

RESEARCH ARTICLE

Identification and Phylogenetic Positioning of *Salmonella* Dublin from Aborted Cattle Materials

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Abstract

This study was aimed to isolate, and characterize the *Salmonella* Dublin in the liver, lung tissues and the abomasum contents of 367 aborted cattle fetal samples obtained from four different cities (Ağrı, Erzincan, Erzurum and Kars) of Turkey by using molecular methods. After proper incubation of the tissues and contents, *Salmonella* spp. identification was performed. Colonies with suspicion of *Salmonella* were boiled on a dry heat block for DNA extraction, while multiplex PCR was used for species identification of *Salmonella* spp. and identification of *S. Dublin*. The multiplex PCR, 16S rDNA sequence, and phylogenetic analysis were performed on an isolate with a band profile specific to *S. Dublin*. The *S. Dublin* identification was performed according to BLAST analysis and similarity scores obtained from NCBI GenBank. Molecular prevalence of *S. Dublin* in the aborted fetal samples of cattle was 0.82%. The 16S rRNA sequence results of the isolate was found to be similar to many *S. Dublin* strains. The partial sequence of the 16S rRNA gene region of the isolate was recorded in the GenBank database with the name strain Erzurum VCRI and access number MZ452230. In conclusion, *S. Dublin* was identified in the aborted cattle fetuses and phylogenetic position was determined for *S. Dublin* using the sequence analysis of 16S rDNA gene region.

Keywords: Cattle, *Salmonella* Dublin, Phylogenetic, Sequence

Sığır Abort Materyallerinden *Salmonella* Dublin İdentifikasyonu ve Filogenetik Pozisyonlandırılması

Öz

Bu çalışmanın amacı Türkiye'nin dört farklı şehirden (Ağrı, Erzincan, Erzurum ve Kars) alınan 367 adet sığır aborte fötüs örneklerine ait karaciğer ve akciğer dokuları ile abomazum içeriklerinde *Salmonella* Dublin'i izole etmek ve moleküler yöntemlerle karakterizasyonunu sağlamaktır. Doku ve içeriklerin uygun inkübasyonu sonrasında *Salmonella* spp. identifikasyonu yapıldı. DNA ekstraksiyonu için *Salmonella* spp. şüpheli kolonilere kuru ısı bloğu üzerinde kaynatma metodu, *Salmonella* spp.'ların tür düzeyinde identifikasyonu ve *S. Dublin* teşhisi için ise multipleks PCR metodu kullanıldı. Multipleks PCR analizi sonucunda *S. Dublin*'e özgü bant profili gösteren bir izolatın 16S rDNA dizi analizi ile sekanslaması ve filogenetik analizi gerçekleştirildi. *S. Dublin* identifikasyonu ise BLAST analizine göre ve NCBI GenBank'tan elde edilen benzerlik skorlarına göre yapıldı. Sığır atık fötal örneklerinde *S. Dublin*'in moleküler prevalansı %0.82 olarak saptandı. İzolatın 16S rRNA sekans bulguları birçok *S. Dublin* suşu ile benzer olarak bulundu. İzolatın 16S rRNA gen bölgesinin sekansı Genbankasına MZ452230 erişim numarası ve Erzurum VKEM suş ismi ile kaydedilmiştir. Sonuç olarak sığır atık fötüsünden *S. Dublin* tanımlanmış ve 16S rDNA gen bölgesinin dizi analizi ile de *S. Dublin*'e filogenetik pozisyon kazandırılmıştır.

Anahtar sözcükler: Sığır, *Salmonella* Dublin, Filogenetik, Sekans

INTRODUCTION

Salmonellosis is a disease which can cause digestive and urogenital system infections in cattle regardless of age^[1]. *Salmonella enterica* subspecies *enterica* serovar Dublin (*Salmonella* Dublin) is a serotype hosted by cattle^[2]. Due to having the *Salmonella* plasmid virulence (spv) gene,

which plays a role in its pathogenesis, *S. Dublin* is different from many other *Salmonella* serotypes and this gene makes the microorganism resistant to phagocytosis^[1]. In cattle establishments, clinically infected animals or asymptomatic carriers cause the bacteria to spread within the farm^[1]. This type of animals shed the bacteria into the environment via discharges such as milk, saliva, faeces,

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vaginal discharge, urine, etc. ^[1,2]. Infection is generally transmitted via faecal-oral route ^[1].

In addition to causing diarrhea or septicemia in calves, *S. Dublin* causes significant economic losses due to the abortions in gestating animals ^[3]. Abortion may occur at any stage of gestation in cattle, however the abortions caused by *S. Dublin* are the most common in the 5th-9th months of gestation. Because *S. Dublin* rarely causes clinical symptoms, it has extremely negative effects on fertility and this causes significant economic losses in the cattle industry ^[4,5]. In clinical terms, abortions are reported to be caused by serotypes B, C and D. There are different mechanisms of abortions caused by *Salmonella* infection in cattle. One of them is the infection of the fetus by the bacteria, which reaches the uterus and the fetus, and eventually causes the death of the fetus. In this case, isolation of the agent from the internal organs of the aborted fetus enables us to put forward this mechanism. Endotoxemia, which causes the release of inflammatory mediators, causes a secondary luteolysis as a result of the release of prostaglandin. The body temperature increase is also responsible from the death of the fetus ^[1].

S. Dublin has been isolated at a prevalence of 5.6% (106/1877) in cattle fetal membrane samples and at a prevalence of 2.1% (4/190) in vaginal swab samples ^[5]. The abortion rate of *S. Dublin* has been reported as 0.66% in Australia (1/150) ^[6]. Anderson ^[7] analyzed 391 aborted cattle fetuses in USA using bacteriological culture, and isolated *Salmonella* spp. from 1.7% (7/391) of the samples while 5 (71.4%) of them has been identified as *S. Dublin*. It has been stated that abortion rate of *S. Dublin* is 26.7% in England ^[8]. Moreover, a previous study has reported the cattle abortions rate caused by *S. Dublin* as 80% throughout the world ^[9]. The aim of this study was to isolate, and characterize *Salmonella* Dublin in the liver and lung tissues, and the abomasum contents of 367 aborted cattle fetal samples obtained from four different cities (Ağrı, Erzincan, Erzurum and Kars) of Turkey by using molecular methods.

MATERIAL AND METHODS

Ethical Approval

The Atatürk University Animal Experiments Local Ethics Committee approved the study protocol of this study (decision no: 164/2021).

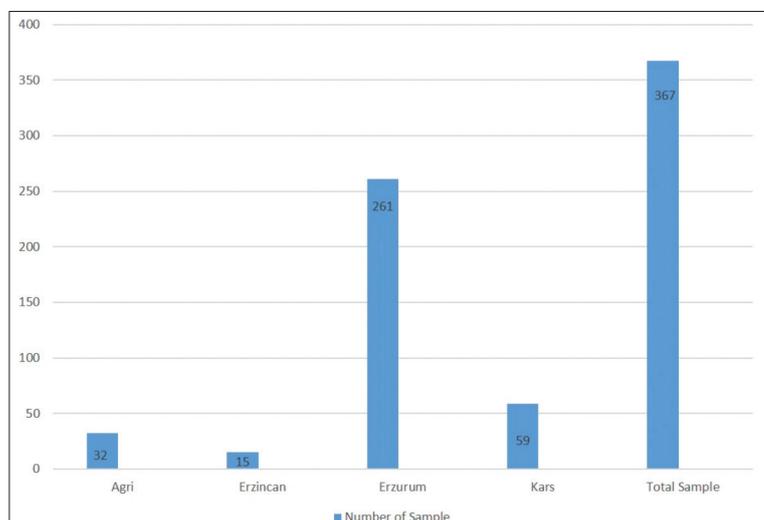
Study Material

The material of the study consisted of liver and lung tissues as well as abomasum contents of 367 aborted cattle fetuses brought to Ağrı, Erzincan, Erzurum and Kars cities (Fig. 1) of Turkey. Samples were collected from February to April, 2021. After necropsy was performed properly on the aborted fetuses, at least 3 grams of the organs were taken into sterile sample containers, while abomasum content was soaked into the transport swab with Stuart's medium. *Salmonella Typhimurium* strain, which was isolated by our institute in the previous years, was used as positive control for bacteriological culture and multiplex PCR analyses.

Bacterial Isolation and Identification

Bacteriological culture was performed on the fetal tissues and abomasum contents for the analysis of *S. Dublin*. For *Salmonella* spp. isolation, fetal lung and liver tissues as well as abomasum contents were directly inoculated onto XLD agar and these media were incubated for 5 days under anaerobic conditions at 37°C. After incubation, the typical H₂S positive black colonies which formed on XLD agar and surrounded by a reddish-pink halo were assessed with suspicion of *Salmonella* spp. and later the preparations obtained from these colonies were gram-stained ^[10]. Bacterial colonies with a gram-negative *Bacillus* morphology were passaged into blood agar and left for incubation for 48 h at 37°C under aerobic conditions. After incubation, *Salmonella* spp. identification was performed using a Vitek-2 Compact (Biomérieux) GN ID card ^[11].

Fig 1. Number of samples studied by province



DNA Extraction

For DNA extraction, 4-5 bacterial colonies from *Salmonella* spp. suspected colonies were taken into sterile microcentrifuge tubes containing 500 µL PBS. DNA extraction was performed by boiling on dry ice block [3].

Multiplex Polymerase Chain Reaction

Multiplex PCR method of previous work was used for species identification of *Salmonella* spp. and *S. Dublin* [12]. The *invA* gene region was used for type identification, and *SeD A1118* and *SeD A2283* primers were used for the identification of *S. Dublin*. PCR mixture was prepared with 12.5 µL Hotstart Master Mix (Qiagen, Hilden, Germany), 7.5 µL deionized ultrapure water, 2 µL primer mix and 3 µL target DNA, and the first denaturation was performed at 95°C for 15 min. Later, denaturation occurred for 30 sec at 95°C, annealing for 30 sec at 60°C and extension for 45 sec at 72°C, making 35 cycles in total. The mixture was kept at 72°C for 5 min for the final extension. Qiaxcel Advanced System (Qiagen, Germany) was used for the determination of the sizes of PCR amplicons. Electroforesis was performed according to OM500 protocol of DNA High Resolution kit. The detection of a 284 bp band for *invA* gene region only for *Salmonella* spp., and the detection of a 284 bp band for *invA* gene, a 378 bp band for *SeD A2283* gene and a 463 bp band for *SeD A1118* gene for *S. Dublin* led to assess these agents as positive.

16S rDNA Sequencing and Phylogenetic Analysis

After the multiplex PCR analysis, 16S rDNA sequence analysis was performed by BM Laboratory Systems. EurX GeneMATRIX Bacterial & Yeast DNA isolation kit (Poland) was used for the isolation of DNA from the colony in the pure culture. Spectrophotometric measurements were performed using Thermo Scientific Nanodrop 2000 (USA) to control the amount and purity of the DNA's. The gene region which was targeted for the identification of the type was amplified using a universal primer pair (27F-1492R) [13]. For amplification, 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.3 µM of each primer, 2 U Taq DNA polymerase and 3 µL template DNA were prepared to make a total volume of 35 µL and the first denaturation was performed for 5 min at 95°C. Later, denaturation occurred for 45 sec at 95°C, annealing for 45 sec at 57°C and extension for 60 sec at 72°C, making 35 cycles in total. The mixture was kept at 72°C for 5 min for the final extension. The amplified PCR product was purified from the single band samples using a MAGBIO "HighPrep™ PCR Clean-up System" (AC-60005) kit. The sequencing of 16S rDNA gene was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730XL Sanger sequencer (Applied Biosystems, Foster City, CA). The readings taken from 27F and 1492R primers were assembled to a contig to create a consensus sequence. CAP contig assembly algorithm in the BioEdit software was used for this process. Sequence analysis results

were assessed using BLAST analysis and similarity scores obtained from NCBI GenBank [14]. The genetic relationships between the 16S rRNA genes of one *S. Dublin* strain and representative strains from the *S. Dublin* were inferred using the maximum likelihood method based on Kimura's two-parameter model with 1000 bootstrap using the MEGA X software (Version 10.2.2) [15].

RESULTS

Isolation and Identification Results

In this study, *Salmonella* spp. was identified in 16 (4.35%) of the liver and lung tissues and abomasum contents of 367 aborted cattle fetuses, which were analyzed using bacteriological culture, with the presence of agents with Gram-negative bacillus morphology, having typical colonies with black color in the middle and surrounded by a reddish-pink halo due to the H₂S positivity in XLD medium. In the analyses of *Salmonella* spp. isolates using Vitek-2 GN ID panel, all of their identifications were confirmed on a genus level (*Salmonella* spp.) (Table 1).

Multiplex PCR Results

As a result of the genus-specific multiplex PCR analysis of 16 isolates, which were identified as *Salmonella* spp. using phenotypic methods and Vitek-2 automated identification system, all of these isolates were confirmed as *Salmonella* spp. with the presence of 284 bp PCR products that showed *invA* gene amplification. In multiplex PCR, 3 of the 16 isolates (18.75%) were identified as *S. Dublin* with the presence of 284 bp product belonging to the *invA* gene region specific to the *Salmonella* genus, as well as the presence of 378 bp *SeD A2283* and 463 bp *SeD A1118* genes (Fig. 2). *S. Dublin* was equally isolated from organs, and these were not statistically different. The molecular prevalence of *S. Dublin* in aborted fetal samples of cattle was found to be 0.82%.

The Findings of 16S rDNA Sequencing and Phylogenesis

After Multiplex PCR, 16S rRNA sequence analysis was performed on one of the three isolates identified as *S. Dublin* positive. PCR analysis with the universal primers (27F and 1492R) showed the presence and successful amplification of the 16S rRNA gene region with the presence of 1465 bp bands in one *S. Dublin* isolate. The 100% similarity was not found between the DNA sequences of *S. Dublin* isolate analyzed and the DNA sequences uploaded to the gene bank. The similarities with the DNA sequences with the closest similarity were evaluated. The 16S rRNA sequence results of the isolate was found to be similar to those of many *S. Dublin* strains including CP032379, CP0324446, CP032449, CP032396, CP032390, CP032393, CP032387, CP032384, LK931502, CP075021, CP074229, CP063756, CP063754, CP019179, with 99.46%, CP0019179, CP0075113, CP074226, CP001144, with 99.39%, FJ997268,

Table 1. Sample distribution and culture positivity rate by province (%)

Province	Total Sample	Positive	Culture Positive (%)	Vitek 2 Diagnosis <i>Salmonella</i> spp.			S. Dublin Positive
				Very Good	Good	Medium	
Ağrı	32	0	0.0	-	-	-	-
Erzincan	15	2	15.4	2	0	0	0
Erzurum	261	8	3.1	6	2	0	2
Kars	59	6	10.2	5	1	0	1
Total	367	16	4.35	13	3	0	3

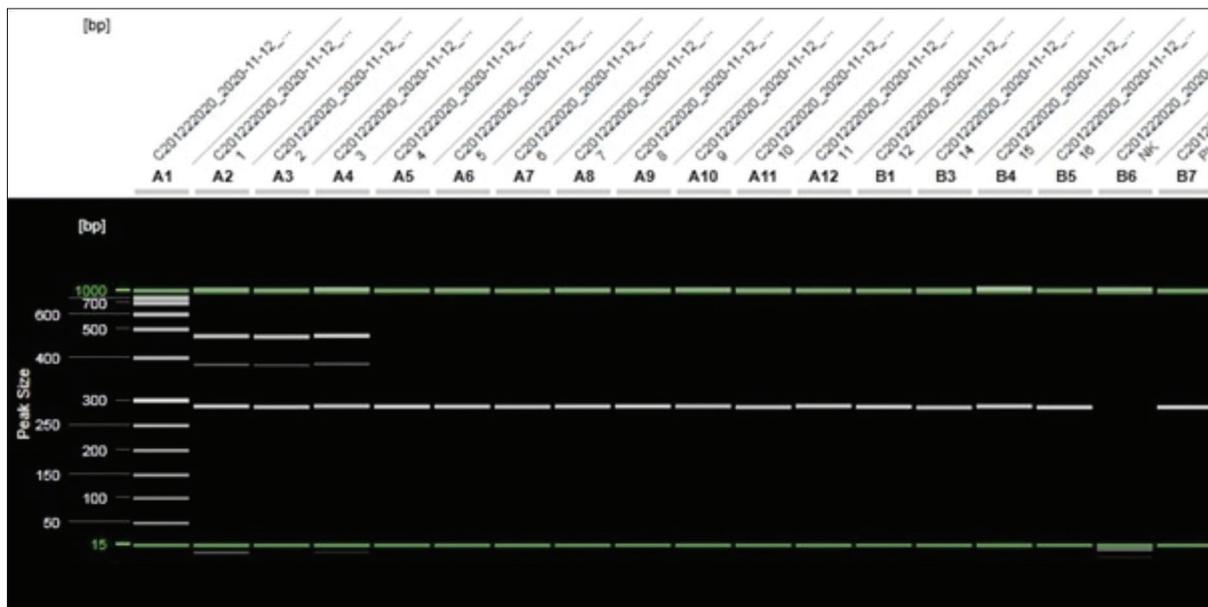


Fig 2. Capillary electrophoresis image of multiplex PCR; A4: *Salmonella* Dublin isolates (*invA*, SeD A1118 and SeD A2283 positive). A5-B5 *Salmonella* spp. isolates (*invA* positive only). B6: Negative Control. B7: Positive Control

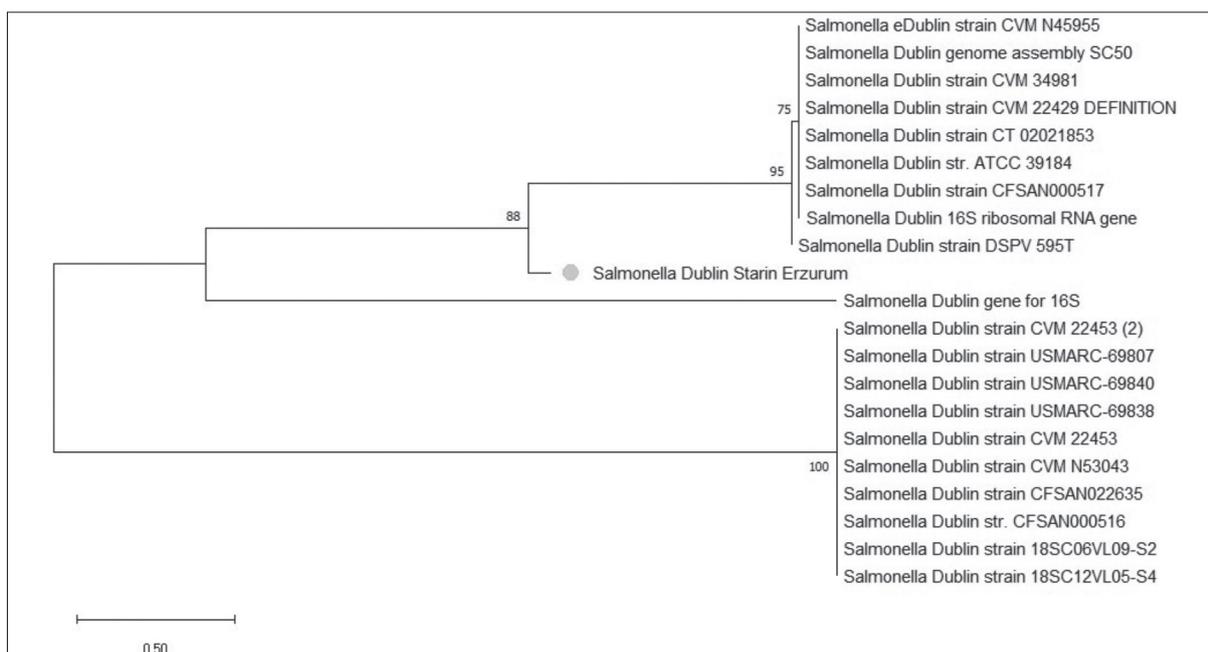


Fig 3. The dendrogram generated by the neighboring joining method for the *S. Dublin* isolate that was designated based on the sequence of the 16S rRNA gene region

with 99.16%, AF227868, with 99.39%, D12810 with 99.09% similarity index. The partial sequence of the 16S rRNA gene region of the isolate was recorded in the GenBank database with the name strain Erzurum VKEM and access number MZ452230.

The *S. Dublin* isolate identified in this study was found to be in the same cluster as the *S. Dublin* strains isolated from cattle. This isolate was positioned in the same branch with cattle and turkey isolates which were defined as cattle stool, ground turkey, ground beef (Fig. 3).

DISCUSSION

Salmonella species play a role in the etiology of abortions in cattle [9]. In this study, the roles of *Salmonella* types in the etiology of cattle abortions were investigated by using cultural method and *Salmonella* spp. positivity was found to be 4.35% (16/367), this finding was consistent with previous studies that reported the rate between 0% and 6.5% [7,16-19]. In this study, *S. Dublin* positivity was determined as 0.81%, this finding was consistent with previous studies that reported this rate as 0.66-5.6% [5,6,20]. In this study, three of the isolates (18.75%) were identified as *S. Dublin* after PCR analysis. A previous study that analyzed 391 aborted cattle fetuses in the United States of America using bacteriological culture isolated *Salmonella* spp. from 1.7% (7/391) of the samples, while 5 (71.4%) of them were identified as *S. Dublin* [17]. Another study has isolated *Salmonella* spp. from 24 (6.5%) of the samples while 2 (8.3%) of them were identified as *S. Dublin* [18]. It has been postulated that the prevalence of cattle abortions caused by *S. Dublin* in England was 3.6% in fattening cattle and 16.6% in dairy cattle [8].

Even though it has been recommended to inoculate fetal stomach content and fetal brain directly for culture [9,21], in this study *S. Dublin* was isolated from aborted fetal liver, lung and abomasum contents. The previous studies have similarly isolated *Salmonella* spp. in fetal tissues [22], placenta discharge [9,23], fetal stomach content and placental samples [24]. PCR-based analyses can be used in the identification of *Salmonella* serovars [10]. A previous study has developed a multiplex PCR which included primers specific to *S. Dublin*, namely *SeD_A1118* and *SeD_A2283*, and primers specific to *invA* gene regions which are specific to *Salmonella* spp. [12]. Although PCR has some advantages in identification, bacteriological culture is necessary for advanced analyses [25].

A 16S rDNA sequence analysis can allow the phylogenetic analysis of the bacteria and it can allow the identification of microorganisms that can rarely be grown. In 16S rDNA sequence analyses, it is necessary to find a similarity rate less than 1% [26]. In the current research, by performing sequence analysis of one isolate of *S. Dublin* with 16S rDNA allowed genomic comparisons of *S. Dublin* strains isolated

in previous studies. *S. Dublin* isolate, which was identified in this study was found to have; 99.46% similarity with the isolates which have been registered to the NCBI GenBank with the access codes CP032379, CP0324446, CP032449, CP032396, CP032390, CP032393, CP032387, CP032384, LK931502, CP075021, CP074229, CP063756, CP063754, CP019179, 99.39% similarity with the isolates which have been registered to the NCBI GenBank with the access codes CP0019179, CP0075113, CP074226, CP001144, 99.16% similarity with the isolate which has been registered to the NCBI GenBank with the access code FJ997268, 99.39% similarity with the isolate which is registered to the NCBI GenBank with the access code AF227868 and 99.09% similarity with the isolate which is registered to the NCBI GenBank with the access code D12810. While *S. Dublin* isolate was in the same set with the *S. Dublin* isolates with the code D12810 obtained from a sample of cattle origin in Japan, it was on the same branch with the *S. Dublin* isolates with the codes CP001144, CP074226, CP075113, CP019179, CP32396, CP032390, CP032387, LK931502, FJ997268 obtained from minced beef, minced turkey meat and cattle faeces samples from USA, France, Argentina, Nigeria and United Kingdom.

As a result, the number of cattle isolates were high on the branch of the phylogenetic tree where the isolate of cattle origin subject to 16S rDNA sequencing. At the same time, the origin of the isolates with phylogenetic relationship goes back to Europe, America, Africa and Asia shows that the geographical distribution of the agent is wide. In conclusion, definitive identification of *S. Dublin* was identified in aborted cattle fetuses. Furthermore, 16S rDNA gene region sequence analysis was performed, and phylogenetic position of *S. Dublin* isolate was determined.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that data supporting the study findings are also available to the corresponding author.

FUNDING SUPPORT

None declared.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Both authors planned, designed and performed the analysis. The manuscript was written by both authors. Both authors have interpreted the data, revised the manuscript for contents, and approved the final version.

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