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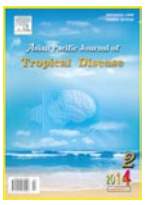

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Brucellosis: presence of zoonosis infection 3 500 years ago in North of Iran

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ABSTRACT

Objective: To investigate the presence of brucellosis in graved human and animal buried in the same graves around 3 500 years ago.
Methods: Two ancient graves in AH2XX1 trench in Gohar Tepe (Behshahr, North of Iran) in which human and animal were buried in single grave were selected for this study. After DNA extraction from human and animal tooth samples, TaqMan real time polymerase chain reaction method was utilized to check and measure the presence of brucellae bacteria.
Results: Brucellosis was found in human with 3.9×10^5 copy/0.05 gr and in animal with 6.5×10^3 copy/0.05 gr in grave one. In grave two, 2.6×10^5 copy/0.05 gr in human and 7.7×10^3 copy/0.05 gr in animal were found.
Conclusions: This study demonstrated the presence of brucellosis infections in this region in 3 500 years ago. Also, it showed that the transmission of brucellosis in Gohar Tepe might be due to the frequent use of milk and meat as the main energy source by the residents. Although previous research claimed that brucellosis was limited to the European continent in the Middle Bronze Age, the current research showed the presence of brucellosis in Middle East region. An advantage of this study over previous research is the use of real time polymerase chain reaction which is more rigorous for studying the presence of brucellae in ancient objects compared to Paleopathological method.

1. Introduction

Brucellosis, a major bacterial zoonosis, is a globally important infection[1]. This small, facultative intracellular, aerobic Gram-negative coccobacilli pathogen can influence a wide range of mammals including man, cattle, sheep, goat, swine, rodents and marine mammals[1,2]. This bacterium is most commonly transmitted to human through the consumption of unpasteurized milk products, direct contacting with infected animal or inhalation of aerosolized droplet[3]. David Bruce identified brucellae for the first time in 1887, and it was accordingly named after him[4]. However, it was found by Shaw and Zammit that brucellosis could be transmitted from animal to human, a point which was doubted by Bruce *et al*.[5]. Brucellosis can annually cause more than 500 000 human infections worldwide and it remains as one of the major public health problems in Mediterranean region, Western Asia, parts of Africa and Latin America[6]. The Middle East has traditionally been considered as an endemic area of brucellosis and Iran, in the center of Middle East, is one of the countries with highest incidence for human brucellosis worldwide. And this disease remains a huge burden for the country[7].

Archeological excavation in Gohar Tepe (a region in the north of Iran) (Figure 1) started in 2005, and was associated with the discovery of an ancient civilization. Based on radiocarbon dating studies, the existence of Gohar Tepe dates back to the Middle Bonze Age and lasted to the Iron Age[8]. One of the interesting findings in excavations was graves with human skeletons buried with animals (goat or sheep) together. In some cases the animals were also covered with clay. Some paleopathological signs of infection were found by Sołtysiak and Mahforouzi[8] in two ancient graves that selected for this study. This study aimed to investigate the possible presence of brucellosis in Gohar Tepe (Behshahr-North of Iran) by molecular approach. Samples collected from human and animal for examining

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the possible presence of brucellosis genome in tooth of both human and animal.

Figure 1. Behshar located at east part of Mazandaran Province in North of Iran where ancient district of Gohar Tepe founded in 2005.

2. Material and methods

2.1. Samples collection

Two graves were selected for this study; they belonged to AH2XX1 trench in Gohar Tepe. Grave 1 (F5AH2XX1) was an eighteen year old female with some pottery and animal (Figure 2) and in Grave 2 (F18AH2XX) was a young female with damaged bones but without the long bones of the hands and feet as well as with an animal covered in clay. Parts of samples were sent to Cambridge University for radiocarbon dating. All necessary permits were obtained for the described field studies from Dr. Farhang Khademi from Tarbiat Modares University and Ali Mahforouzi (Head of research team of culture heritage organisation in Gohar Tepe, behshahr-Iran). The samples were carried out separately to prevent any contaminations. Tooth surfaces were drilled and ground using disposable equipment.

Separate mortar and pestles were utilized to grind the samples until a fine ground powder was created.

Figure 2. Grave 1 of AH2XX1 trench that contained a skeleton of a woman with a goat buried below her.

2.2. DNA extraction

A total of 10 mL of extraction solution (containing 0.45 mol/L Ethylene Diamine Tetraacetic Acid and 0.25 mg/mL proteinase K, pH 8.0) was added to 500 mg of sample powder and the capped tubes sealed with parafilm were incubated with slow rotation for 24 h and at the following day, they were further incubated in 56 °C for 1-3 h[9]. Samples were centrifuged for 2 min at 5000 r/min and the supernatant was used for further processes.

DNA extraction process was done by Genejet™ silica bead DNA extraction kit (Fermentas GmbH, Germany). In summary, supernatant transferred to a new tube, 30 mL of binding buffer and 20 μL silica suspension were added to the solution. pH was also adjusted by sodium acetate, then tubes were incubated in 55 °C for 1 h in the dark. Samples were centrifuged for 15 seconds at 16000 r/min and supernatant was discarded completely. A total of 1 mL of washing buffer (ice-cold) was added to silica pellet and the silica was re-suspended and the washing procedure was repeated for 2 times. They were centrifuged again for 15 seconds at 16000 r/min and the remaining liquid was removed and dried at room temperature for 15 min with open lids. Finally, 50 μL elution buffer was added to the dried silica and resuspended and incubated at 55 °C for 5 min. After centrifuged for 30 seconds at 16000 r/min, supernatant was transferred into a fresh 1.5 mL tube.

DNA quality was measured by spectrophotometer (Nanodrop 2000) and stored at -20 °C until tested. DNA extraction process and polymerase chain reactions (PCRs) were done in separate rooms.

2.3. Real time PCR assay

TaqMan probe and primers were designed using Allele ID 7.0 (PREMIER Biosoft). TaqMan real-time PCR assay was designed based on genus-specific BCSP31 gene which encodes an immunogenic membrane protein of 31 kDa. A 166-bp amplicon was amplified by: sense primer, Mbm1 (5′-ATC GTT CTT GAA GCC TAC-3′) and antisense primer, Mbm2 (5′-AAA TAC CGT TCG AGA TGG-3′). A 24-bp TaqMan probe was designed, Mbm (5′-ATA TCA AGG CTG AAC ACC TGA AGC-3′) and 3′-end is blocked with a phosphate group to prevent extension of probe in the PCR reaction. Mbm TaqMan probe was fluoresce labeled at the 5′-end with 6-carboxyfluorescein phosphoramidite (FAM) as the reporter dye, and the 3′-end with 5-carboxytetramethylrhodamine (TAMRA) as the quencher. The theoretical specificity of the primers and probe was determined by comparison with the Gene Bank database using the Basic Local Alignment search tool (Blast). Reactions were carried out in a total reaction volume of 20 μL containing 10 μL premix Ex Tag™ (Perfect Real time) reagent (Takara Bio Inc, Shiga, Japan), a 100 nmol/L concentration of each primer, a 250 nmol/L concentration of the probe and 5 μL of DNA extract. The real-time PCR was performed on a 7500 real-time PCR system (Applied Biosystems) using the following profile: initial template denaturation at 95 °C for 30 seconds followed by 40 cycles of 95 °C for 5 seconds and 60° C for 34 seconds. Results confirmed by conventional allele specific PCR by above mentioned protocols in Molecular Biology lab of Tarbiat Modares University and PCR amplicons were sent for sequencing. Sequencing results were investigated by Blast and comparing with GenBank sequence number: M20404.1.

3. Results

Radiocarbon dating analysis showed that samples in this research belonged to 3550 years ago. According to analytical sensitivity assay, the method used in this research was

sensitive to detection of brucellae equivalent to 2 genome of *Brucella melitansis (B. melitansis)* 16M. Amplification of 5-fold serial dilution of a plasmid with the cloned fragments of 166 bp from *B. melitansis* 16M showed a linear detection range of $10-10^5$ copy per reaction mixture, with an R^2 value of 0.99 and a PCR efficiency of 2. The Linear regression was Ct=-3.20 log (copy no)+37.5.

We found brucellae in the animal and human in both graves. In grave 1 (Fi5-AH2XX1) copy number of bacteria in human was 3.9×10^5/0.05 gr of tooth, and in animal it was 6.5×10^3 copy/0.05 gr of tooth. In grave 2 (Fi18-AH2XX) copy number of brucellae in human and animal was 2.6×10^5 copy/0.05 gr and 7.7×10^3 copy/0.05 gr of tooth, respectively.

4. Discussion

In this study, for the first time, the presence of brucellosis was demonstrated in ancient world by molecular approach and this bacterium was found in human and animal that were buried together. The DNA extraction method used in this study yield high pure DNA and specific primers and probs were helpful to identify and quantify the bacterium in the samples.

Among the previous research on brucellosis in ancient times, D'Anastasio reported the oldest possible evidence of brucellosis by paleopathological analysis in partial skeleton of the late Pliocene hominin (2.5 million years ago)[4,10]. Capasso described vertebral lesions observed in the skeleton of adults and two millennia-old cheese at Herculaneum (Roman population, 1 900 years ago)[11,12]. In the studies in the Middle East, Ortner reported a case of possible brucellosis in the spine of an adult skeletal individual from Jordan dated back to around 3 100 BC[13]. Also, Ortner *et al.*[14] and Rashidi *et al.*[15] reported possible brucellosis in human remains from Bronze Age in Jordan and Bahrain.

Findings of this study are one of the oldest approved brucellosis so far that show presence of brucellosis in human and animal in Middle Bronze Age in this area. The fact of putting human and animal in the same grave can indicate the importance of meat and dairy products consumption for these populations as basic energy sources. Presence of brucellosis in these samples shows convenient transmission of brucellosis among this population.

In the animal samples, there were more copy numbers of bacteria compared with human samples. This can be due to more persistence of DNA in animal tooth samples or due to more accumulation of bacteria in animal tooth compared to human tooth.

In conclusion, the results confirmed the transmission of brucellosis from animal to human in ancient world. Also, brucellosis seems to be prevalent in Middle East during the Middle Bronze Age (3 500 years ago). The findings further imply that contrary to D'Anastasio[4], brucellosis distributions were not limited to the European continent in Middle Bronze Age.

An advantage of this study over previous research is the use of real time PCR which is more rigorous for studying the presence of brucellae in ancient samples compared with paleopathological method.

Conflict of interest statement

We declare that we have no conflict of interest.

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