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REGULAR ARTICLES

Serological, molecular detection and potential risk factors associated with camel brucellosis in Pakistan

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Abstract Brucellosis is one of the most important zoonoses in developing countries and was considered the most widespread zoonosis in the world. Brucellosis was reported in camels and has been reported from all camel-keeping countries.

The present study was performed in three districts (Jhang, Chiniot, and Bhakkar) of Punjab province of Pakistan. A total of 200 camel (*Camelus bactrianus*) sera were collected using random and multistage cluster sampling from different areas. Fifty samples were collected from one organized governmental farm. One hundred fifty samples were collected randomly from nomadic/pastoral production systems. All sera were tested with Rose Bengal plate agglutination test (RBPT) and confirmed by ELISA. Genomic DNA was extracted from all serum samples and tested by real-time PCR. Various potential risk factors (season, rearing with other animals, and abortion or orchitis history) recorded through questionnaires were statistically analyzed by Chi-square test.

In total, 5 % of investigated sera were positive by RBPT. Only 2 % of the camel sera were CELISA positive. *Brucella abortus* DNA was detected in 1.5 % of the investigated animals. Season, rearing of camels with other ruminants, abortion, and orchitis history were found to be statistically significant ($p < 0.05$) disease for determinants.

Camel brucellosis is a zoonotic disease in the Pakistani Punjab with various risk factors maintaining and perpetuating its spread. Therefore, there is a need for implementing control measures and raising public health awareness in prevention of brucellosis in Pakistan.

Keywords Camel · Brucellosis · RBPT · ELISA · PCR · Prevalence · Risk factors · Pakistan

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Introduction

Brucellosis is a disease of livestock including cattle, goats, sheep, camels, and pigs and has also been reported in wildlife. It is caused by bacteria of the genus *Brucella* (B) including the species *Brucella abortus* (cattle), *Brucella melitensis* (small ruminants), and *Brucella suis* (pigs). In livestock, it basically causes reproductive illness characterized by late abortion, retained fetal membranes, orchitis, and impaired fertility (Gumi et al. 2013). *B. abortus* is transmitted via contact with the placenta, fetus and fetal and vaginal fluids from infected animals. Animals become infectious after abortion or full-term parturition. *B. abortus* may also be found in milk, semen, feces, and hygroma fluids. In milk it is shed for a prolonged time. Few infected cattle become chronic carriers. *B. abortus* can be transmitted by ingestion, via the intact mucous membrane and skin abrasions.

Camel brucellosis was first recognized in 1931 (Abbas and Agab 2002). Since then, it has been reported from all camel-

keeping countries including Egypt, Libya, Sudan, Ethiopia, Nigeria, Somalia, Kenya, and Jordan.

Areas or countries where camel production is of little, medium, and of major economic importance are South Asia, the Near-East, Egypt, Libya, Central Asia, Pakistan, and Iraq (2–5 %); Algeria, Kenya, and Ethiopia (5–10 %); and Sahelian countries (eg. Mauritania and Somalia) and those of the Arabian Peninsula (≥25 %), respectively (Faye 2013). Moreover, camel farming is developing as a new livestock activity for tourism on the Canary Islands, in Spain and Egypt, dairy production in Netherland and USA, diversification of agricultural activities in France, or new actively in desert areas like in Namibia. The highest camel densities (number of camels per km^2) are observed in countries of the Horn of Africa and the United Arab Emirates (more than 2 camels/km^2) and in Sahelian countries (1 camel/km^2). The density is usually lower in Asia except for Pakistan and Afghanistan (Faye 2013).

In Pakistan, brucellosis in camels has not received much attention from researchers, yet. Camelids are not known to be primary or main hosts of *Brucella* spp., but they are susceptible to both *B. abortus* and *B. melitensis* and do not develop obvious clinical signs (Abbas and Agab 2002; Wernery 2014).

Brucella infection in humans may be caused by contact with infected animals or consumption of contaminated camel milk (Gautret et al. 2013; Guanche Garcell et al. 2016; Rhodes et al. 2016).

Seroprevalance in camel using RBPT has been reported from all camel-rearing countries except Australia. The incidence appears to be closely related to breeding and husbandry practices and ranges from: 2 % (Pakistan), 3.8 % (Chad), 3.9 % (Somalia), 4.9 % (Egypt), 5.7 % (Ethiopia), 1.4 to 8 % (Saudi Arabia), 10.3 % (Kenya), 14.8 % (Kuwait), 15 % (Union of Soviet Socialist Republics), 21 to 40 % (Sudan) and 0.01 to 60 % (United Arab Emirates) (Wernery 2014). The prevalence of camel brucellosis is mainly dependent upon the primary host in contact to camels as camels can contract infection from infected (cattle, buffaloes, sheep, and goats) (Gwida et al. 2012).

Although vaccination of brucellosis can be used to minimize the prevalence of brucellosis in camel herds, the success remains questionable (Dawood 2008; Roth et al. 2003; Treanor et al. 2010; Yang et al. 2013). Lack of camel vaccination in Pakistan may contribute to infection of herds.

Different serological tests like Rose Bengal plate agglutination test (RBPT), complement fixation test (CFT), serum agglutination test (SAT), competitive enzyme-linked immunosorbent assay (CELISA), and fluorescence polarization assay (FPA) have been used for detection of brucellosis in camels (Gwida et al. 2012). RBPT is a cheap conventional test used for individual animal and herd screening (Ali et al. 2013). Indirect i-ELISA is the most sensitive test available and is considered as confirmatory test (Gumi et al. 2013; Yawoz et al. 2012). Risk factors associated with human brucellosis have been studied in Pakistan (Mukhtar 2010). Seroprevalance of brucellosis in humans having direct contact with animals has been reported to be 14 and 11 % tested with RBPT and ELISA, respectively (Hussain et al. 2008), and 6.79 % sera investigated were positive using agglutination test (Rashid et al. 1999). However, possible risk factors for camels have not been studied yet.

The economic and public health impact of camel brucellosis remains of concern in developing countries. To the best of our knowledge, little is known on economic losses caused by camel brucellosis. The disease may generally cause significant loss due to late first calving, long calving intervals, low herd fertility, and low milk production. Brucellosis hinders international livestock trade (Zinsstag et al. 2011).

The objective of the study was to determine the seroprevalence of brucellosis in camel (*Camelus bactrianus*) herds and potential risk factors in three districts of Punjab, Pakistan.

Materials and methods

Study area

The study was conducted in three districts (Jhang, Chiniot, and Bhakkar) of Central Punjab, Pakistan. Jhang is located on the east bank of the Chenab River (Fig. 1). Chiniot city lies on the left bank of the Chenab River amidst small rocky hills. Bhakkar area lies on the planes along the Indus, called Kaccha, but most of the district area is located in the desert Thal. Samples were collected from nomadic/pastoral and organized production systems. According to Pakistan livestock census 2006, the number of camel heads in Jhang-Chiniot (previously one district) and Bhakkar were 8289 and 19,339, respectively. The study started in December, 2014 and was finished in May 2015.

Study design and data collection

A total of 200 blood samples were collected. One hundred and fifty blood samples ($n = 150$) were collected using multistage cluster sampling method. At the first stage, three districts (primary units) of Punjab were randomly selected, i.e., Chiniot, Jhang, and Bhakkar. At the second stage, various groups of camel population or clusters or nomadic production systems (secondary units) were randomly selected; 50 samples were sampled from various clusters of each district. Cluster sampling is opted when there is an incomplete list of all members of a population (Thrusfield 2007). Fifty ($n = 50$) blood samples were randomly collected from an organized governmental camel farm (Camel Breeding and Research Station RakhMahni) in the district Bhakkar. It is located about 30 km south of Hyderabad Thall, district Bhakkar and is considered to be the only organized (governmental) camel farm in Pakistan. Total camel population is 155 including 5 adult bulls. All animals

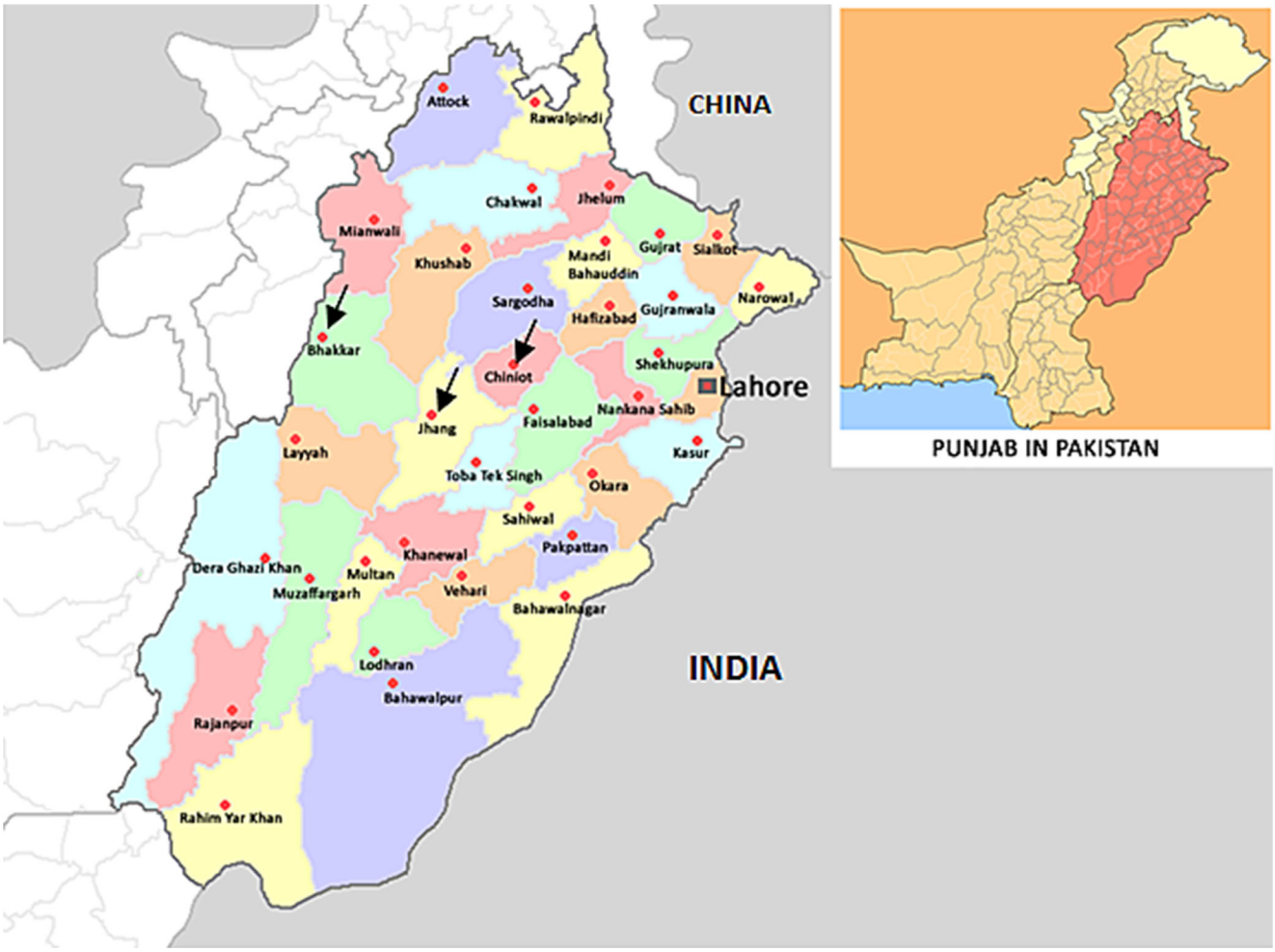

Fig. 1 Map of the study area in Pakistan

are Maracha breed. Camels are not vaccinated against brucellosis. Fifteen camel cows were in lactation.

Ethical statements

The study design was approved by the Ethics Committee, College of Veterinary and Animal Sciences, Jhang, Pakistan.

Collection of samples

Approximately 5.0 mL of blood was collected in sterile syringes (Star plus, China) from the jugular vein of each animal (National Animal Ethics Advisory Committee 2009) and then transferred to a blood vacutainer (Bio-One, China) with gel containing clot-activating factors. These samples were stored immediately at 4 °C in an ice-box and transported to the Epidemiology and Public Health (EPH) laboratory, College of Veterinary and Animal Sciences, Jhang. Sera were separated by centrifugation (select spin spectra 6c, China) at 5000 rpm for 5 min and stored in cryotubes (Grainer, Germany) at −20 °C till further analysis. Standard operating procedures (SOPs) were followed for collection of blood samples (Zewolda and Wereta 2012).

Serological evaluation of serum samples

Rose Bengal plate agglutination test

Serum samples were initially screened using RBPT antigen (IDEXX Pourquier, France). Briefly, 25 μL of serum was mixed with an equal volume of antigen preparation on a glass plate; the plate was agitated gently for 4 min. A serum sample was considered positive if agglutination occurred (Alton 1988; OIE 2009).

ELISA

All serum samples were investigated using a commercial available CELISA kit (Svanovir®, Sweden) as confirmatory test (Zewolda and Wereta 2012). The ELISA was done according to the manufacturer's instructions (SVANOVIR®,

Sweden). This kit is a multispecies assay used to detect antibodies against brucellae in serum. It is designed to detect antibodies in various animal species in situations of low and high prevalence and for confirming results from herd screening.

The results were expressed as percentage inhibition (PI), calculated from the optical density (OD) of the samples and conjugate controls, respectively, using the following formula:

$$PI = \frac{100 - \text{Mean OD sample}}{\textit{Mean OD Conjugate control}} \times 100$$

Serum samples with <30 % PI were considered negative.

Molecular detection of Brucella DNA

DNA preparation DNA was extracted and purified using DNeasy Blood and Tissue Kit (QIAGEN, Germany) according to the instructions of the manufacturer. Eluted DNA concentration was determined photometrically using a Nano Drop ND-1000 UV–Vis spectrophotometer (Nano-Drop Technologies, Wilmington, USA).

Real-time PCR assay Multiplex real-time PCR used for detection of the genus-specific *Brucella* cell surface salt extractable *bcsp31* gene, the *B. abortus alk*B gene, and the *B. melitensis BME*I1162 gene was used (Probert et al. 2004). PCR was performed using the following primer and probe set (Jena Bioscience GmbH, Germany) (Table 1). The 25-μL multiplex PCR mixture consisted of 2 TaqMan™ Environmental master mix (Applied Biosystems, New Jersey USA), 200 μM of each primer, 100 μM of each probe, and 5 μL of extracted DNA. Amplification and real-time fluorescence detection were performed on a Mx3000P thermocycler (Stratagene, Canada) using the following reaction conditions: 2 min denaturation at 50 °C, a polymerase activation at 95 °C for 10 min, followed by 50 cycles of 95 °C for 25 s and 57 °C for 1 min. A sample with a fluorescence signal 30 times greater than the mean standard deviation in all wells over cycles 2 through 10 was considered a positive result, whereas a sample yielding a fluorescence signal less than this threshold value was considered negative. Cycle threshold values below 38 cycles were interpreted as positive. The threshold was set automatically by the instrument. The samples scored positive by the instrument were additionally confirmed by visual inspection of the graphical plots showing cycle numbers versus fluorescence values.

Results

Brucella seroprevalence and molecular detection

In total, 5 % of the investigated samples (10 of 200) were seropositive by RBPT. The 10 (6.67 %) positive camel sera were collected from the nomadic rearing system. The samples collected from the organized governmental camel farm were negative. Further confirmatory testing using CELISA revealed that 2 % of the tested samples were positive (Table 2). Four serum samples (2 %) were positive in both tests (Table 3). *Brucella* DNA was detected in three serological positive serum samples which were also positive *B. abortus* in the assay (1.5 %) (Table 3). However, real-time PCR could not detect *Brucella* DNA in seronegative samples.

Potential risk associated with seroprevalence of brucellosis

The associated risk factors (rearing system, season, type of production, and health conditions) and prevalence of brucellosis are summarized in Table 3. In general, it was observed that the prevalence of brucellosis was significantly higher in nomadic rearing system compared to the organized farm. The prevalence of brucellosis in winter season was higher than in spring or in summer. Mixed keeping with other

Table 1 Oligonucleotide primers and probes used in the real-time multiplex PCR assay for the detection of *Brucella spp*., *B. abortus*, and *B. melitensis*

Target	Primer	
Brucella spp.	5′GCTCGGTTGCCAATATCAATGC 3′	Forward
	5′GGGTAAAGCGTCGCCAGAAG 3′	Reverse
	FAM-AAATCTTCCACCTTGCCCTTGCCATCA-BHQ1	Probe
B. abortus	5′GCGGCTTTTCTATCACGGTATTC 3′	Forward
	5′CATGCGCTATGATCTGGTTACG 3′	Reverse
	HEX-CGCTCATGCTCGCCAGACTTCAATG-BHQ1	Probe
B. melitensis	5′AACAAGCGGCACCCCTAAAA 3′	Forward
	5′CATGCGCTATGATCTGGTTACG 3′	Reverse
	CY5-CAGGAGTGTTTCGGCTCAGAATAATCCACA-BHQ2	Probe

FAM carboxyfluorescein, *HEX* hexachlorofluorescein, *BHQ1* Black Hole Quencher 1, *BHQ2* Black Hole Quencher 2

Table 2 Prevalence of camel brucellosis in Punjab, Pakistan, using RBPT, CELISA, and real-time PCR

Test used		Positives	Prevalence
Conventional screening test	RBPT	10/200	5 %
Confirmatory test	CELISA	4/200	2 %
Molecular detection	rt PCR	3/200	1.5 %

livestock appeared more at risk than separate animals. Among the female animals, the results showed higher prevalence of brucellosis in aborted camels followed by non-pregnant and pregnant. There were no positive cases among apparently health males while one male camel was seropositive (33.3 %).

Discussion

According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO), and the Office International des Epizooties (OIE), brucellosis is one of the most important zoonoses in the world. Brucellosis is known from but not well investigated in Pakistan (Gul et al. 2014). It poses a high risk to professionals in livestock breeding and to the consumers of milk or milk products (Gwida et al. 2010; Wernery 2014). In the current study, three independent serological tests were employed to detect the presence of *Brucella* antibodies in serum samples of camels.

The sensitivity and specificity of RBPT using commercially produced IDEXX or locally produced (VRI, Pakistan) antigens were previously discussed in small ruminants in Pakistan (Ali et al. 2015).

There is no compensation for culled animals in Pakistan. The majority of camel farmers is poor and cannot afford to cull *Brucella*-positive animals. As a result, these farmers tend to sell infected animals. These animals are then spreading the infection to healthy populations. It can be assumed that the prevalence of camel brucellosis is increasing day by day in Pakistan. The situation is comparable to the situation in small ruminants (Ali et al. 2015).

In the present study, the prevalence of brucellosis in camels was 5 % using RBPT. Previous studies reported that the prevalence of camel brucellosis may range from 0 to 20 % in Pakistan (Azwai et al. 2001; Gameel et al. 1993; Ghanem et al. 2009; Gul et al. 2014; Moustafa et al. 1998; Siddiqui 2009; Tassew and Kassahun 2014; Teshome et al. 2003; Wernery 2014). In this study, the prevalence of camel brucellosis using CELISA was found to be 2 %. This is in close agreement with previous studies showing a prevalence ranging from 2.3 to 3.1 % (Alshaikh et al. 2007; Azwai et al. 2001; El-Sawalhy et al. 1996; Ghanem et al. 2009). The higher results of the conventional screening tests may be caused by cross-reactivity due to antigens of *Yersinia enterocolitica* O:9, *Escherichia hermannii*, *E. coli* O:157, *Stenotrophomonas maltophilia*, *Vibrio cholera* O:1, or *Salmonella* serotypes (Azwai et al. 2001; Gwida et al. 2011; Wernery 2014).

Brucellosis diagnosis may also be achieved using *bscp*31 PCR for molecular detection of *Brucella* DNA in a serum sample (Gwida et al. 2010; Gwida et al. 2011; Ullah et al. 2015). In this study, *B. abortus* DNA was detected only in 1.5 % of pregnant camels during the winter season.

In the present study, prevalence of brucellosis can be correlated to the origin of the sample ($P < 0.05$). Geographically, the districts Jhang and Chiniot are both river flooded areas with transhumant nature while the district Bhakkar is characterised as an area of sand dunes in the South Punjab. This finding of the current study is in concordance with previous report in Zambia (Muma et al. 2006). Differences in management and husbandry practices, environmental conditions, lack of veterinary and extension services in remote areas of developing countries, lack of farmer's knowledge about camel diseases,

Table 3 Association between seroprevalence of brucellosis in camel herds and potential risk factors

Risk factors		No. of animals	RBPT	CELISA	rt-PCR
Rearing system	Nomadic	150	6.67 %	2.67 %	2.0 %
	Organized	50	0	0	0
Season	Winter	87	11.5 %	4.6 %	3.44 %
	Spring	40	0	0	0
	Summer	73	0	0	0
Herd type	Mixed	106	9.4 %	3.8 %	2.83 %
	Unispecies	94	0	0	0
Condition	Pregnant	48	2.08 %	0	0
	Non-pregnant	92	3.26 %	0	0
	Aborted	10	50 %	30 %	20 %
	Orchitis	3	33.3 %	33.3 %	0
	Apparently healthy male	47	0	0	0

and virulence and existence of pathogenic organism in the area have been described as risk factors as well (Azwai et al. 2001; Gwida et al. 2011).

In this study, a higher prevalence was recorded in the nomadic production system than in the organized production system. This prevalence might be caused by the cohabitation of camels with infected small and large ruminants favoring cross-transmission of infection. Sharing the same pastures and watering points with infected cattle and small ruminants has enhanced the transmission of brucellosis to camels (Ghanem et al. 2009; GUL and KHAN 2007; Gwida et al. 2011; Ullah et al. 2015).

Brucellosis was more often seen in female camels as in males, but this finding was statistically not significant ($P > 0.05$). In most studies conducted on camel brucellosis earlier, susceptibility of male and female camels has not been significantly investigated (Azwai et al. 2001; Gul et al. 2014; Gwida et al. 2012). The season was found to be statistically significant ($P < 0.05$) as shown by higher prevalence (11.5 %) in the winter (rainy) season when immunosuppression could be supposed. It is in close agreement with the findings of Sprague et al. (2012). It is supposed that the numbers of cases decreases in summer and spring because brucellae will not survive warm weather and cannot withstand direct exposure to sunlight. Increased activity of predators and scavengers will result in the fact that fetuses or infected material will also not persist in the environment beyond mid-May (Hollingsworth 1998).

In camels reared with other livestock, prevalence was statistically significantly higher ($P < 0.05$). Our findings are in accordance with previous studies (Boukary et al. 2013; Teklue et al. 2013).

Another risk factor, i.e., abortion history, was statistically significant ($P < 0.05$), which is in accordance to the findings of similar studies (Mohammed et al. 2011; Teklue et al. 2013). History of orchitis in bulls was statistically significant ($P < 0.05$) which is in close agreement with the findings of previous studies (Akbarmehr and Ghiyamirad 2011; Wiesch et al. 2010).

Conclusion

In the present study, the rearing system (nomadic and organized), the season, abortion and orchitis history were found to be risk factors and should be taken in consideration when counter measures are taken. The control of brucellosis in livestock and humans also depends on the reliability of the methods used for detection and identification of the causative agent. It is recommended that testing and re-testing are performed on routine basis and stamping out is restricted to confirmed cases. The use of CELISA has to be validated for its use in camels carefully. PCR may amend the diagnostic tool box if needed.

Our findings point to the fact that camels get chronically infected and may serve as reservoir and source of infection even if co-herding is no longer practiced.

A large-scale epidemiological study is needed to gain a clear picture on the situation of camel brucellosis in Pakistan. *Brucella* isolates should be investigated using modern molecular techniques to unravel epidemiological links.

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Authors' contributions SF, IK, MY, and AN participated in the conception and design of the study and SF performed the field work. IK, HE, FM, and HN analyzed the data and wrote the manuscript. SF, IK, HE, FM, and HN were contributed to the analysis and helped in the manuscript discussion. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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