ORIGINAL ARTICLE

Molecular detection of *Tropheryma whipplei*, *Cryptosporidium* spp., and *Giardia lamblia* among celiac disease samples

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Background: Celiac disease (CD) is one of the most common disorders, resulting from both environmental (gluten) and genetic factors. The clinical features of the Iranian CD are still unknown and there is insufficient information about the atypical presentation of CD from Iran. As, many previous reports revealed an association between controlled protozoal infections and the CD according to cytokines production, the aim of this study was to determine the prevalence of CD and possible co-infection with the most prevalent protozoal infections including *Tropheryma whipplei, Cryptosporidium*, and *Giardia duodenalis* among CD samples. **Materials and Methods:** In this study, from April 2014 to November 2016, 524 samples were obtained from small intestine of patients with gastrointestinal diseases referring to the Pathology Department of Namazi Hospital, Shiraz, Iran. Multiplex polymerase chain reaction assay was then performed on the histological positive CD samples for the prevalence of the microorganisms. **Results:** Sixty-four (12.21%) patients were diagnosed as having CD by histopathological examination. The prevalence of *T. whipplei* and *Cryptosporidium* spp. was 19 (29.69%) and 8 (12.5%) respectively, among CD positive samples there was no positive sample for *Giardia lamblia*. **Conclusion:** The prevalence of CD among the southwestern Iranian population was high and comparable with other areas of Iran as well as many other countries. Furthermore, no significant association between the presence of *T. whipplei*, *Cryptosporidium* spp., and level of the histopathological changes of villi in the CD was observed ($P > 0.05$).

Key words: Celiac disease, Cryptosporidium, Giardia lamblia, Tropheryma

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INTRODUCTION

The typical autoimmune disorder of the small intestine is referred to as celiac disease (CD) which affects many organs and also causes malabsorption. The CD occurring in all age groups of the working population. Both intestinal and extra-intestinal symptoms of the CD are probably induced by the consumption of wheat, rye, and barley proteins. Classical CD and severe malabsorption, diarrhea, abdominal discomfort, and general malaise have been observed in some patients.[1,2] The active form of CD is closely associated with mild gastrointestinal (GI) symptoms, iron deficiency, and autoimmune diseases. The similarity of the GI symptoms of CD with Giardia have been formerly addressed in the literature.[3] *Cryptosporidium* species are also causative agents of self-limiting diarrhea which may last for a few months, with children under 5 years old enduring the highest burden.[4] These pathogenic parasites were detected simultaneously with the CD.[5] However, the laboratory diagnosis of the infection is mainly based on staining techniques, ELISA, and various Polymerase chain reaction (PCR) assays.[6,7] PCR assay showed high sensitivity and specificity and was successfully employed for the Giardia identification in stool specimens.[8]

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Whipple's disease (WD) has been cultivated as a chronic infectious disease caused by the bacterium *Tropheryma whipplei*.[9] A carefully developed diagnostic procedure to differentiate the WD is critical since the untreated cases are potentially lethal in humans.[10] None of the previous works were simultaneously employed the possible role of the three micro-pathogens (*T. whipplei, Cryptosporidium*, and *Giardia lamblia)* using multiplex-PCR assay. Finally, the iliac disease is considered as a complicated and multifactorial metabolic disorder with many GI complications. As such, this study aimed to include the samples with a broad spectrum of different levels of CD and to investigate co-infection with the most prevalent protozoal and bacterial infections among the pathologically confirmed cases of celiac in Southern, Iran. Furthermore, the possible association between such infections among the confirmed celiac cases was probed.

SUBJECTS AND METHODS

Sample preparation

From April 2014 to November 2016, a total of 525 samples taken from the small intestine of the patients referred to the Pathology Department of Nemazi Hospital, Shiraz University of Medical School, Iran; which were then chosen for the presence of CD. The mean age of patients was 14.46 ± 1.82 years ranged from 0.5 to 78 years. The specimens were pathologically investigated for any evidence of CD. Patients were divided into male ($n = 24$) and female ($n = 40$) groups. Tissue sections (5 mm thickness) were prepared in the paraffin blocks at − 20°C. The first section was discarded and the other sections were displaced in a clean 1.5 ml tube using a sterile toothpick or tweezers. Alternatively, the tissues could have been previously laser capture micro-dissected directly from the sections.

Deparaffinization

One milliliter of xylene was added to the vortex for 10s; then, the tube was maintained at room temperature for approximately 5 min. The tubes were spun for 5 min at maximum speed (14,000 rpm) and the supernatants were discarded. Then, the wash with a fresh aliquot of xylene was performed and repeated. The pellet was washed by adding 1 ml of absolute ethanol. The tubes were flicked to dislodge the pellet; then, the tubes were spun for 10s. Next, they were left at RT for approximately 5 min. The tubes were spun for 5 min at maximum speed (14,000 rpm) and then carefully removed. Then, the supernatants were discarded. The washes were repeated using once 90% and then 70% ethanol. Finally, the tissue allowed the pellet to air dry in a thermoblock at 37°C for about 30 min.

DNA extraction and polymerase chain reaction amplification

The histopathologic celiac positive samples were subjected to a multiplex PCR assay for three intestinal bacterium and protozoa including *T. whipplei*, *Cryptosporidium* spp., and *G. lamblia*. Total DNA was extracted from the samples (100–500 ml of the packed pellet) by lysis in 50 mM Tris-HCl, 20 mM EDTA, containing 2 mg of proteinase K per ml and 0.5% Sarkosyl, incubated at 37°C for 1 h. Then, 5 M NaCl was added to give a final concentration of 1 M, and CTAB was added to a concentration of 1%. Following the incubation at 65°C for 30 min, one freeze-thaw cycle, and phenol-chloroform extraction, the DNA was precipitated by the addition of 0.6 volumes of isopropanol, and the DNA pellet was washed with 70% ethanol. After desiccation, the DNA pellet was resuspended in 100 ml of sterile distilled water. The PCR assays were performed with the specific primers to amplify *Cryptosporidium* spp. (F: GAGGTAGTGACAAGAAATAACAATACAGG, R: CTGCTTTAAGCACTCTAATTTTCTCAAAG), *G. lamblia* (F: CGAGACAAGTGTTGAGATGC, R: GGTCAAGAGCTTACAACACG), T. whipplei (F: AGAGAGATGGGGTGCAGGAC, R: AGCCTTTGCCAGACAGACAC).[11-13]

Mucosal histology

Multiple mucosal biopsies were obtained from the second part of the duodenum or proximal jejunum. Duodenal biopsy specimens were paraffin-processed, formalin-fixed, serially sectioned and stained by hematoxylin and eosin staining. The presence of parasitic elements (trophozoites of Giardia, coccidian oocysts, and spores of microsporidia) was carefully investigated. Modified Marsh classification was used for the grading of the mucosal changes: Grade 0, normal histology; Grade 1, mild increase in the intraepithelial lymphocytes (IEL), crypt-villous (CV) ratio 1:1; Grade 2, moderate villous atrophy with CV ratio more than 1; Grade 3, flat mucosa with no recognizable villi[14] [Table 1].

Statistical analysis

The results were analyzed using the SPSS software Version 10.1 (IBM, New York, USA). The Chi-square test was used to determine the relationship between the occurrence of CD and the pathogen microorganisms. The differences were considered statistically significant when the $P < 0.05$.

Table 1: Marsh modified histologic classification used for diagnosis of celiac disease

Marsh type	IEL/100 enterocytes-duodenum	Crypt hyperplasia	Villi
0	<30	Normal	Normal
1	>30	Normal	Normal
2	>30	Increased	Normal
3a	>30	Increased	Mild atrophy
3b	>30	Increased	Marked atrophy
3c	>30	Increased	Complete atrophy

IEL=Intraepithelial lymphocytes

RESULTS

Histological findings and polymerase chain reaction assay

Of a total of 524 samples examined by microscopy in the Department of Pathology of Nemazi Hospital in Shiraz, Iran, 64 samples (12.21%) were confirmed the occurrence of CD. Mucosal histological findings were studied in the celiac patients including increased intraepithelial T lymphocyte (IEL) in 53 samples of, villous atrophy in 40 samples, flattening of villi in 10 samples, shortening of villi in 4 samples, and increased lymphoplasmacytic cells in 4 samples [Figure 1 and Table 1].

The positive celiac samples (40% males and 60% females) were subjected to the multiplex PCR assay to amplify *T. whipplei*, *Cryptosporidium* spp., and *G. lamblia.* The result of PCR (for *T. whipplei*, *Cryptosporidium* spp., and *G. lamblia*) are shown in Figure 2. Of a total of celiac cases (64 samples), 25 samples (39.06%) were PCR positive [Table 2]. Of the 25 positive samples, Marsh type of 3b, demonstrating marked atrophy of villi, revealed the highest rate (15 samples (60%) ($P < 0.05$). The frequency of 3a and 3c the Marsh types was also 2 (8%) and 8 (32%) samples, respectively.

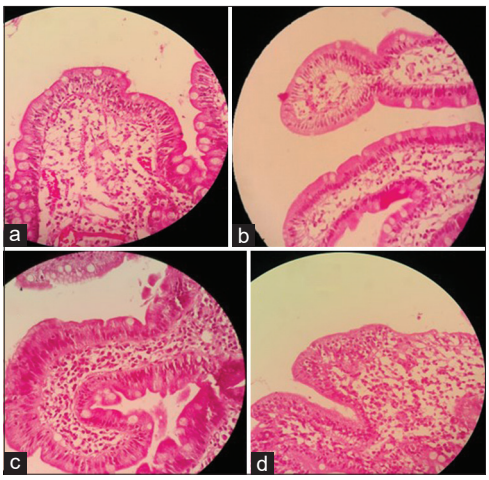

Figure 1: (a) Marsh 0: Normal duodenal mucosa with unremarkable villus and loos connective tissue in lamina propria. (b) Marsh 1: increased intraepithelial lymphocytes > 30/100 enterocytes (×400). (c) Marsh 2: normal villus morphology with crypt hyperplasia and increased, and intraepithelial lymphocytes. (d) Marsh 3b. Moderate villus atrophy with increased intraepithelial lymphocytes >30/100 enterocytes

Among all the celiac positive specimens (64 samples), 19 samples (29.69%) were positive for *T. whipplei*, and 8 samples (12.5%) were positive for *Cryptosporidium* spp. *G. lamblia* was not detected in the samples.

The morphological aspects of the small intestinal in 19 *T. whipplei* PCR positive with light microscopy showed that 18 of them had IEL, 12 had villous atrophy, 4 had flattening of villi, 2 had shortening of villi, and 1 had increasing lymphoplasmacytic cells. There was no significant association between the PCR positive and the histological changes of patients with *T. whipplei*. Moreover, the morphological changes in 8 *Cryptosporidium* spp. PCR positive included 7 IEL, 6 villous atrophy, 1 flattening of villi, and 1 increasing lymphoplasmacytic cells. There was no significant association between the PCR positive and histological changes of patients with *Cryptosporidium* spp. [Table 2].

Numerical differences in the frequency of the diseases (*T. whipplei*, *Cryptosporidium* spp., and celiac) were observed within the ages of 1–9 years old which was not significant. Besides, no significance association was found between the sex and occurrence of the diseases [Table 3]. Surprisingly, of a total of 25 PCR positive patients, two cases were simultaneously infected with *T. whipplei* and *Cryptosporidium* spp.

DISCUSSION

CD is a life-long gluten-sensitive autoimmune disease of the small intestine affecting genetically susceptible individuals, worldwide. The prevalence of CD varies based on diagnostic methods and geographical locations.[15] Biopsy method is remarked as a gold standard for the CD diagnosis.[16] The occurrence of the CD in Asia 0.6%, South America 0.4%, and Europe 0.8% was reported using the biopsy method.[15] In the present study, according to the Modified Marsh Criteria, 12.21% of cases (including 64 samples out of 524 samples) were found to be positive for CD. Nikpour and Mohammad Hosseini[17] reported the histopathological features of CD in 8 (6.3%) of the patients (6 Marsh IIIA; 2Marsh IIIC). A distinct association was also reported between helminthic

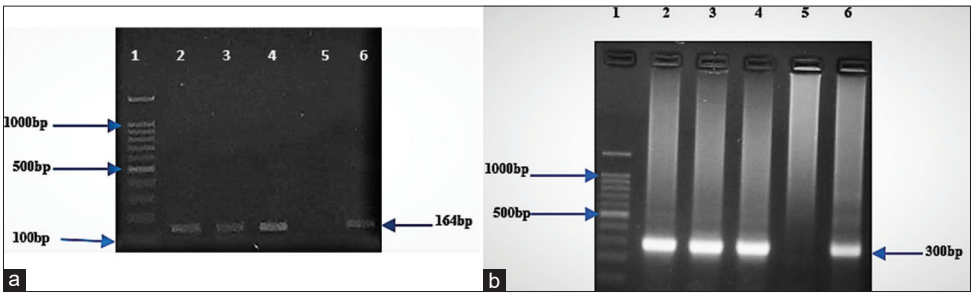

Figure 2: Detection of *Tropheryma whipplei* DNA (a) and *Cryptosporidium* spp. DNA (b) from CD samples on polyacrylamide gel electrophoresis. Lane 1: DNA ladder (100 bp), 2, 3 and 4: Positive samples, 5: Negative control, 6: Positive control

Table 2: Frequency of the bacterial and protozoal diseases in the celiac positive cases

CD	*Tropheryma whipplei*		*Cryptosporidium* spp.		*Giardia duodenalis*	
	PCR positive	PCR negative	PCR positive	PCR negative	PCR positive	PCR negative
3a	1	1	1	1	0	2
3b	12	3	5	10	0	15
3c	6	2	2	6	0	8
Total	19	6	8	17	0	25

PCR=Polymerase chain reaction; CD=Celiac disease

Table 3: The frequency of polymerase chain reaction positive samples and the celiac patients (25 samples) according to the age and sex classification

	Tropheryma whipplei		*Cryptosporidium* spp.		*Giardia duodenalis*		CD		
	PCR positive	PCR negative	PCR positive	PCR negative	PCR positive	PCR negative	3a	3b	3c
Age									
1-9	14	3	4	13	0	17	1	10	16
10-19	3	3	3	3	0	6	1	3	2
20-40	2	0	1	1	0	2	0	2	0
Total	19	6	8	17	0	25	2	15	18
Sex									
Male	7	3	4	6	0	10	0	7	3
Female	12	3	4	11	0	15	2	8	5
Total	19	6	8	17	0	25	2	15	8

PCR=Polymerase chain reaction; CD=Celiac disease

or protozoal infections and the immune-mediated intestinal disorder such as CD. Parasites infections are common in the developing country. They mainly affect host immune response and take part in the progress of autoimmune diseases. Alongside, parasites may colonize as opportunistic pathogens in the intestinal tissues of celiac patients revealing remarkable GI disorders.[3] Severe villous atrophy, malabsorption, dyspepsia, and secretory diarrhea were reported following the infection of small intestine epithelial tissues by *Cryptosporidium* spp. and *Giardia* spp. in the celiac and nonceliac cases.[3,18] Cryptosporidiosis is a zoonotic protozoal disease usually causing self-limited diarrhea in the immunocompetent patients; chronic lethal forms of the infection were also reported in the immunocompromised hosts.[19] Coincidence of *Cryptosporidium* spp. infection (in stool) with the CD (in the duodenal biopsy) was also diagnosed in children.[20,21] According to the study conducted by Stuppy and Garcia, 10.6% of patients with GI complaints showed *cryptosporidium* spp. oocytes in stools; meanwhile, 23.2% indicated the serologically confirmed cases of CD. In addition, the parasitic treatment led to mitigating the occurrence of CD.[22] In a recent study performed by Khalaf Ali, *et al*., no evidence of *G. lamblia* infection was also shown in the CD patients.[23] 12.5% *Cryptosporidium* spp. and 0% *G. lamblia* in the celiac patients were also identified in our study.

T. whipplei is the causative agent of a rare chronic infectious disease responsible for GI disorders such as malabsorption, diarrhea, weight loss, and villous atrophy which may be misdiagnosed with CD. In some cases, this enteropathy caused by *T. whipplei* may be severe and lethal.[24-26] Furthermore, a considerable association between WD and the immunomodulatory condition was indicated in the literature.[27] In the study conducted by Amsler *et al*., 4.2% of saliva and stool samples were positive for *T. whipplei* in GI patients.[28] In our study, a high rate (29.69%) of celiac specimens were found positive for WD.

Various causative agents play roles in the villi atrophy as well as the CD. They comprise autoimmune disorders including Crohn's disease, neoplasia, drug associated enteropathy, infiltrative amyloidosis, collagenous sprue, and parasitic infectious including Whipple's disease, giardiasis. As the villi atrophy is a multifactorial disorder, it is difficult to differentiate CD with other causative agents. As such, it is necessary to employ both serological and hematological tests coincident with taking a proper biopsy to confirm the disease.[29] The confirmation of the disease is also crucial to apply accurate strategy for treatment. In addition, the high prevalence of WD in the celiac patients was remarkable which need more investigations in this area.

Based on previous published data, possible association between the lymphocytic reduction and triggering the immune response to activate this inflammatory reaction was reported. In this cases, the occurrence of GI pathogens was suggested.[30] Furthermore, a considerable atrophy of GI villi was observed in this study. These changes were not directly taken part in the occurrence of CD, but may explain

the presence of the inflammatory diseases which resulted from the GI pathogens.

CONCLUSION

The coincident occurrence of CD with other infectious agents was observed in the present study. Therefore, the application of other confirmatory laboratory tests is essential to differentiate the causative agents and finally to choose a better strategy of treatment. Since no association between the occurrences of histopathological level of destruction of villi and those parasitic and bacterial infections was found, more investigations are suggested to find the association between the intensity of villi atrophy and the infectious agents.

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Conflicts of interest

There are no conflicts of interest.

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