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Presence of *Helicobacter cinaedi* in Atherosclerotic Abdominal Aortic Aneurysmal Wall

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Recently, the relationship between *Helicobacter cinaedi* (*H. cinaedi*) infection and several diseases, including cardiovascular and central nervous system disorders, bone and soft tissue disorders, and infectious abdominal aortic aneurysms (AAAs), has been reported. Moreover, *H. cinaedi* may be associated with arteriosclerosis. In the present study, we investigated the association between *H. cinaedi* infection and clinically uninfected AAAs. Genetic detection of *H. cinaedi* in the abdominal aneurysm wall was attempted in 39 patients with AAA undergoing elective open surgery between June 2019 and June 2020. DNA samples extracted from the arterial wall obtained during surgery were analyzed using nested polymerase chain reaction (PCR). The target gene region was the *H. cinaedi*-specific cytolethal distending toxin subunit B (*cdtB*). Nine (23.1%) of 39 patients showed positive bands corresponding to *H. cinaedi*, and further sequencing analyses demonstrated the presence of *H. cinaedi* DNAs in their aneurysm walls. In contrast, all the non-aneurysm arterial walls in our patients were negative for *H. cinaedi*. In conclusion, this is the first report of the detection of *H. cinaedi* in the walls of a clinically non-infectious AAA.

Keywords: abdominal aortic aneurysm (AAA); arteriosclerosis; cytolethal distending toxin subunit B (*cdtB*); *Helicobacter cinaedi*; nested PCR
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Introduction

In many cases, abdominal aortic aneurysms (AAAs) progress asymptomatically; once ruptured, the mortality rate is reported to be nearly 70-80% (Chaikof et al. 2018). In 2017, the mortality rates of AAA were surveyed among patients in 18 countries (15 EU countries, Australia, Canada, and the USA) and were estimated to be 5.0 and 2.1 per 100,000 men and women, respectively (Al-Balah et al. 2020). Methods to reduce the risk of AAA progression include smoking abstinence, blood pressure control, and statin use. However, these methods are insufficient to prevent AAA rupture. Currently, surgical operations, either open surgery or endovascular aneurysm repair (EVAR), are the only methods that can save patients.

The initiation and progression of AAA remain unclear. Arteriosclerosis, inflammation, oxidative stress, bacteria, trauma, and heredity are risk factors for AAA (Lawrence and Rigberg 2019; Tracci et al. 2019).

Among these, we focused on bacteria and AAA because the infection is a frequent cause of inflammation, which leads to many pathogenic conditions. *Chlamydia pneumonia* (*C. pneumonia*) has been detected in noninfectious aneurysmal walls and has been suggested to be related to aneurysms (Juvonen et al. 1997; Karlsson et al. 2000; O'Connor et al. 2001). In this context, clinical trials using antibiotics, such as roxithromycin, targeting *C. pneumonia* have been conducted, and their effectiveness in inhibiting

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aneurysm progression has been observed (Vammen et al. 2001). However, this finding is controversial, and a report by Kokje et al. (2015) did not support the results of Vammen et al. (2001). Therefore, it is worth studying other bacterial infections.

We have previously detected *Helicobacter cinaedi* (*H. cinaedi*) in three patients with infectious AAA (Kakuta et al. 2014). *H. cinaedi* is a gram-negative spiral bacillus that is mainly detected in the hepatic and intestinal tracts and is classified as an enterohepatic *Helicobacter* species (Kawamura et al. 2014). During the last two decades, improvements in detection methods have led to the frequent identification of *H. cinaedi* irrespective of immune status (Kawamura et al. 2014). *H. cinaedi* is difficult to grow *in vitro*; however, recent improvements in polymerase chain reaction (PCR)-based detection methods have resulted in several reports of bacteremia by *H. cinaedi* (Matsumoto et al. 2007; Araoka et al. 2014; Kawamura et al. 2014). Oyama et al. (2012) reported *H. cinaedi* in the feces of healthy individuals using a highly sensitive nested PCR-based method and suggested the existence of asymptomatic *H. cinaedi* carriers. Khan et al. (2014) reported on the mechanisms of arteriosclerosis induced by *H. cinaedi* in a mouse model. They hypothesized that *H. cinaedi* is involved in the development of atherosclerosis through the phagocytosis of *H. cinaedi* monocyte-derived macrophages and an increase in intracellular fat droplets. In addition, *H. cinaedi* has the unique effect of changing the expression of cholesterol metabolic proteins, is involved in foam cell formation, and is associated with atherosclerosis. *H. cinaedi* is thought to be the most frequent bacteremia-causing strain among *Helicobacter* species with high vascular invasive ability, and relapse rates are estimated to be 30-60% after infection (Kawamura et al. 2014).

In this study, we focused on *H. cinaedi*, which is associated with atherosclerosis. We investigated the presence of *H. cinaedi* in clinically non-infected AAA using a nested PCR method.

Materials and Methods

Patients analyzed in the present study

All patients with atherosclerotic AAA who were surgically treated at Tohoku University Hospital or Iwate Prefectural Isawa Hospital between June 2019 and June 2020 were enrolled in the present study. Patients with suspected infections, ruptures, or inflammatory aneurysms were excluded. Patients who had undergone EVAR were excluded from the study. All patients underwent open aortic repair of the aneurysms. A normal central arterial wall is an ideal control that is impossible to obtain ethically. Therefore, we analyzed the iliac artery as the second-best control in this study. The study conformed to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from each patient and the study was approved by the Ethics Committees of both institutions (Tohoku University School of Medicine: 2019-1-076 and 2020-1-159; Iwate Prefectural Isawa Hospital: 2020-5).

Sample collection and DNA extraction

Samples were collected during surgery according to the maximum diameter of the AAA, immediately after aortic cross-clamping, and followed by an incision of the aneurysm. Each sample was cut into 3-5 mm square pieces from the aneurysmal wall. They were snap-frozen as quickly as possible and stored at −80°C in aseptic conditions until use.

We purified DNAs from the arterial walls. We used the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), and DNA concentrations were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The quality of each extracted DNA sample was assessed by PCR using a human beta-actin (*ACTB*) primer set (Lejmi et al. 2008) as shown in Table 1.

Polymerase chain reaction (PCR)

To detect the faint DNA of *H. cinaedi*, nested PCR was performed as previously described (Oyama et al. 2012). The first PCR was performed using the DNA polymerase KOD FX Neo (Toyobo, Osaka, Japan) following the manufacturer's recommendations, with some modifications. In brief, the final concentrations of reagents were 3.1 mM $MgSO_4$, 0.2 mM dNTP, 0.2 μM of the primer set, 0.5 U of KOD FX Neo, and an appropriate amount of template DNA. PCR volume was 25 μL, and Veriti 200 Thermal Cycler (Thermo Fisher Scientific) was used. PCR conditions were an initial 3 min denaturation at 95°C, followed by 40 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 30 s, and then a final extension at 68°C for 7 min. These PCR products, including the negative control, were kept at 25°C until use. For the nested PCR, 2 μL of each 100-fold diluted product of the first PCR was used as the template,

Table 1. Nucleotide sequences of primers used in this study.

Gene name (accession number)		Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Annealing temp.	Product size
cdtB (NC_020555.1)	First PCR	GGCTCTTCGGCAACATCTGA	CAGCTGCAATAAGCGGTTCT	60°C	637-bp
	Nested PCR	GGATTTAGGCTCTCGCTCT	CTTCCAGAGTTCCCTATCAC	60°C	432-bp
ACTB (NG_007992.1)		AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG	60°C	279-bp

cdtB, cytolethal distending toxin subunit B; ACTB, human beta-actin; PCR, polymerase chain reaction.

and other conditions were identical to those for the first PCR. At this step, the negative control of the first PCR was also used to detect faint contamination of *H. cinaedi* DNA. At least two independent experiments were performed to check reproducibility. Purified *H. cinaedi* DNA (approximately 1 pg corresponding to 500 copies) was used as a positive control, and distilled water was used as a negative control. Because the target region should be specific to *H. cinaedi*, we set the cytolethal distending toxin subunit B (*cdtB*) gene (NC_020555.1) as the target. A comparison of the *cdtB* genes in the *Helicobacter* family members is summarized in Fig. 1; the sequences in the blue box are specific to *H. cinaedi*. Hence, we designed primers that span this region. The nucleotide sequences of the primer sets are listed in Table 1.

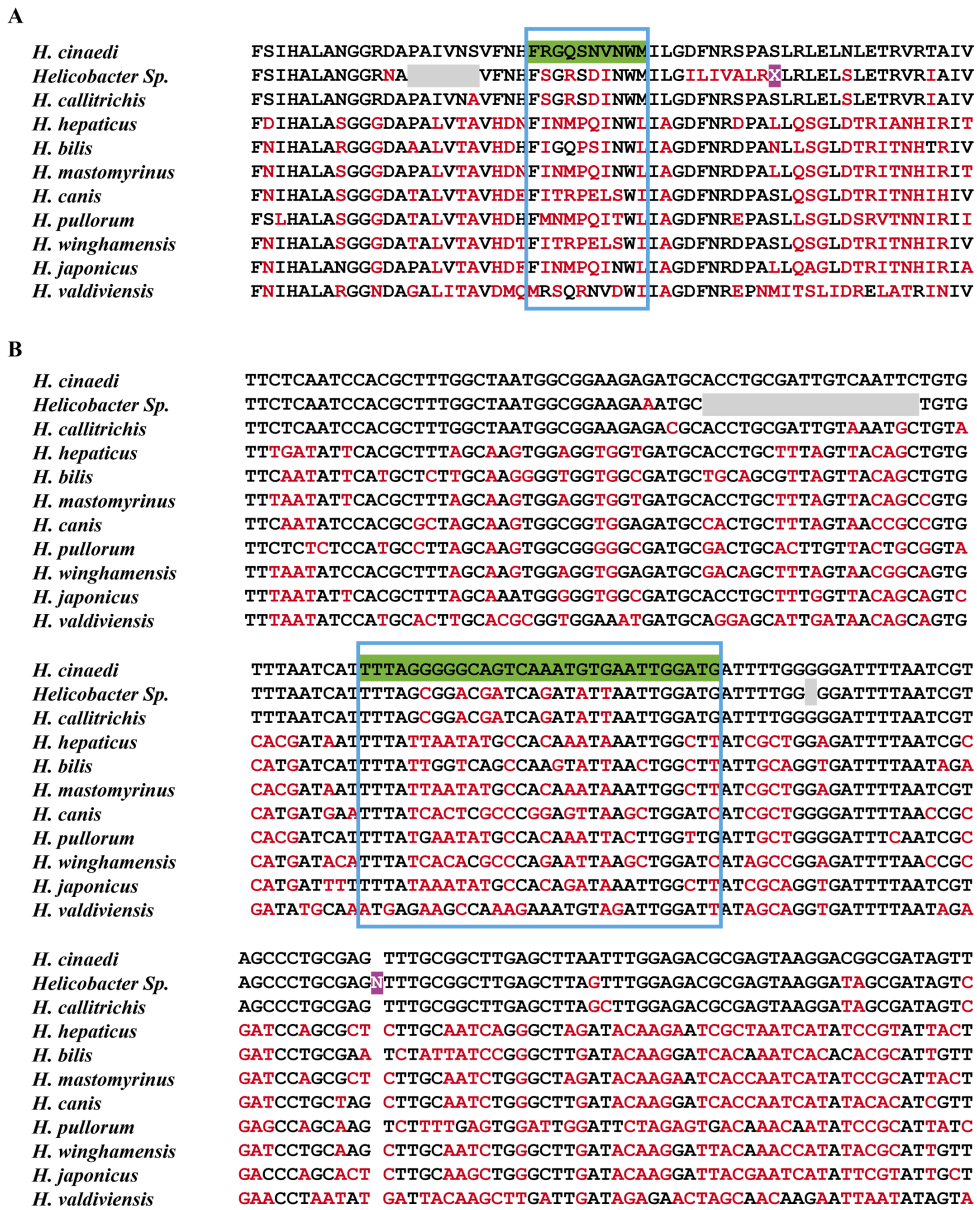

Fig. 1. Comparison of *Helicobacter* family members harboring the *cdtB* gene.
(A) Amino acid sequences of the most divergent part between 161st and 220th of *Helicobacter cinaedi* (NC_020555.1) are shown, and amino acid residues different from those of *Helicobacter cinaedi* are shown in red. Highlights in green denote specific amino acid sequences of *Helicobacter cinaedi* in this study as the landmark, and corresponding sequences of other members are boxed in blue. Highlights in gray denote deletion in *Helicobacter Sp*. (AF243080.1), and in pink, unspecified amino acid. (B) Nucleotide sequences corresponding to amino acid sequences in (A) are shown, and nucleic acids different from those of *Helicobacter cinaedi* are shown in red. Blue box corresponds to a nucleotide sequence in blue-boxed amino acid sequences in (A), highlights in gray denote deletion, and a highlight in pink denotes one-base insertion of an unspecified nucleotide. Note that this one-base deletion followed by a one-base insertion at 24-nt downstream caused frame-shift for 8 amino acids (GILIVALR) in *Helicobacter Sp*.

Electrophoresis

PCR products were electrophoresed on a 2% agarose gel, and GelRed Prestain Buffer with Orange Tracking Dye (Biotium, San Francisco, CA, USA) was used for visualization. After electrophoresis, PCR products were detected using a ChemiDoc XRS+ System (BioRAD Laboratories, Hercules, CA, USA).

Nucleotide sequencing analyses

For DNA sequencing, the nested PCR products were purified using ExoSAP-IT Express PCR Product Cleanup (Thermo Fisher Scientific). Nucleotide sequences were determined using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3500xL Genetic Analyzer (Applied Biosystems).

Statistical analyses

The *t*-test for normally distributed data and the Wilcoxon signed-rank test for non-normally distributed data were analyzed. Fisher's exact test was used for contingency table analysis. We used univariate analysis because of the limited number of cases. JMP Pro 14.0.0 software (SAS Institute Inc., Cary, NC, USA) was used for these analyses. $P < 0.05$ was considered statistically significant for all analyses.

Results

Patients analyzed in this study and detection of H. cinaedi DNA by nested PCR

We analyzed 39 aneurysmal walls in 39 patients with AAA. The clinical characteristics of the patients are summarized in Table 2. In this study, 20 non-aneurysmal iliac arteries obtained simultaneously during surgery were analyzed as controls. The concentrations of obtained DNAs of aneurysms and iliac arteries were 35 ng/μL (interquartile range: 20-55 ng/μL), which were within the appropriate amount of DNA for PCR analysis. The quality of each

Table 2. Clinical characteristics and results of the nested PCR of the 39 patients analyzed in this study.

	Nested PCR		*P*-value
	Positive: n = 9	Negative: n = 30	
Age (years)	72 [68-75.5]	69.5 [67.75-76.25]	0.76
Sex (Male)	8	25	1.0
Family history of AAA	2	2	0.23
Smoking history			0.56
Current or ex-smoker	8	28	
Never smoked	1	2	
Periodontal disease	9	27	1.0
Type of AAA			0.17
Suprarenal	0	2	
Juxtarenal	3	3	
Infrarenal	6	25	
Diameter of the aneurysm (mm)	50 [45.5-52.5]	52 [49-56.25]	0.30
Comorbidity			
Hypertension	9	24	0.55
Hyperlipidemia	7	18	0.45
Diabetes mellitus	2	4	0.61
Coronary artery disease	2	10	0.69
Cerebrovascular disease	1	6	1.0
Chronic obstructive pulmonary disease	3	13	0.71
Dialysis	1	0	0.23
Peripheral artery disease	1	3	1.0
Medication			
Oral steroids	0	2	1.0
Antiplatelet agents	3	8	0.69
Antidiabetic agents	2	4	0.61
Antihypertensives	7	22	1.0
Statins	6	16	0.70

Data are presented as n or median [interquartile range].
AAA; abdominal aortic aneurysm.

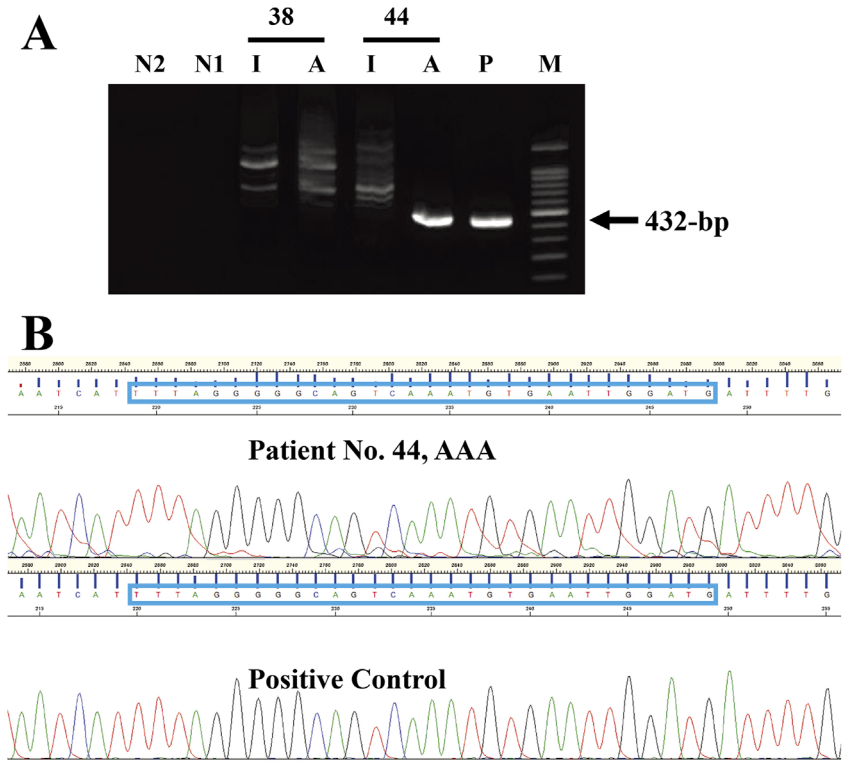

Fig. 2. Detection of *Helicobacter cinaedi* DNA by nested PCR, followed by nucleotide sequencing analysis. (A) Representative results of nested PCR after electrophoresis in a 2% agarose gel. N1, negative control for the second PCR using the negative control at the first PCR as the template to detect the faint contaminated *H. cinaedi* DNA. N2, negative control for the second PCR. I and A denote iliac artery and AAA, respectively, and the results of patients 38 and 44 are shown. P, purified *H. cinaedi* DNA used as the positive control; M, size marker. The expected product size is 432-bp as indicated on the right of the gel. Only 44A showed a clear band comparable to the positive control. (B) Results of nucleotide sequencing analyses of nested PCR products (44A and P) are shown. Nucleotide sequences of both strands were analyzed, and sequencing results of the sense strand are shown; these products harbored sequences identical to those of the *cdtB* gene of *H. cinaedi* (NC_020555.1). The specific sequences in *cdtB* of *H. cinaedi* are boxed in blue.

extracted DNA was assessed by PCR with a human *ACTB* primer set using 2 μL DNA, and all the analyzed DNAs showed a band corresponding to *ACTB* (data not shown). Two microliters of DNA were used for PCR-based *H. cinaedi* analysis. None of the 39 patients showed bands in the first PCR, whereas nine (23.1%) showed bands corresponding to *H. cinaedi* after nested PCR. Typical examples are shown in Fig. 2A. The AAA sample (number 44) showed a clear band equivalent in size to that of the positive control (lane P, purified *H. cinaedi* DNA). The bands obtained from these nine patients and the positive control were purified and their nucleotide sequences were determined. Non-aneurysmal iliac arteries as controls were available from six of these nine positive patients, and none of these six samples showed a band corresponding to *H. cinaedi*. Non-aneurysmal iliac arteries were available in 14 of the 30 PCR-negative patients, and none of these iliac arteries showed positive bands. The results and clinical characteristics of the patients are summarized in Table 2. No significant associations were observed between the two groups.

Sequence analyses of PCR products

Typical examples of the sequencing results are shown in Fig. 2B; DNA from AAA sample number 44, shown in Fig. 2A, clearly demonstrated a DNA sequence identical to *cdtB* of *H. cinaedi* (top column in Fig. 2B). Purified *H. cinaedi*, used as a positive control (lane P in Fig. 2A), also showed the same sequence (bottom column in Fig. 2B). The nucleotide sequence specific to *cdtB* in *H. cinaedi* is shown.

Discussion

In this study, we examined clinically non-infectious AAA using a highly sensitive and specific detection method and observed *H. cinaedi* DNA in nearly one-fourth of the aneurysmal wall. To the best of our knowledge, this is the first report of the detection of *H. cinaedi* in the walls of clinically non-infectious AAA.

The sensitivity of *H. cinaedi* detection has recently improved from bacterial culture (Totten et al. 1985) to PCR-based genetic methods, where the initial targets for PCR were *16S rDNA* (16S ribosomal RNA) or *gyrB* (gyrase protein B-subunit) (Pena et al. 2002; Minauchi et al. 2010).

Oyama et al. (2012) improved the sensitivity and specificity using nested PCR targeting *cdtB*, which resulted in a significant reduction in the risk of misidentifying other *Helicobacter* species.

The common symptoms of *H. cinaedi* infection are diarrhea, abdominal pain, enteritis or proctitis (Kawamura et al. 2014); however, extraintestinal infections, such as cellulitis, erysipelas, arthritis, infectious endocarditis, cerebral meningitis, and sepsis, also occur (Kawamura et al. 2014; Okubo et al. 2014; Nishida et al. 2016; Kushimoto et al. 2017; Hase et al. 2018); these are possible consequences of secondary bacteremia after asymptomatic infection.

Khan et al. (2012) identified *H. cinaedi* in arteriosclerotic tissues during autopsy. Khan et al. (2014) proposed a mouse model in which the development of *H. cinaedi*-mediated arteriosclerosis caused by monocyte-derived macrophage-mediated mechanisms, along with changes in cholesterol metabolism, could cause arteriosclerosis. We previously reported the detection of *H. cinaedi* in infectious AAA (Kakuta et al. 2014), which suggests that *H. cinaedi* may invade the arterial walls. These recent findings suggest that *H. cinaedi* is one of the strongest candidates associated with arterial disorders. Hence, it is important to study the association between *H. cinaedi* infection and atherosclerotic AAA.

Several factors are implicated in the initiation and progression of AAAs, including proteases associated with macrophages, genetic alterations, and local hypoxia of the arterial walls. One probable hypothesis is that macrophages, coupled with inflammation and/or oxidative stress, induce matrix metalloproteinase 9 (MMP-9) expression in the arterial wall, and this proteinase-driven degeneration in the aortic tunica media triggers further progression to aneurysms (Longo et al. 2002; Raffort et al. 2017). Based on these observations, we focused on atherosclerotic AAA and hypothesized that a preceding infection with *H. cinaedi* can cause asymptomatic bacteremia and/or intravascular inflammation that induces cascades, including inflammatory cell-derived mechanisms that are associated with the initiation and/or progression of AAA. We detected *H. cinaedi* DNA exclusively in aneurysmal walls and not in non-aneurysmal arterial walls. These results are consistent with our initial hypotheses. If infection with *H. cinaedi* is indeed one of the crucial pathogeneses of atherosclerosis and/or aortic dilatation, patients with small AAA may be treated by eradicating this bacterium.

The present study had some limitations. First, we could not use healthy aortic walls as the control because of ethical issues. Therefore, we used the iliac artery as a control whenever possible. The possibility of false negatives and false positives cannot be completely excluded because the number of bacteria contained differed among samples. In addition, the purity of the extracted DNA and its concentrations may vary. Furthermore, the number of samples was limited because of the recent shift in surgical methods from open surgery to EVAR. However, this study is significant, as it is the first to investigate the importance of *H. cinaedi* in AAA. Further investigations, including animal models or liquid biopsies from AAA patients, are necessary to identify the yet-unknown pathogeneses of AAA and to guide the clinical management of patients with AAA.

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Author Contributions

Conception and design: S.H., H.S., H.G., and D.A.; Sample collection: S.H., H.S., H.G., M.H., D.A., Y.T., and M.U.; Analysis and interpretation: S.H., H.S., H.G., T.M., and T.A.; H.G. and T.K. provided the final approval of the version to be published.

Conflict of Interest

The authors declare no conflict of interest.

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