Probable Role of *Bilophila wadsworthia* in Appendicle Infection

Mohammad Tabatabaei (PhD)
Microbiologist, Department of pathobiology, Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran

Aslam Dehvary (MSc)
Department of Pathobiology, Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran

Bita Geramizadeh (PhD)
Pathologist, Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Mohammad Hadi Niakan (MD)
Surgeon, Trauma Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Corresponding author: Mohammad Tabatabaei

Tel: +987136138696

Email: mtabatabaei2003@yahoo.co.uk

Address: Trauma Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Received: 02 Jan 2018
Revised: 02 Jan 2019
Accepted: 20 Jan 2019

ABSTRACT

Background and Objective: *Bilophlia* 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.

This paper should be cited as: Tabatabaei M, Dehvary A, Geramizadeh B, Niakan MH [Probable Role of *Bilophila wadsworthia* in Appendicle Infection]. mljgoums. 2020; 14(1):

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 dH_2O, 2.5 µl PCR buffer (Cinnagen, Iran), 1.5 µl (1.5 mM) $MgCl_2$ (Cinnagen, Iran), 1.5 µl (200 mM) dNTPs (Cinnagen, Iran), 1.5 µl (100 nmol) of both BWf (5′GAATATTGCGCAATGGGC3′) and BWr (5′TCTCCGGTACTCAAGCGTG3′) 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

product of the first reaction. The final reaction mixture solution was the same as in the first step, but its reverse primer was BWn (5′CGTGTGAATAATGCGAGGG3′).

Following the initial denaturation (at 94°C for 4 min), amplification conditions were: denaturation at 94°C for 1 min, annealing at 49°C for 1 min, and extension at 72°C for one minute. This was repeated for 30 cycles in a Block assembly 96G thermocycler with a hot top assembly (Analytic Jena, Germany), with a final extension of 72°C for 7 min; the PCR products remained in the thermal cycler at 4°C until they were collected. The preparation of the reagents for PCR, addition of the DNA extracts to the mixture, and PCR thermocycling were performed in three separate rooms to prevent crossover contamination by extraneous nucleic acids. A duplex PCR with all three primers and the same amplification conditions was further performed as a complimentary reaction.

The purified DNA of *B. wadsworthia*, kindly donated by Dr. David Schleheck (University of Konstanz, Konstanz, Germany), were used as appropriate positive controls, and sterile nuclease free distilled water was employed as a negative control. Control positive DNA samples were used to test PCR validity, consistently resulting in the expected PCR products of 305 and 207 bp, respectively. Electrophoresis was performed to examine the PCR products. PCR amplicons 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 *16SrRNA* 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

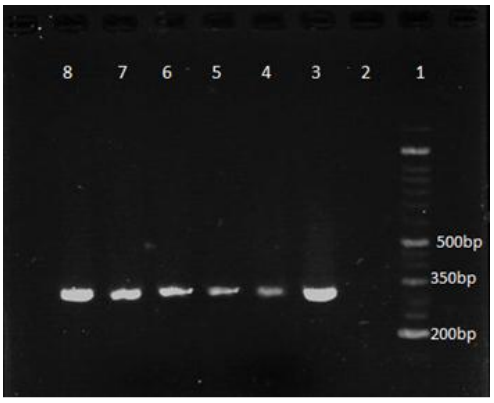


Figure 2- Amplification fragment of 16S rDNA gene by BWr and BWn primer in semi nested PCR (207 bp). Lane 1: 50bp DNA size marker, Lane 2: negative control, Lane 3: positive control, Lanes 4-8: some positive samples

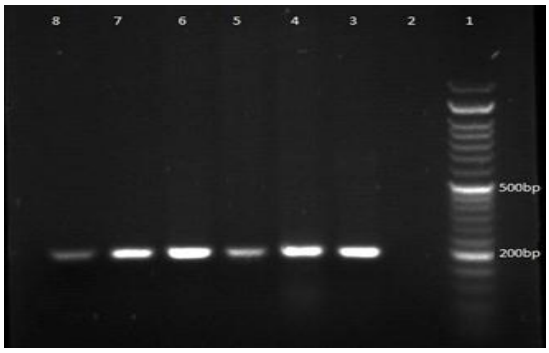
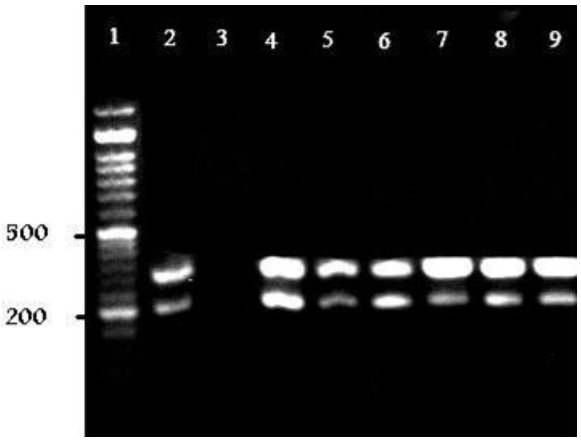


Figure 3- A duplex PCR amplification of 16SrDNA gene by BWf and BWr primers (305 bp) and BWr and BWn primers (207 bp). Lane 1: 50bp DNA size marker, Lane 2: positive control, Lane 3: negative control, Lanes 4-8: some positive samples



RESULTS

Attempts were made to isolate *B. wadworthia* 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).

Table 1- The PCR results on the basis of sex

		PCR		Total
		Negative	Positive	
Sex	Male	25	29	54
	Female	18	19	37
Total		43	48	91

Table 2- The PCR results on the basis of pathologic diagnosis

Pathologic Diagnosis	PCR results		Total
	Negative	Positive	
No specific pathologic changes	6	2	8
Acute suppurative appendicitis and periappendicitis	0	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).

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

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

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-).

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 first time, as normal flora or pathogen, exactly in appendisitis. For this reason, 25 normal and 25 infected appendical 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.

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 appediceal 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 appendicle samples regarding the presence of *Bilophila* DNA. Therefore, based on the pathological and molecular examination of positive appendicle tissues, *B. wadsworthia* is capable of acting as an important cause of significant lesions in the appendicle 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.

REFERENCES

1. Laue H, Friedrich M, Ruff J, Cook AM. *Dissimilatory sulfite reductase (desulfoviridin) of the taurine-degrading, non-sulfate-reducing bacterium Bilophilawadsworthia RZATAU contains a fused DsrB-DsrD subunit.* J Bacteriol. 2001; 183(5): 1727-1733. DOI: 10.1128/JB.183.5.1727–1733.2001.

2. Baron EJ, Bennion R, Thompson J, Strong C, Summanen P, McTeague M, Finegold SM. *A microbiological comparison between acute and complicated appendicitis.* Clin Infect Dis. 1992; 14(1): 227-231. doi.org/10.1093/clinids/14.1.227.

3. Baron EJ, Summanen P, Downes J, Roberts MC, Wexler H, Finegold SM. *Bilophila wadsworthia, gen. nov. and sp. nov., a unique gram-negative anaerobic rod recovered from appendicitis specimens and human faeces.* J Gen Microbiol. 1989; 135(12): 3405-11.

4. Baron EJ, Curren M, Henderson G, Jousimies-Somer H, Lee K, Lechowitz K, Strong CA, et al. *Bilophila wadsworthia isolates from clinical specimens.* J Clin Microbiol. 1992; 30:1882-1884.

5. Summanen PH, Jousimies-Somer H, Manley S, Goldstein EJC, Finegold SM. *Bilophila wadsworthia isolates from clinical specimens.* Clin Infect Dis. 1995; 20 (Suppl 2): S210-1

6. Finegold S, Summanen P, Gerardo SH, Baron E. *Clinical importance of Bilophila wadsworthia.* Eur J Clin Microbiol Infect Dis. 1992; 11(11): 1058-63.

7. Laue H, Smits TH, Schumacher UK, Claros MC, Hartemink R, Cook AM. *Identification of Bilophila wadsworthia by specific PCR which targets the taurine: pyruvate aminotransferase gene.* FEMS Microbiol Lett. 2006; 261(1): 74-79.

8. Urbán E, Hortobágyi A, Szentpáli K, Nagy E. *Two intriguing Bilophila wadsworthia cases from Hungary.* J Med Microbiol. 2004; 53(11): 1167-1169.

9. McOrist AL, Warhurst M, McOrist S, Bird AR. *Colonic infection by Bilophila wadsworthia in pigs.* J Clin Microbiol. 2001; 39(4): 1577-9.

10. Da Silva SM, Venceslau SS, Fernandes CL, Valente FM, Pereira IA. *Hydrogen as an energy source for the human pathogen Bilophila wadsworthia*. Antonie Van Leeuwenhoek. 2008; 93(4): 381-390.

11. Sapico FL, Reeves D, Wexler HM, Duncan J, Wilson KH. and Finegold SM. *Preliminary study using species-specific oligonucleotide probe for rRNA of Bilophila wadsworthia*. J Clin Microbiol. 1994; 32 (10): 2510-2513.

12. Laue H, Denger K. and Cook AM. *Taurine reduction in anaerobic respiration of Bilophila wadsworthia.* Appl Environm Microbiol. 1997; 63(5): 2016-2021.

13. Bennion RS, Baron EJ, Thompson JE Jr, Downes J, Summanen P, Talan DA, Finegold SM. *The bacteriology of gangrenous and perforated appendicitis-revisited*. Annual Surgery. 1990; 211(2): 165-171.

14. Bennion, RS, Thompson JE, Baron EJ, Finegold SM. *Gangrenous and perforated appendicitis with peritonitis: treatment and bacteriology.* Clin Ther. 1990; 12 (Suppl C): 31-44.

15. Gerardo SH, Marina M, Citron DM, Claros MC, Hudspeth MK, Goldstein EJC. *Bilophila wadsworthia clinical isolates compared by polymerase chain reaction fingerprinting.* Clin Infect Dis. 1997; 25(Suppl 2): S291–4.

16. Finegold SM, Bennion RS, Thompson JE, Wexler HM, Baron EJ. *Gangrenous and/or perforated appendix: clinical outcome and in vitro susceptibility testing.* Hospital Practice. 1990; 25(S4): 3-12.

17. Schumacher UK, Eiring P, Häcker FM. *Incidence of Bilophila wadsworthia in appendiceal, peritoneal and fecal samples from children.* Clin Microbiol Infect. 1997; 3(1): 134-136.

18. Feng Z, Long W,Hao B, Ding D, MaX,Zhao L, Pang X. *A human stool-derived Bilophilawadsworthia strain caused systemic inflammation in specific-pathogen-free mice.* Gut Pathology. 2017; 9: 59. DOI 10.1186/s13099-017-0208-7.