			
No	221 (68)	160 (96)	<0.001
Yes	102 (32)	7 (4)	
Treatment of water			
Not boiling	220 (68)	76 (46)	<0.001
Boiling	103 (32)	90 (54)	
Keep animals			
No	256 (80)	103 (62)	<0.001
Yes	66 (20)	64 (38)	
Previous episodes of diarrhoea			
No	127 (39)	113 (68)	<0.001[C]
Yes	196 (61)	54 (32)	

Risk factors for cryptosporidiosis

In a multivariate analysis, residence (high or low density), water treatment and preceding episodes of diarrhoea were the only significant predictors of *Cryptosporidium* infection. Children from high-density areas within urban Lusaka had increased odds of *Cryptosporidium* infection (Table 3). Not boiling water also increased the odds of *Cryptosporidium* infection and children who had a preceding diarrhoea episode were more likely to be infected (Table 3).

Species and genotype of isolates

Of the total 50 positive samples, high quality amplification of the *gp60* gene was obtained from 16 samples (2 rural, 14 urban).

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Table 3. Univariate and multivariate analysis of risk factors for *Cryptosporidium* infection

Characteristic	COR* (95% CI)	p	AOR# (95% CI	p
Age	0.97 (0.95 to 0.99)	0.03	1.007 (0.97 to 1.04)	0.659
Site				
Urban	Ref			
Rural	0.06 (0.01 to 0.28)	<0.001	0.37 (0.11 to 0.26)	0.114
Residence				
Low	Ref			
Medium	1.11 (0.22 to 5.48)	0.894	1.96 (0.41 to 10.06)	0.419
High	9.96 (2.34 to 42.38)	0.002	6.66 (1.29 to 34.22)	0.023
Water treatment				
Not boiling	4.39 (1.98 to 7.9)	0.009	2.5 (1.29 to 5.17)	0.007
Boiling	Ref			
Keeping animals				
No	0.42 (0.18 to 0.95)	0.38	1.01 (0.40 to 2.52)	0.972
Yes	Ref			
Diarrhoea complaint				
No	Ref			
Yes	18.3 (5.6 to 59.64)	<0.001	9.31 (3.02 to 28.73)	0.001

Abbreviation: Ref, reference category.
*COR, crude OR.
#AOR, adjusted OR.

Table 4. Species and genotype of *Cryptosporidium* isolates

Species	Subtype families	No. of subtypes	Subtype distribution
C. hominis	Ia	Ia	1
		IaA27R3	1
		IaA30R3	1
		IaA31R3	3
	Ib	IbA9G3R2	2
		IbA9G3	5
	Ie	IeA11G3	1
C. parvum	IIc	IIcA5G3a	1
		IIcA5G3R2	1

Both rural isolates and 12 of the urban isolates were *C. hominis*; only 2 (urban) isolates were *C. parvum* and these were both the anthroponotic genotype IIc (Table 4). Phylogenetic analysis (Figure 1) revealed that these isolates from Zambian children were genetically diverse and do not constitute a geographically isolated clade.

Discussion

This study reports a *Cryptosporidium* prevalence of 10% (95% CI 8 to 13%) in children aged <5 y. Previous Zambian studies have reported relatively higher prevalence,[10,15] consistent with studies in Egypt and Côte d'Ivoire,[27,28] in which a higher prevalence rate was also reported in children with diarrhoea. However, in another Egyptian study, low prevalence (1%) was reported in the tested children.[29] It is evident that, worldwide, *Cryptosporidium* infection in children varies considerably, due to many factors such as seasonal variations, sanitation hygiene and health education.

The children enrolled in this study were largely resident in high-density housing, which in univariate analysis was significantly associated with infection. High-density residential areas in developing countries are characterised by poor sanitation, rationed water supply and increased human-to-human interaction, which contribute to increased risk of infection.[30] Open defaecation by children also occurs. The highest number of cases in this study (only urban samples) was obtained in March, which corresponds to the late rainy season in Zambia. A previous study on seasonal influence on *Cryptosporidium* infection[10] reported similar findings with a high prevalence at the end of the rainy season. Increased incidence of cryptosporidiosis following rains was also reported by studies conducted in Botswana[31] and Tanzania.[32] Of note is that in the current study, there were very few samples collected from rural sites during this period and no cases were detected. As farming is the main activity in rural settings, it is possible that taking children to a health facility was regarded as lower priority than attending to crops needing attention, which might explain the low number of reported cases.

Cryptosporidium infection was associated with failure to boil drinking water. *Cryptosporidium* infection has been correlated with the type of water used for consumption per household.[33,34] Many studies have emphasised that *Cryptosporidium* species

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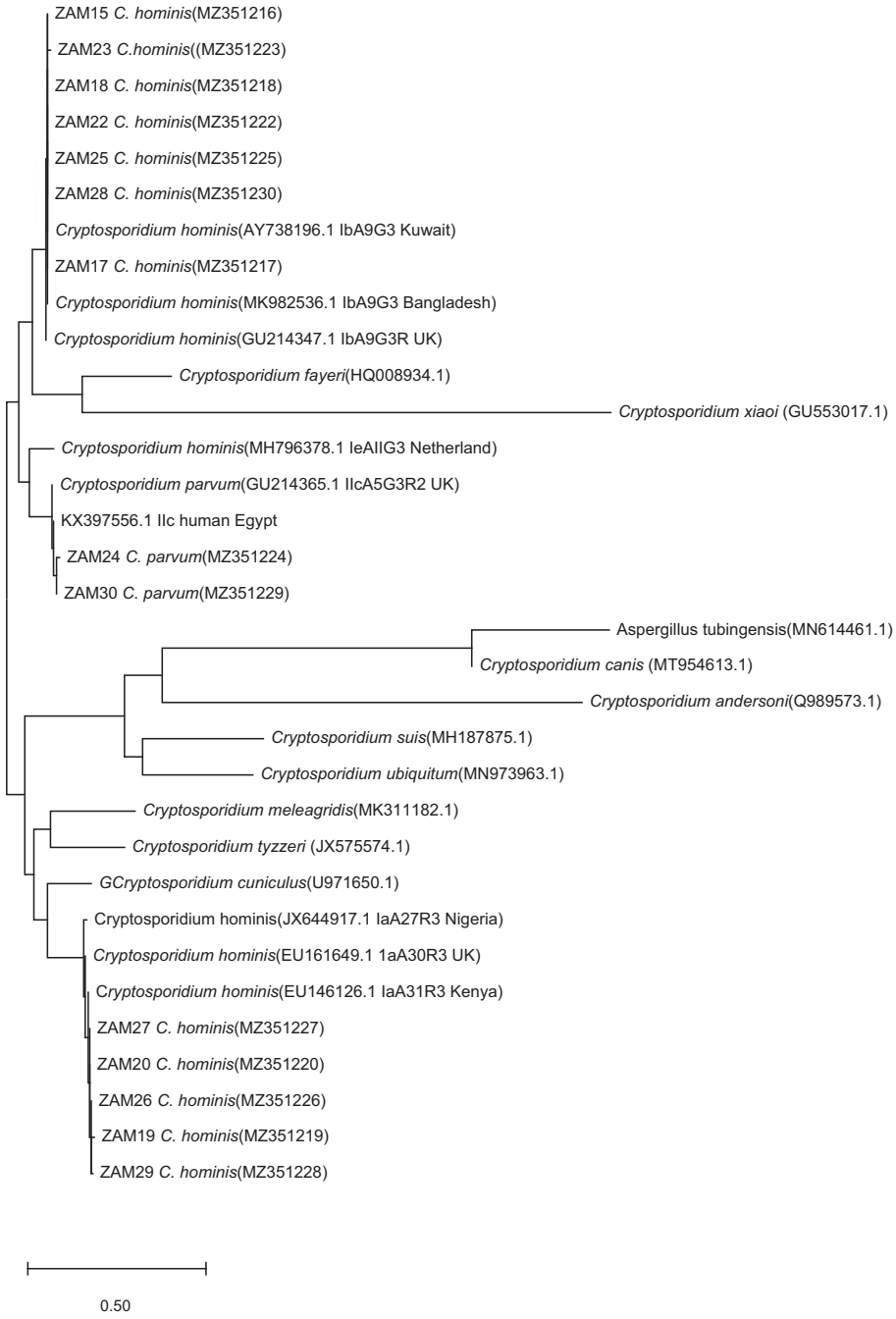

Figure 1. The evolutionary history was inferred using the neighbour-joining method.[26] The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method[24,25] and are in the units of the number of base substitutions per site. This analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1787 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.[23]

are mostly found in untreated water.[35] However, other studies have reported that children who were drinking water directly from the tap were at risk of having cryptosporidiosis, which may reflect the fact that chlorination of municipal drinking water does not inactivate oocysts.[36–38] Khalifa et al.[39] showed that children who were coming from households where water was not treated were more likely to have *Cryptosporidium* infection.

Diarrhoea is the commonest symptom in *Cryptosporidium*-infected patients, and infection in children is associated with

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acute and persistent diarrhoea.[40,41] Persistent diarrhoea is a worrying feature in children with *Cryptosporidium* infection[42–44] as it is often associated with mortality.[16]

Molecular characterisation of the isolates identified *C. hominis* as the predominant species (88%) and *C. parvum* was only identified in two (13%) samples. Similar findings have been reported by Mulunda et al.,[17] El-Badry et al.[45] and Ghallab et al.[46] in diarrhoeic children. Of the six *C. hominis* subtype families described to date, this study identified three common subtypes, Ia, Ib and Ie. These subtype families have been reported in children in a number of developing countries.[47,48] Within the subtype family, subtype Ib has been especially associated with abdominal pain, vomiting and diarrhoea.[49–51] The present study identified nine different subtypes (Table 4) with subtype IbA9G3 being the most frequent. In a recent Zambian study conducted by Mulunda et al.,[17] *C. hominis* accounted for the majority of cases, with IeA113T3 being predominant. However, in that study the urban/rural source of the isolates was not ascertained. The zoonotically transmitted *C. parvum* subtype families IIa, IId, IIe, IIl were not identified in this study, and only subtype family IIc was detected from two children. The subtype family IIc has previously been detected in human samples and its anthroponotic transmission has also been confirmed in the USA, Canada, Europe and Australia.[13] This subtype family was also found to be dominant in the samples from Nigerian children.[52]

This study has important limitations. First, because only one stool sample was submitted for investigation by each participant, this could have led to underestimating the prevalence of *Cryptosporidium*. Second, initial detection using microscopy, known to be insensitive,[20] would underestimate the overall prevalence among diarrhoea cases. If case definition had included PCR detection, the number of cases would presumably have been higher. Third, incidence in rural areas would be underestimated if farming activities at certain times of year were deemed to have a higher priority than healthcare and families did not bring their children for treatment. Fourth, we only used the *gp60* gene for genotyping *Cryptosporidium* species, which is only useful for *C. hominis* and *C. parvum*; other species require different protocols. For example, new primers have been published for amplification of *C. meleagridis,*[53] *Cryptosporidium ubiquitum*,[54] *C. felis*,[55] *Cryptosporidium viatorum*[56] and *Cryptosporidium ryanae*.[57] Although it is likely that anthroponotic transmission is the primary route of infection, as these other species are generally much less common in Zambia, other possible zoonotic transmission events may have occurred and other species may have contributed to the parasites identified by microscopy.

In conclusion, this study reports a cryptosporidiosis prevalence of 10% in children with diarrhoea, with a peak in March that corresponds to the late rainy season in Zambia. Children who came from households where drinking water is not boiled, or who had reported recurrence of previous diarrhea, were more likely to have *Cryptosporidium* infection. Sequence analysis of the *gp60* gene from 16 positive samples (14 from urban and 2 from rural sources) revealed *C. hominis* (14/16) and *C. parvum* (2/16) and suggests that anthroponotic transmission is dominant. Other species could have been detected by other genotyping protocols now available.

Authors' contributions: BB, JS and PKe designed the study protocol. BB carried out sample collection and analysis. BB, JS, Pke, SC, ANM, Pka, KC and ES analysed and interpreted the data. JS, NS and PKe analysed and interpreted molecular data. PK drafted the manuscript and all the authors participated in preparing subsequent drafts, read and approved the final manuscript. BB is the guarantor of the paper.

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Ethical approval: Approval to carry out the study was obtained from University of Zambia Biomedical Research Ethics Committee (UNZABREC) (1 February 2018, ref. 002–11–17). Written consent was obtained from all parents/caregivers.

Data availability: The data underlying this article are available in the article.

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