**Probable Role of *Bilophila wadsworthia* in Appendicle Infection**

**ABSTRACT**

**Background and Objective:** *Bilophila* spp. are gram-negative, pleomorphic rod, obligate anaerobe, oxidase-negative, catalase-positive and non-motile bacteria. *B. wadsworthia* is type species of genus *Bilophila* with the additional characteristic of urea hydrolysis. *B. wadsworthia* can be found in a variety of anaerobe infections, particularly appendicitis and intra-abdominal infection that are considered as important opportunistic pathogens.

**Methods:** This study was designed to identify *Bilophila* spp. in clinical specimens by culture and PCR. We examined 91 DNA samples extracted from infected appendix tissues with specific primers.

**Results:** Data showed that *Bilophila* spp. DNA existence in 53.85% (n=49) provided appendiceal tissue.

**Conclusion:** The pathological and molecular examination of infected appendiceal tissues revealed that *B. wadsworthia* is able to act as the primary cause of significant lesions in the appendicle tissues.

**Key words:** *Bilophila* spp., Appendectomy, Appendicle specimens, PCR, Nucleotide sequencing.
INTRODUCTION
The genus *Bilophila* belongs to Desulfobibrionacea in Delta subdivision of protobacteria (1). The strictly anaerobic bacterium *Bilophila wadsworthia* is a gram-negative, bile tolerant, strong catalase positive, non-motile, indole negative, urease positive and non-spore forming rod. The preferred ecological niche of *Bilophila* spp. is unknown but presumably located in the lower gastrointestinal tract. *Bilophila* spp. is considered as virulent because it is the third most common anaerobic isolate in the studies of gangrenous and perforated appendicitis; however, it is present in the feces of about 50% of humans in the mean counts of only 10^5 to 10^6/g (with total flora counts of 10^11/g) (2). *B. wadsworthia*, originally recovered from infections in patients with gangrenous and perforated appendicitis (3), was also isolated from a variety of other clinical specimens, including pleural fluid, joint fluid, blood and pus from a scrotal abscess, mandibular osteomyelitis and axillary hidradenitis suppurativa (4, 5, 6). *B. wadsworthia* was named for its apparent requirement for 20% bile in the growth medium (7). *B. wadsworthia* is also found in intra-abdominal infections, especially appendicitis and extra-abdominal infections, gastrointestinal tract, oral cavity, vaginal fluids of asymptomatic adults, and even the periodontal pockets of dogs (8). The first report on the isolation of *B. wadsworthia* from non-human sources was in pigs (9). *B. wadsworthia* is considered as an opportunistic pathogen in appendicitis and other intra-abdominal infection due its presence in advanced stages of infection (10, 13). Therefore, this study was designed to investigate the occurrence and probable role of *B. wadsworthia* in the appendectomy specimens of patients who had undergone surgery to treat the initial diagnosis of acute appendicitis.

MATERIALS AND METHODS
A total of 91 appendiceal tissues and contents were obtained aseptically by surgery from patients who had undergone appendectomy to treat an initial diagnosis of acute appendicitis in Namazi hospital (Shiraz, Iran) over a 9-month period (October 2015-June 2016). The diagnosis of appendicitis was made by the pathologic examination of the appendiceal tissue. The landmark of pathology or the diagnosis of appendicitis as the gold standard is the presence of inflammatory cells in the wall, lumen and serosa of the appendix. The samples were immediately transferred to microtube containing thioglycollate media; after that, they were transferred beside the ice.comperes to the laboratory of the Bacteriology Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran. To obtain the strains and compare the culture and PCR results, cultures were initially performed as enrichment cultures. All microtubes containing samples were vortexed, placed beside Anaerocult A (Merck, Germany) in an anaerobic jar, and incubated for 72h at 37°C. Initial enriched cultures were transferred to the enrichment culture plates by serially streaking the cultures on Bacteroides Bile Esculin (BBE) agar and blood agar plates; they were then incubated under anoxic conditions at 37°C. *Pseudomonas aeruginosa* were used for testing the anaerobic condition.
A 100µl sample of enrichment culture was used for DNA extraction. Total DNA was prepared from enrichment cultures using the Gram negative DNA extraction kit (Cinnagen, Iran). For extractions, the protocol for Gram negative bacteria was followed as described in the kit. The extracted DNA were determined to be of good quality, and DNA concentration was measured using Nanodrop (10000 V 3.52). DNA concentrations were adjusted to 15ng µL⁻¹ prior to PCR amplification. Ultimately, the extracted DNA samples were stored at -20°C for further use.
Primers targeting the 16S rRNA gene of *B. wadsworthia*, obtained from previously published work, amplified the 16S rDNA gene of the type strain (ATCC 49260, Gen-Bank accession no. L35148) (11).
PCR was carried out in two steps as semi nested reaction. In the first step, the final reaction mixture solution was prepared in 25 µl volume for each reaction which contained 13.8 µl dH₂O, 2.5 µl PCR buffer (Cinnagen, Iran), 1.5 µl (1.5 mM) MgCl₂ (Cinnagen, Iran), 1.5 µl (200 mM) dNTPs (Cinnagen, Iran), 1.5 µl (100 nmol) of both BWf (5’GAATATTGCAGCAATGGGCC3’) and BWr (5’TCTCCGGAATCTCAAGGTG3’) oligonucleotide primers, 1 U of *Taq* DNA polymerase (Cinnagen, Iran), and 3 µl template DNA samples. In the second step, semi Nested PCR was done on the PCR
products of 305 and 207 bp, respectively. Electrophoresis was performed to examine the PCR products. PCR amplimers were assessed by loading 8 µl of the PCR product plus 2 µl of loading buffer into separate wells of a 2% (w/v) agarose gel (Agarose I; Cinnagen, Iran) containing ethidium bromide (0.5 µg/ml). A molecular weight marker (50 bp; Cinnagen, Iran) was loaded into the first well so as to detect the PCR product size. The gel was immersed in TBE buffer and subjected to a voltage difference of 100V which separated the fragments. Visualization was undertaken using an ultraviolet transilluminator (BTS-20, Japan), and the resulting image was captured by a computer software program (AlphaEase; Alpha Innotech). For final confirmation, the partial nucleotide sequencing of nucleotide (nt) 38 to 492 of the 16S rRNA gene was performed by PCR amplification using primers 5′GAATATTGCGCAATGGGC3′ and 5′TCTCCGGTACTCAAGCGTG3′ and subsequent sequence analysis on an ABIPrism 3100 Genetic Analyzer (Applied Biosystems, Inc.). Sequences were further compared with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool) (GenBank accession no. U82813.1) (12).

Figure 1- Amplification fragment of 16S rDNA gene by BWf and BWr primer (305 bp). Lane 1: 50bp DNA size marker, Lane 2: negative control, Lane 3: positive control, Lanes 4-8: some positive samples

Figure 2- Amplification fragment of 16S rDNA gene by BWr and BWn primer in semi nested PCR (207 bp). Lane 1: 50bp DNA size marker, Lane 2: negative control, Lane 3: positive control, Lanes 4-8: some positive samples
RESULTS
Attempts were made to isolate *B. wadsworthia* from appendicle tissues through culture and PCR methods. According to our findings, no colonies were grown out of the 91 cultures enriched on BBE and blood agar plates. DNA extracted from 91 enrichment cultures were used as the template for the 16S rRNA specific primer pairs. We used two primer sets for semi nested PCR which amplified the fragments of 16S rDNA gene of *B. wadsworthia* and yielded 305 and 207 bp PCR fragments (Figure 1 and 2). The primer set was tested on the *B. wadsworthia* purified DNA sample donated by Dr. David Schleheck (University of Konstanz, Konstanz, Germany). A duplex PCR with all three primers and same amplification conditions was done as a complimentary reaction (Figure 3). The presence of *B. wadsworthia* DNA in the enrichment cultures was, therefore, indicated by the positive PCR reactions for 16S rDNA gene, and corresponded well with the results obtained by DNA sequencing (see above).

| Pathologic Diagnosis                                | PCR results | Total |
|----------------------------------------------------|-------------|-------|
| No specific pathologic changes                     | Negative 6  | 2     | 8     |
| Acute suppurative appendicitis and periappendicitis| Zero        | 3     | 3     |
| Early acute appendicitis and periappendicitis      | 2           | 2     | 4     |
| Acute appendicitis with periappendicitis           | 23          | 28    | 51    |
| Follicular hyperplasia                             | 7           | 6     | 13    |
| Fibrotic appendix                                  | 2           | 0     | 2     |
| Fecalith                                           | 0           | 3     | 3     |
| Gangrenous appendicitis                            | 1           | 4     | 5     |
| Perforated gangrenous appendicitis                 | 1           | 1     | 2     |
| Total                                              | 42          | 49    | 91    |

Furthermore, 25 samples of infected appendicles tissue were compared with 25 pathologically normal tissues; 10 out of 25 normal tissues were positive by PCR while from the 25 infected appendicles, 17 were positive by PCR (Table 3).
owing to its presence in a variety of clinical infections such as anaerobic infection, intra-abdominal infection, and appendicitis. Investigations have shown that this species exists in advanced stages of infection (2, 5, 13). Scientists in Helsinki, Finland (13) isolated 28 strains from patients with appendicitis (approximately half of the studied subjects), which is in accordance with the experiments of a group at the Wadsworth Anaerobe Laboratory (2, 13, 14-16). Based on the results of the first experiment, we were not sure about the exact role of B. wadsworthia in appendicitis; therefore, we decided to specify it, for the first time, as normal flora or pathogen, exactly in appendicitis. For this reason, 25 normal and 25 infected appendiceal specimens were compared; the same sex and age were almost kept in view to determine the presence of Bilophila spp. in non-infected tissues specified by the gold standard of pathologic diagnosis. Schumacher et al., evaluated the incidence of B. wadsworthia in appendiceal, peritoneal and fecal samples obtained from children. B wadsworthia was.

Table 3- The Results of PCR on the basis of infected or normal appendicles tissues

| PCR results | Infection Negative | Positive | Total |
|-------------|--------------------|----------|-------|
| Negative    | 15                 | 10       | 25    |
| Positive    | 8                  | 17       | 25    |
| Total       | 23                 | 27       | 50    |

DISCUSSION
Whereas biochemical methods are still employed to identify the rapidly growing bacterial strains in the clinical microbiology laboratories, molecular methods are becoming more common for the identification of slowly growing strains. These methods are further utilized in identifying the strains in the case of inconclusive phenotypical data. There are currently several molecular methods for identification and differentiation of bacterial species, among which 16S rRNA gene sequencing is regarded as the “gold standard” for bacterial identification.

The preferred ecological niche of Bilophila spp. is unknown, but it is presumably the lower gastrointestinal tract. However, this organism is occasionally found in the oral and vaginal floras. Bilophila spp. is considered to be virulent because it is the third most common anaerobic isolate in studies of gangrenous and perforated appendicitis; however, it is present in the feces of about 50% of humans in the mean counts of only 10^5 to 10^6/g (with total flora counts of 10^{11}/g) (3). B. wadsworthia also has a clinical importance owing to its presence in a variety of clinical infections such as anaerobic infection, intra-abdominal infection, and appendicitis. Investigations have shown that this species exists in advanced stages of infection (2, 5, 13). Scientists in Helsinki, Finland (13) isolated 28 strains from patients with appendicitis (approximately half of the studied subjects), which is in accordance with the experiments of a group at the Wadsworth Anaerobe Laboratory (2, 13, 14-16). Based on the results of the first experiment, we were not sure about the exact role of B. wadsworthia in appendicitis; therefore, we decided to specify it, for the first time, as normal flora or pathogen, exactly in appendicitis. For this reason, 25 normal and 25 infected appendiceal tissues were compared; the same sex and age were almost kept in view to determine the presence of Bilophila spp. in non-infected tissues specified by the gold standard of pathologic diagnosis. Schumacher et al., evaluated the incidence of B. wadsworthia in appendiceal, peritoneal and fecal samples obtained from children. B wadsworthia was.

Figure 4-Alignment results of Bilophila spp. partial 16S rRNA gene with 16S rRNA gene sequence of some Bilophila isolates

The nucleotide sequences of 16S rRNA gene PCR product of some recently identified samples showed 100% homology with the previously published B. wadsworthia sequences in GenBank (accession no. U82813.1) (Figure 4-).
recovered from 25% and 28.5% of the patients with non-infected and chronically inflamed appendices, respectively, whereas 53.8% and 64.2% of the patients with acute and complicated appendicitis showed *B. wadsworthia* growth, respectively (17). In two recent studies focused on the bacteriology of appendicitis, *B. wadsworthia* was recovered in 46% of the tissue samples, as well as *Bacteroides fragilis*, *Bacteroides thetaiotaomicron* and *Peptostreptococcus micros*, with a mean recovery rate of more than seven anaerobes in complicated appendicitis cases (2, 13). Baron et al. reported that the recovery rate of *B. wadsworthia* increased according to the grade of appendicitis (2).

Feng et al. observed that a human stool-derived *B. wadsworthia* strain inoculated in specific-pathogen-free mice caused systemic inflammation, adding new evidence to the pathogenicity of this bacterium and implying its potential role in chronic inflammation related metabolic diseases such as diabetes (18).

In the present research, we evaluated 91 samples of inflamed appendix which were appendiceal tissues with acute inflammatory cells in the wall within their lumens. The presence of *B. wadsworthia* was investigated by culture and PCR, which is the highest number of appendical tissue evaluated to find the foot print of *B. wadsworthia*. Data showed the significant connectedness between infection and presence of *Bilophila* spp. in appendix (*P*=0.047). Therefore, *Bilophila* spp. can surely be introduced as an opportunistic pathogen in appendicitis.

**CONCLUSION**

Clear differences were observed between normal and infected appendiceal samples regarding the presence of *Bilophila* DNA. Therefore, based on the pathological and molecular examination of positive appendiceal tissues, *B. wadsworthia* is capable of acting as an important cause of significant lesions in the appendiceal tissues. According to the literature, the frequent occurrence of *B. wadsworthia* in inflamed appendices, its association with other known anaerobic pathogens, and its recovery from infectious processes such as appendicitis are suggestive of pathogenic properties in this species.

**ACKNOWLEDGEMENTS**

The authors wish to thank the staff members of the Department of Pathobiology and all those who collaborated with the authors, particularly the laboratories of microbiology and biotechnology, Faculty of Veterinary Medicine, Shiraz University, Iran. The research was funded by Shiraz University (Grant no. S9331242).

**CONFLICTS OF INTEREST**

None to declare.
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