academicJournals

Vol. 7(17), pp. 1723-1728, 23 April, 2013
DOI: 10.5897/AJMR12.1482
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Relationship between aneurysm and microorganism: Is *Helicobacter pylori* a primer agent or has an affinity to the tissue?

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Accepted 29 March, 2013

Aneurysm and microorganism relationship, which is urged to be a cause of atherosclerotic and non atherosclerotic pathogenesis, is a subject that has been discussed for years. In addition to many known microorganisms about this relationship, in recent years *Chlamydophila pneumoniae*, anaerobic bacteria and *Helicobacter pylori* are the most emphasized microorganisms suggested as a possible factors influencing the development and expansion especially of abdominal aortic aneurysm. In this study, we aimed to evaluate the potential etiopathogenetic relationship between abdominal and ascending aneurysm and *H. pylori*. The study was conducted between January 2010 and December 2010 as a cross-sectional, case-control study with 50 cases admitted to TR Ministry of Health, Dr. Siyami Ersek Thoracic and Cardiovascular Surgery Training and Research Hospital, Department of Cardiovascular Surgery and diagnosed with abdominal aortic aneurysm (AAA) and ascendıng aortıc aneurysm (AsAA) according to their clinical findings and their radiodiagnostic data. Laboratory studies of the research were performed in Serology-enzyme linked immunosorbent assay (ELISA) and Molecular Biology laboratories of the Depatment of Medical Microbiology in Istanbul University, Cerrahpasa Faculty of Medicine and in private Burc Molecular Diagnostic Center. The study was performed in three groups. The first group was the patient group (PG) including 50 patients with aneurysm (40 ascending, 10 abdominal), the second group was the patient control group (PCG) including 30 patients with post-stenotic aneurysm (PSAG) and the third group was the healthy control group (HCG) with 47 healthy individuals selected among the people admitted to Computed Tomography (CT) laboratory of the department of radiodiagnostics of Istanbul University (IU) Cerrahpasa Faculty of Medicine (CFM) according to their particular complaint, but not having abdominal or cardiovascular complaints resembling the AAA and AsAA. *H. pylori* IgG test was applied to the serum samples taken from all people of the three groups by enzyme immunoassay method. Additionaly, DNA extraction was applied using a commercial tissue extraction kit in the tissue specimens of the patient group and in the tissue specimens of patients with post-stenotic aneurysm. *H. pylori* DNA was determined by real-time PCR method using a commercial kit (WAY2GENE® *Helicobacter pylori*, Genmar, Turkey) according to the manufacturer's protocol. *H. pylori* IgG positivity was detected in PG, PSAG and HCG as 76, 57 and 57%, respectively. *H. pylori* DNA was detected only in one male patient with atherosclerotic abdominal aneurysm and could not be detected in other 49 cases. While these strain's genotype was identified as VagA s1b/m1, it was found to be negative for all other genes. In conclusion, however, VagA s1b1 type *H. pylori* was detected in vascular tissue of a case with atherosclerotic abdominal aneurysm and not detected in PCG with post-stenotic aneurysm, these data are not enough to support an etiopathogenetic relationship between aneurysm and *H. pylori*. The presence of *H. pylori* DNA in only one case suggests that *H. pylori* is not a primer agent and probably has an affinity to the vascular tissues.

Key words: *Helicobacter pylori*, aneurysm, PCR.

INTRODUCTION

There is evidences that certain microorganisms have an etiopathogenic role in the development of aneurysm. *Chlamydophila pneumoniae* and anaerobic bacteria have been suggested as a possible factors influencing the development and expansion of abdominal aortic aneurysm (AAA) (Mazur et al., 2004; Brook, 2009) . *H. pylori* which is a *G*ram-negative rod colonised in the stomachs of humans was considered as a bacteria causing chronic gastritis and playing important roles in peptic ulcer disease, gastric carcinoma and gastric lymphoma. Additionnaly virulence factors, such as c*ag* A, v*ac* A and i*ce* A genes related to pathogenicity, as well as host factors were defined (Dunn et al., 1997; Gatti et al., 2005). According to the findings of previous studies, subjects harbouring Cag A positive strains of *H. pylori* have been considered to be in risk of carotid atherosclerosis and coronary heart diseases (Nyberg et al., 2008; Ziver et al., 2010). In the pathogenesis of AAA and ascending aortic aneurysm (AsAA), inflammatory response is considered significant and it is also suggested that generally microorganisms may be triggering factors for the initiation of inflammatory process in the host. The most frequently discussed of these microorganisms was initially *Chlamydophila pneumoniae* as well as herpesviruses (Kotze and Ahmed, 2011). Recently, a number of studies investigating whether Cag A positive *H. pylori* may increase the risk of atherosclerosis and coronary heart disease have been increased. A case with abdominal aortic aneurysm infected with *Helicobacter pylori* was presented (Hırose and Kugimiya, 2000) and additionnaly epidemiologic studies detecting *H. pylori* especially in carotid atherosclerotic plaques were published (Ameriso et al., 2001; Singh et al., 2002; Mayr et al., 2003; Karlsson et al., 2000) but until now, a precise relationship between *H. pylori* virulence factors and the pathogenesis of aneurysms was not revealed.This study aims to evaluate the role of *H.pylori* in the etiopathogenesis of AAA and AsAA.

MATERIALS AND METHODS

A total of 50 cases admitted to the department of Cardiovascular Surgery of TR Ministry of Health, Dr. Siyami Ersek Thoracic and Cardiovascular Surgery Training and Research Hospital and diagnosed with AAA and AsAA according to their clinical findings and radiodiagnostic data were enrolled to this study.The study was performed between January 2010 and December 2010 as a cross-sectional, case-control study .

Cases with abdominal aortic aneurysm (AAA) consisted of 10 patients having an abdominal aortic diameter larger than 3 cm determined upon radiodiagnostic methods. Three (3) (30%) of them had ruptured AAA, whereas 7 (70%) had non-ruptured AAA, while both groups had an aneurysm diameter of 5 cm. All of the cases with AAA included in the study had a past surgical procedure or had undergone endovascular intervention.

Cases with ascending aortic aneurysm (AsAA) consisted of 40 patients having an ascending aortic diameter larger than 3 cm. Two (2) (5%) of them had ruptured AsAA and from the non ruptured AsAA group, 25 (6.5%) had a minimum of 5 cm aneurysm diameters and 13(32.5%) had maximum 5 cm aneurysm diameters. All patients included in AsAA group had undergone a surgical procedure in the past.

Cases with post stenotic aneurysm consisted of 30 patients. This group was used as a patient control group (PCG). The healthy control groups (HCG) was formed by selecting 47 individuals among people admitted to Computed Tomography (CT) laboratory of the department of radiodiagnostics of Istanbul University (IU) Cerrahpasa Faculty of Medicine (CFM) according to their particular complaint, but not having abdominal or cardiovascular complaints resembling the AAA and AsAA. Abdominal and ascending aortic diameters were within normal limits (<3 cm) in individuals of this group.

The data and results obtained from both groups, such as age, gender, education status, occupation, smoking habits (smokers among the study groups were considered smokers), hypertension, diabetes, cardiovascular disease in family history, total cholesterol, triglyceride, HDL and LDL values, diameter of aneurysm, whether it is ruptured and potential stomach complaints were collected and recorded. Laboratory studies of the research were performed in Serology-ELISA laboratories and molecular biology laboratories of IU CFM department of medical microbiology and in Private Burc Molecular Diagnostic Center.

Blood and aneurysm tissues sampling and test methods were performed by the following procedures. Blood (10 ml) samples were collected by venous puncture from each of the patient and control group cases. The blood samples were centrifuged 5 min at 3000 rpm and the preserved serums were stored at -80°C until laboratory studies. *H. pylori* specific IgG was determined by ELISA method. Aneurysm tissue samples were stored at -80°C until the test day.

For the determination of *H. pylori* IgG, 1/101 diluted serum samples were tested using *H. pylori* ELISA IgG kit (Euroimmun, Lübeck-Germany). A fresh lysate of the standard strain of *H. pylori* ATCC 43504 was used to perform the test with the kit. A negative test result was evaluated when the absorbance was <0.8. An intermediate test result was evaluated when the absorbances were between 0.8-1.1 and a positive test result was evaluated when the absorbances was >1.1.

Tissue samples were collected during operations from 50 patients with AAA and AsAA and from 30 patients with post-stenotic aneurysm.They are immediately frozen at -*70*°C until the test day. For the DNA extraction of H. pylori, collected tissue samples were minced using surgical scalpales under sterile conditions. Genomic DNA was extracted from 25-30 mg of minced tissue using a commercial DNA extraction kit (High Pure PCR Template Preparation Kit, Roche Diagnostic GmbH Mannheim, Germany) following the manufacturer's instructions. All extracts were stored at -80°C until usage. *H. pylori* DNA was determined by real-time PCR method using a commercial kit (WAY2GENE® Helicobacter pylori, Genmar, Turkey) according to the manufacturer's protocol.

Real-time PCR mixture contained 2 µl of sample DNA, 1.6 µl (25 Mm) $MgCl_2$, 14.4 µl agent mix (primers and prob), 2.0 µl enzyme mix in 20 µl finale volume. The assay run on the Roche Light Cycler. The reaction was performed with preliminary denaturation for 10 min at 95°C (slope, 20°C/s), followed by 45 cycles of denaturation at 95°C for 10 s (slope, 20°C/s), annealing at 55°C 10 s (slope, 20°C/s), extension at 72°C for 15 s (slope, 20°C/s). During PCR reaction this process, fluorescences signals was collected in

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Table 1. The primer set used in this study.

Primer set	Sequence (5'-3')	Nucleotide	Product size
HP1	gcc aat ggt aaa tta gtt cc	304	
HP2	ctc ctt aat tgt ttt tac at	714	410 bp
HP3	agt tcc tgg tga gtt gtt ct	318	
HP4	agc gcc atg aaa acc acg	678	360 bp

HP: *H. pylori*

Table 2. Demographic details of Patient and Healthy Control Group.

Study group	(n)	Age	Gender		Smoking	
			Female	Male	Smoker	Non-smoker
Total Aneurysm (Abdominal/Aort)	50	(34-78)	17	33	12	38
Patient Control Group	30	(38-72)	11	19	14	16
Healthy Control Group	47	(29-67)	15	32	19	28

single including mode continuously and melting curves were generated automatically by LightCycler.

Standard nested PCR protocols were used for amplification of *H.pylori*. Previously tested clinical samples for *H. pylori* were used as positive control (urea breath test). Primers choosen from urease A gene region of *H.pylori* were used for the PCR amplification as previously described (Wang et al., 1993) (Table 1). The PCR mixture contained 0.5 μM each primer, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 0.2 mM each dNTP (Fermentase®, Lithuania), and 1.25 U of *Taq* DNA polymerase (Fermentase®, Lithuania). The final reaction volume was 50 μl containing 10 μl of DNA sample. Nested PCR was performed by transferring 2 μl of the first PCR product to the second round reaction mixture. The cycling conditions used in both rounds consisted of an initial denaturation at 95°C for 2 min, 40 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s followed by a final extension step of 5 min at 72°C. The amplification products were run on a 1.5% agarose gel electrophoresis and visualised on an UV transilluminator.

For genotyping analysis, the cagA, cagE, vacA s1/s2, vacA m1/m2, iceA1/A2, and babA2 genotypes were carried out utilizing PCR assay by using specific primers as described previously (Erzin et al., 2006). Briefly, PCR reaction was performed in a total volume of 25 µl containing approximately 100 ng DNA, 2.5 µl of 10X polymerase buffer, 2.0 mmol/l $MgCl_2$, 0.2 mmol/L dNTPs, 0.4 µmol/l of each primer, and 1 U of Taq polymerase (MBI Fermentas). The PCR program on the icycler™ (BioRad) thermal cycler was as follows: an initial denaturation step at 94°C for 4 min, followed by 40 cycles of 30 s at 94°C, 30 s at 52°C, 1 min at 72°C, and a final extension step of 8 min at 72°C. The amplification products were electrophoresed on 2% agarose gels at 100 Volt for 30 min. The gel and running buffers were 1X TBE (0.89 M Tris-Base, 0.89 M Boric Acid, 20 mM Na2EDTA). The fragments were visualized by ethidium bromide under UV transilluminator.

This study was approved by the ethical committee of Istanbul 1. No Clinical Studies in Deanery of Cerrahpasa Faculty of Medicine by decision no; C-011 on 12.01.2010.

Statistical analysis

SPSS 17.0 statistical program was used for the assessment of the data obtained from the study. Chi-square, Fisher, T test and variance analysis were used in the assessment; $p<0.05$ was found to be significant.

RESULTS

The patient group with 50 cases (10 diagnosed with AAA, and 40 diagnosed with AsAA), the PCG with 30 cases diagnosed with post stenotic aneurysm and the HCG with 47 person were included in this study by being matched in terms of age, gender and smoking habits (Table 2).

In the patient group of 50 cases, 33 were female and 17 were male. Their age were between 34-78 and 12 were smoker and 38 non smoker. In the PCG of 30 case, 11 were female and 19 were male. Their age was between 38-72 and 14 were smoker and 16 non smoker. In the HCG group of 47 individuals, 15 were female and 32 were male. Their age was between 29-67 and 19 were smoker and 28 non smoker. Hypertension was determined in 87% of our total cases diagnosed with aneurysm (AAA,AsAA,PSAG).

The systolic blood pressure of these patients was >160 mmHg, and the diastolic blood pressure was >95 mmHg. 89% of our total cases diagnosed with aneurysm were receiving diabetes treatment. Hypertension and diabetes was not detected in individuals of our healthy control group. In 76% of the PG with 50 cases, in 56.7 % of the PCG with 30 cases and in 60% of HCG with 47 person *H. pylori* IgG was found positive. A statistically significant difference was not found for *H. pylori IgG* positivity in comparisons between all study and control groups. ($p>0.05$) (Table 3).

H. pylori DNA was detected in one tissue sample out of 50 patient group with AAA by real-time PCR (Figure 1). This case belong to a 75 years old male patient with AAA secondary to atherosclerosis pathology. The same patient sample was found to be positive with nested PCR method. The size of the PCR product was 360 bp (Figure 2). That positive sample was genotyped by PCR analyses. PCR analysis revealed that the *H. pylori* strain

Table 3. Distribution of ELISA results in Patient, Patient Control and HCG cases.

Study group	n	*H. pylori* IgG (+)	*H. pylori* IgG (-)
		n(%)	n(%)
Aneurysm[(1)]	50	38(76)	12(24)
Patient Control Group[(2)]	30	17 (56.7)	13(43.3)
Healthy Control Group[(3)]	47	28(60)	19(40)

1x2= p>0.05; 1x3= p>0.05.

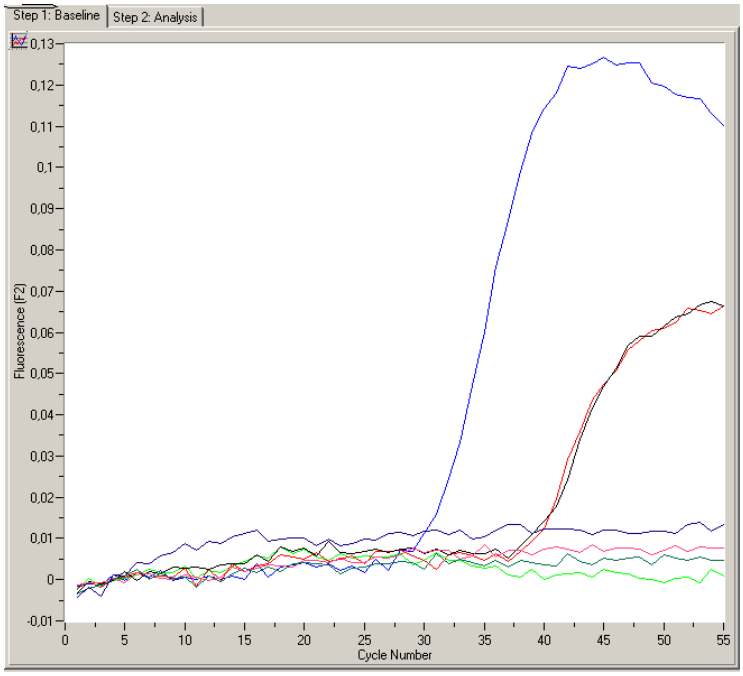


Figure 1. The figure shows amplification curves of positive control and *H. pylori* positive patient.

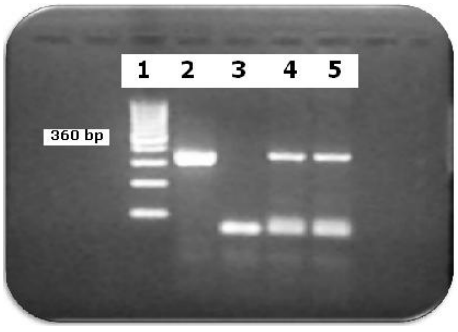


Figure 2. PCR product of *H .pylori* positive case. Lane1:100 bp ladder, lane 2: positive control lane 3: negative control, lane 4: patient sample, lane 5: same patient sample.

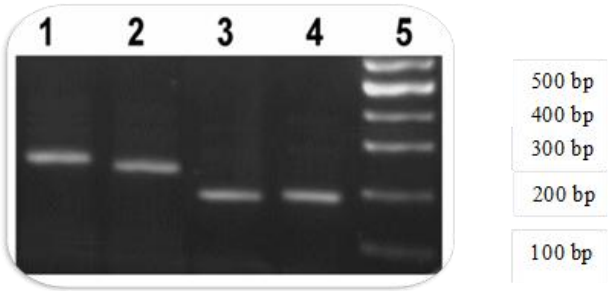


Figure 3. Agarose gel electrophoresis showing the PCR based *H. pylori* genotyping. Lane 1: VacA s1(259 bp) ; Lane 2: VacA m1 (230 bp); Lane 3: Vac S1b (187 bp); Lane 4: *H.pylori* specific amplification (190 bp); Lane 5: 100 bp DNA ladder.

has the genotype of VacA S1b/m1 (Figure 3). Other tested virulance genes were negative in our patient. It was reported that type s1/m1 strains produced a higher level of cytotoxin activity *in vitro* than type s1/m2 strains; none of 19 type s2/m2 strains produced detectable

cytotoxin activity (Atherton et al., 1995). *H. pylori* DNA was not detected in any tissue samples of the other control groups.

DISCUSSION

Atherosclerosis is the mostly emphasized factor among the factors suggested to be related to the pathogenesis in both types of aneurysm. Unlike the risk factors, such as gender, age, weight, smoking habits, hyperlipidemia known to be involved in atherosclerotic plaque development, it is claimed that the trigger factor may be the inflammatory response of microorganisms in vessel tissue (Mehta et al., 1998; Ross, 1993; Alexander, 1994).

It is also known that mycotic aneurysms may develop due to bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella* spp. and *Streptococcus* spp. reproducing on atherosclerotic plaques (Kumara et al., 2009). In 1997, Saikku, (1997) reported a review particularly the role of *Chlamydophila pneumoniae*, suggesting that this bacterium may be the trigger factor in the development of atherosclerosis and arterial diseases.

After this introduction, as suggested in *Chlamydophila pneumonia* atherosclerosis relationship, *H. pylori* may colonize to the atheromatous tissues instead of normal aorta wall and may cause a lipid accumulation in vessel lumens. There are remarkable studies conducted in recent years on *H. pylori* suggested to induce aneurysms and to trigger atherosclerosis due to the constant and mild inflammatory response. Acute phase reactants including CRP and fibrinogen, T cell response synthesizing interferon-gamma and proinflammatory cytokine levels are seriously increases by *H. pylori* in the host, as well as virulence factors such as CagA, VacA, after *C. pneumoniae*, which are known to be the primary pathogen of gastrointestinal area, particularly gastric tissue (Mendall et al., 1994; Pasceri et al., 1998; Murray et al., 1995; Mendall et al., 1996; Whincup et al., 1996). This probable hypothesis needs to be clarified with new prospective experimental animal studies.

Nyberg et al. claimed for the first time until our previous study (Ziver et al., 2010) that a pathogenesis mechanism can play a role between *H. pylori* and coronary hearth disease and AAA and AsAA.They investigated on a seroepidemiological basis study the fact that *H. pylori* was associated with coronary heart disease but they did not detected any statisticaly significant differences between their patient and control groups in term of *H. pylori*-IgG positivity and additionnaly they did not found any connection between *H. pylori* CagA seropositivity and abdominal aortic aneurysm rupture. According to our *H. pylori*-IgG results, we did not detect also any statistically significant difference for *H. pylori* IgG positivity between our study and control groups. This result matches with the result of the seroepidemiological study reported by Nyberg et al. Hovewer, Ziver et al. (2010) detected a statisticaly significant difference for VacA seropositivity but not for CagA between their study and control groups. According to our *H. pylori*-IgG results of this present study, we can not state that there is a relation between aneurysm and *H. pylori* .

In molecular based studies concerning aneurysm and *H. pylori* relationship; Hirose and Kugimiya (2000) reported in a case study, a relevance between AAA and *H.pylori*, Morre et al. (2000) could not detect evidences supporting the presence of *H. pylori* in atherosclerotic plaques but detected seropositivity for *H. pylori* IgG. Hovewer, contrary to these studies, Falkensammer et al. (2007) reported that no *H. pylori* DNA was presented in tissue samples of 98 AAA cases. Martinez and Martinez, (2002) concluded that *H. pylori*' may be an indirect agent in atherothrombi development and cardiovascular diseases in their consideration "*H. pylori;* a new cardiovascular risk factor?". Mannacio et al. (2011) detected no *H. pylori* DNA in aortic aneurysm samples of 100 cases but detected a significant statistical difference for CagA and VacA positivity between patient and control groups. They detected VacA and the sub-genotype of *H. pylori* strain was detected as VacA s1m. Hovewer, in the present study, we could not detect *H. pylori* DNA in 49 AAA and AsAA (40 non-atherosclerotic and 9 atherosclerotic) and 30 post sterotic aneurysm cases, we detected *H. pylori* DNA in a 75 years old male patient with AAA developed secondary to atherosclerosis. AAA was detected in this patient by abdominal ultrasonography in the cours of the diagnosis of an acute appendicitis, two years ago before his application to our center with AAA. This patient was followed-up by computed tomography (CT) and the diameter of AAA was reached to 63 mm with calcified mural thrombus in 2008. Atheroms were also detected in aort and other arteriols. The genotype of this *H. pylori* strain was VacA s1b/m1 and any of the *cagA*, *cagE*, *iceA* and *babA2* genes were presented. Hovewer, even this case with no significant statistical difference, our results were in accordance with the study of Mannico et al. (2011) and Ziver et al. (2010). There was an accordance of our results with the consideration of "VacA may have a role in the pathogenesis of aneurysms" in the seroepidemiological based on the study of Ziver et al. (2010) that was aimed to detect virulance factors.

According to these results, we suggested that, VacA protein which is coded by VacA gene may induct vacuole formation in the membranes of the host cells and also induct apoptosis. VacA protein may also induct the proinflamatuary cytokines and also cause immunosuppression. VacA protein may also induct the activation of CagA protein by activating itself. All of the above suggestions may lead us to think, *H. pylori* strains harboring VacA may trigger aortic aneurysms based on atherosclerosis in such persistant, chronical *H. pylori* infections. Hovewer, we can not definitely say, *H. pylori* is

a definitely triggering agent in aneurysm cases based on atherosclerosis and we can only suggest, *H. pylori* may be a secondary agent which has an affinity to the vascular lesions.

In conclusion, however, VagA s1b1 type *H. pylori* was detected in vascular tissue of a case with atherosclerotic abdominal aneurysm, these data is not enough to support an etiopathogenetic relationship between aneurysm and *H. pylori*. Presence of *H. pylori* DNA in only one case suggests us that *H .pylori* is not a primer agent and probably has an affinity to the vascular tissues. Hovewer, the detection of this type of *H. pylori* in a AAA patient with atherosclerosis, suggested us that new, matched, large serial clinical prospective and case-control based seroepidemiological/molecular studies with atherosclerotic patients were needed in order to clarify this relationship.

ACKNOWLEDGEMENTS

This study was supported by Istanbul University, Research Fund (project No: 4131). We are thankful for this support. We also would like to thank Msc. Mutaz Almuhtaseb (Department of Medical Microbiology, Cerrahpasa Faculty of Medicine) for his translation support and Private Burc Molecular Diagnostic Center for their technical support.

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