Kirkuk University Journal /Scientific Studies (KUJSS)
Volume 13, Issue 1, March 2018, pp. (289-303)
ISSN 1992 – 0849

Gene sequencing of Blastocystis hominis and its association with H. pylori in the development of irritable bowel syndrome

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ABSTRACT

Blastocystis hominis is a parasites that causes problems in human's digestive tract. Helicobacter pylori is a dangerous bacteria that causes gastric ulcer and may even lead to cancer. To assess the synergistic association of B hominis and H pylori with irritable bowel syndrome among patients attending hospitals of Kirkuk. A total of 344 stool samples were examined by direct examination for B hominis and detection of H pylori antigen during the period from 1st September 2016 to 1st May 2017.

The rate of infection with B. hominis was 38.66%, H. pylori 35.47%, while mixed infections were 67.2%. Infection with Blastocystis and Helicobacter were higher in females than males. Regarding IBS patients about 51.95% and 47.7% were infected with Blastocystis and Helicobacter, respectively. The main clinical symptoms associated with Blastocystis and Helicobacter were flatulence, abdominal pain and nausea. PCR amplified small-subunit rDNA was used to study the genetic diversity of Blastocystis isolates by 3 different restriction enzymes (HinfI, RsaI & Sau3AI). Three distinct genotypes out of 20 Blastocystis isolates were identified (subtypes 1, 3 and 7). The nucleotide sequencing showed that mutation occurred in subtype 3, in the region of TC in the position of T601C and of T661C and converse from ATA to ATC with reverse and forward primer.

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The rate of mixed infections were greater than each infection alone. B. hominis and H. pylori was predominant in females than males. Three distinct genotypes were isolated (subtype 1, 3 and 7).

Keywords: synergism, B. hominis. H. pylori, gene sequence, mutatio

التتابع الجيني للمتبرعمة الكيسية البشرية وعلاقتها مع بكتريا الملوية البوابية في تطور متلازمة تهيج القولون

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الملخص

تعد المتبرعمة الكيسية البشرية من الطفيليات التي تسبب مشاكل في القناة الهضمية للانسان. الملوية البوابية هي بكتريا خطرة تسبب قرحة المعدة وقد تؤدي الى السرطان. لتقييم العلاقة التازرية بين المتبرعمة الكيسية البشرية و بكتريا الملوية البوابية مع متلازمة تهيج القولون بين المرضى الوافدين مستشفيات كركوك، تم فحص 344 عينة براز بطريقة الفحص المباشر للمتبرعمة الكيسية والكشف عن مستضد الملوية البوابية للفترة من الاول من ايلول 2016ولغاية الاول من ايار 2017. كانت نسبة الاصابة بالمتبرعمة الكيسية البشرية (38.66٪) والملوية البوابية (35.47٪) بينما كانت الاصابة المزدوجة (67.2٪). كانت الاصابة بالمتبرعمة الكيسية والملوية البوابية اعلى لدى الاناث مقارنة بالذكور. وفيما يتعلق بمرضى القولون العصبي كان (51.95٪) و(47.7٪) منهم مصابا بالمتبرعمة الكيسية والملوية البوابية على التوالي.

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وكانت الاعراض السريرية المرتبطة بالمتبرعمة الكيسية والملوية البوابية هي الانتفاخ والم البطن والغثيان. تم استخدام الوحيدات الصغيرة للحامض النووي الرايبوزي المضخمة بتفاعل البلمرة المتسلسل لدراسة التنوع الجيني لعزلات المتبرعمة الكيسية بواسطة ثلاث انزيمات تقييد مختلفة (HinfI, RsaI & Sau3AI). تم تحديد ثلاث انماط وراثية مختلفة من اصل 20 عزلة متبرعمة كيسية (الانماط الجينية 1و3و7). واظهر تسلسل النيوكليوتيدات ان الطفرات وقعت في النمط الجيني 3 في منطقة TC في الموقع T601C و T661C وعكسها من ATA الى ATC مع البادئات العكسية و الامامية.

كان معدل الاصابة المزدوجة اكبر من كل اصابة لوحدها. وكانت المتبرعمة الكيسية والملوية البوابية سائدة في الاناث مما للذكور. تم عزل ثلاث انماط وراثية محددة (النمط الفرعي 1و3و7).

الكلمات الدالة: التازر, المتبرعمة الكيسية البشرية, الملوية البوابية , تتابع جيني, طفرة

1. Introduction

Blastocystis hominis (B. hominis) is one of parasites that was regarded as a non-pathogenic yeast, and it is more than a harmless commensal, or it is a potential pathogen. It should be borne in mind that when it causes of intestinal disorder it is sought and persistent diarrhea with irritable bowel syndrome [1]. The pathogenic potential of B. hominis in the human intestine is controversial because the organism has been found in both symptomatic and asymptomatic individuals [2]. Clinical symptoms attributed to Blastocystis infections include recurrent watery diarrhea, mucous diarrhea, vomiting, abdominal cramps and flatulence. Blastocystis can infect both children and adults [3]. It was postulated that hard stools of narrow caliber, painful or infrequent defecation, results in constipation and intractability to laxatives. Diarrhea usually was described as an evacuation preceded by urgency or frequent defecation of small volumes of loose stool. Postprandial urgency is common, as is alternation between constipation and diarrhea. Characteristically, one feature is predominates in a single patient, but significant variability exists among patients [4]. Regarding molecular technologies, now it is divided into 17 small subunit ribosomal RNA (SSU rRNA). Subtypes (STs; ST1–17) have been identified within the B. hominis.

Nine of them (subtypes) [(ST1)- (ST9)] were detected in humans [5]. H. pylori is a very important bacteria that causes stomach ulcer and may lead to cancer [6]. H pylori are one of risky bacteria may lead to cancer because of its penetration into the gastric and tissue leads to

ulcers [7]. There may be synergies between these bacteria with the B. hominis in irritable bowel syndrome which has become a problem for millions of people [8]. This depends on the leaving of this bacteria from the stomach to the colon causing many problems and symptoms [9]. The involvement of these bacteria with B. hominis in irritable bowel irritation is still under study [10]. The aim of our study is to identify this synergy between these two organisms.

2. Materials and Methods

Three hundred forty four stool samples were collected from patients with irritable bowel syndrome. The period of this study lasted from 1st September 2016 to 1st May 2017. H pylori fecal antigens were measured by stool antigen test. DNA genotypes of B. hominis isolates were detected by Restriction fragment length polymorphism (RFLP) analysis of PCR amplified small-subunit rDNA (SSU rDNA) while, DNA Sequencing was applied with Bio System. Big Dye TM termination V 3.1 cycle sequencing kit was used to perform automated DNA sequencing. The B. hominis DNA samples for SB83, SB155, and SB227 genes. An assessment of DNA Sequencing by computer based program sequencer TM was used to edit all data obtained from automated sequences. To determine the nature of mutations it was compared with the reference sequence (NCBI database, accession number: AB091234) to these sequences.

3. Statistical Analysis

SPSS Microsoft Office Excel program was used for statistical analysis of data mean ± SEM (standard error of mean) was used for expression of numeric data, while comparison of the numeric data for healthy control and patient groups was done by using the Paired t-test **[11]**.

4. Results and Discussion

Irritable bowel syndrome is one of the abnormal phenomena that occurs in the human colon and may be shared by many microorganisms such as bacteria and parasites causing diarrhea accompanied by pain with the generation of gases. This study looked for the potential of synergy between B. hominis parasites and H. pylori bacteria. Table (1) showed that out of 133 B hominis infected patients the rate of infection was higher in females (54.9%) than males (45.1%) although there was no significant difference between the two gender (p>

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0.05). This finding is in agreement with those reported in Samarra city [12], in Erbil city [13], in Iran [14] and in Thailand [15], but disagree with those in Kirkuk [16], in Duhok [17]. Also in patients infected with H pylori the rate of infection was higher in females (51.6%) than in males (48.4%). These results were consistent with those reported in Kirkuk [18], in Erbil [19], in Diyala [20], in Baghdad [21], in Basrah [22]. In contrast, other studies reported that males were more infected than females such as study in Baghdad [23,24], in Diyala [25].

This high rate of infection in females with B hominis and H pylori can be explained by the design of our study in investigating IBS patients which is more common in females as a result of fluctuation in sex hormone.

Table (1): Distribution of Blastocystis hominis and Helicobacter pylori according to gender

Gender	Blastocystis hominis		Helicobacter pylori	
	No. positive (%) (n=133)	No. negative (%) (n=211)	No. positive (%) (n=122)	No. negative (%) (n=222)
Male	60 (45.1%)	88 (41.7%)^{NS}	59 (48.4%)	89 (40.1%)^{NS}
Female	73 (54.9%)	123 (58.3%)	63 (51.6%)	133 (59.9%)

NS ($p > 0.05$) = non significant

Table (2) showed that out of 344 subjects, 133 (38.66%) were positive for Blastocystis hominis, while 122 (35.47%) were positive for Helicobacter pylori. Samples with mixed infections with both Blastocystis hominis and Helicobacter pylori were in 82(67.2%) samples which shows a highly significant difference ($p<0.01$).

The present study revealed that the main clinical symptoms associated with B hominis were abdominal pain (59.4%), followed by flatulence (58.6%) and vomiting (11.3%). This result was in accordance with that reported in Dohuk [26] and disagreed with th results obtained in Iran [27] that abdominal pain (86.84%) was the common symptoms. Also in Turkey [28]. reported that abdominal pain and diarrhea were the common symptoms, followed by vomiting While in H pylori infected patients the main clinical symptoms were flatulence (70.5%) followed by abdominal pain (59.0%) while vomiting (12.3%) was the clinical symptom with the lowest rate. There was highly significant difference ($p< 0.01$)

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between patients and controls. This finding was in agreement with that reported in Baghdad [29] that p. value was < 0.05 in patients complaining from gases. In Kirkuk [18] reported that no significant association was found between H pylori with abdominal pain and diarrhea, while there was significant relationship between H pylori and vomiting. This finding contradicts another study reported in Al-Qurna, Basrah that the main gastrointestinal symptoms observed were heart burn (60.6%) followed by vomiting (28.3%) and abdominal distention (24%) [30]. In Sulaimanya [31] reported that there was no relation between patient symptoms and H pylori (p> 0.05) except vomiting, loss of appetite and weight loss (p< 0.05) .

Table (2): The clinical symptoms associated with Blastocystis hominis and Helicobacter.

Parameters		Blastocystis hominis		Helicobacter pylori	
		No. positive (%) (n=133)	No. negative (%) (n=211)	No. positive (%) (n=122)	No. negative (%) (n=222)
Diarrhea	N(%)	38 (28.6%)	56 (26.5%)^{NS}	37 (30.3%)	57 (25.7%)^{NS}
Abdominal pain	N(%)	79 (59.4%)	78 (37.0%)**	72 (59.0%)	85 (38.3%)**
Flatulence	N(%)	78 (58.6%)	96 (45.5%)*	86 (70.5%)	88 (39.6%)**
Nausea	N(%)	45 (33.8%)	49 (23.2%)*	46 (37.7%)	48 (21.6%)**
Vomiting	N(%)	15 (11.3%)	19 (9.0%)^{NS}	15 (12.3%)	19 (8.6%)^{NS}
Weight loss	N(%)	21 (15.8%)	32 (15.2%)^{NS}	22 (18.0%)	31 (14.0%)^{NS}
Anemia	N(%)	19 (14.3%)	26 (12.3%)^{NS}	21 (17.2%)	24 (10.8%)^{NS}
Total		344			

NS= non significant * < 0.05 = Significant ** <0.01= highly significant

Regarding IBS patients, out of 344 subjects, 256 (74.42%) were infected by the syndrome, while 88(25.58%) were uninfected. The result was highly significant (p<0.01). Table (3) revealed that (51.95%) were infected with B hominis. The association between B hominis and IBS was reported by several studies. In Kirkuk [16] found that 33.7% of IBS patients were infected with B hominis. In Al-Muthanna, Iraq [32]. The rate of infection was 33.86%, 44.09% and 45.67% by microscopy, culture, and PCR technique respectively. Similar results obtained in Turkey [33], in Iran [34], and in India [35].

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On the other hand, other studies showed that B hominis was not associated with IBS. In study conducted in Thailand on 126 subjects, including 66 with IBS and 60 as healthy control, they found no significant difference was present between the two groups [36]. In Iran [37] revealed that the rate of B hominis was higher in the control group than in the case group.

Concerning the association between H pylori and IBS. The result showed that (47.7%) of IBS patients infected with H pylori. This association may be related to H pylori virulence factors that are predisposing to IBS, besides the host genetic factors and environmental factors. Moreover, the clinical symptoms are determined by the interplay of these factors [38,39]. These findings are in agreement with those in Diyala, Iraq [40] in Pakistan [41], in Egypt [42], but disagreed with those reported in China [43, 44] that H pylori was not associated with IBS.

These findings were agreed with [45] who determined that the bloating occurs in nearly all patients with irritable bowel syndrome, and it also occurs in patients with other functional and organic disorders, and may occur with the participation of some microorganisms including B. hominis and H. pylori. There is a relationship that may be found through our study that indicate there is a help shown by both organisms and this cooperation between them aggravates the patient's condition and the persistence of symptoms of irritable bowel syndrome. Both organisms help each other to secrete enzymes that provide the other with a suitable environment for reproduction, metabolism and gene expression [46].

Table (3): Distribution of B. hominis and H. pylori according to irritable bowel syndrome

Irritable bowel syndrome		*B hominis*		*H pylori*	
		Positive (n= 133)	Negative (n= 211)	Positive (n= 122)	Negative (n= 222)
Yes	N (%)	133(51.95%)	123 (48.04%)	122 (47.7%)	134 (52.3%)
No	N (%)	0 %	88 (100%)	0 %	88 (100%)

Total= 344 subjects

A genetic diversity would be a powerful tool for identification or classification of B. hominis subtypes. In this study, 20 B. hominis isolates were collected. Restriction fragment length polymorphism (RFLP) analysis of PCR amplified small-subunit rDNA (SSU rDNA)

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was used to study genetic diversity of B. hominis isolates by 3 different restriction enzymes (HinfI, RsaI & Sau3AI). Cluster analysis of the ribo-print patterns showed 3 distinct genotypes out of 20 B. hominis isolates.

The primers used:

Subtype	Product size, bp	Primer sequence 5′-3′	GenBank Acc. No.
SB83 Sub1	351	F: GAAGGACTCTCTGACGATGA R: GTCCAAATGAAAGGCAGC	AF166086
SB227 Sub3	526	F: TAGGATTTGGTGTTTGGAGA R: TTAGAAGTGAAGGAGATGGAAG	AF166088
SB155 Sub7	650	F: ATCAGCCTACAATCTCCTC R: ATCGCCACTTCTCCAAT	AF166087

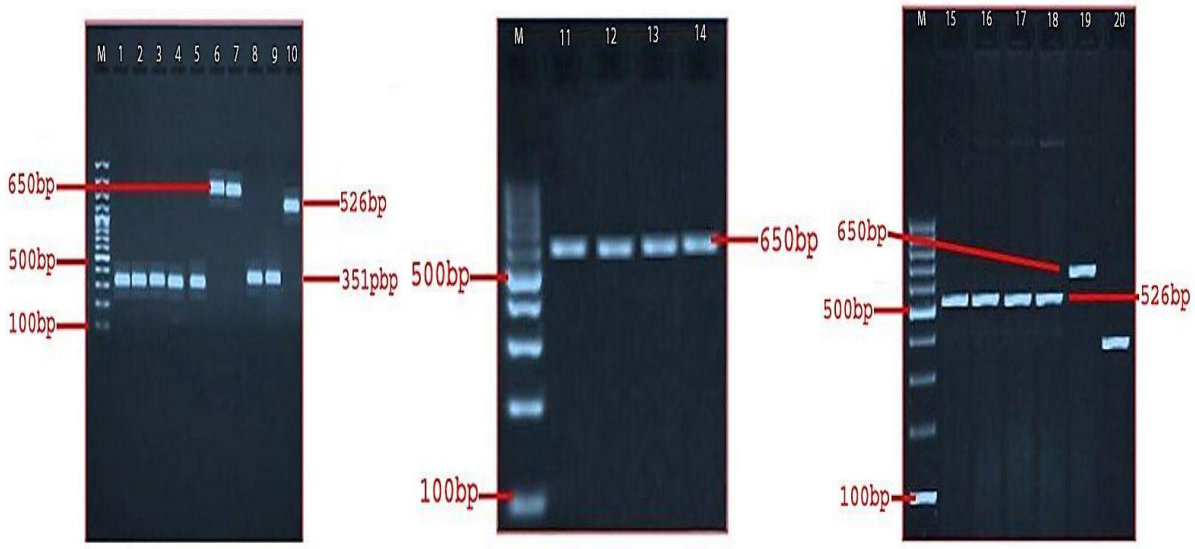

Fig. (1) : Restriction digests in lanes 1,2,3,4 and Lane 5 for SB83 subtype I, lanes 6,7 for SB155 subtype VII, lanes 10 for SB227 subtype III. DNA, Restriction digests in lanes 11-14 for SB155 subtype VII DNA, Restriction digests in lanes 15-18 and Lane 5 for SB83 subtype I, lanes 19for SB155 subtype VII, lanes 10 for SB227 subtype III. DNA In lanes 1-10 was digested with HinfI, DNA in lanes 11-14, was digested with RsaI and DNA in lanes 15-20 was digested Sau3AI.

The Sequence of Subtypes of B. hominis genes:

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The nucleotides sequencing showed that mutation occurred in subtype 3 in the region of TC in the position of T601C and converse from ATA to ATC with forward primer and mutation occurred in the region of TC in the position of T601C with reverse primer.

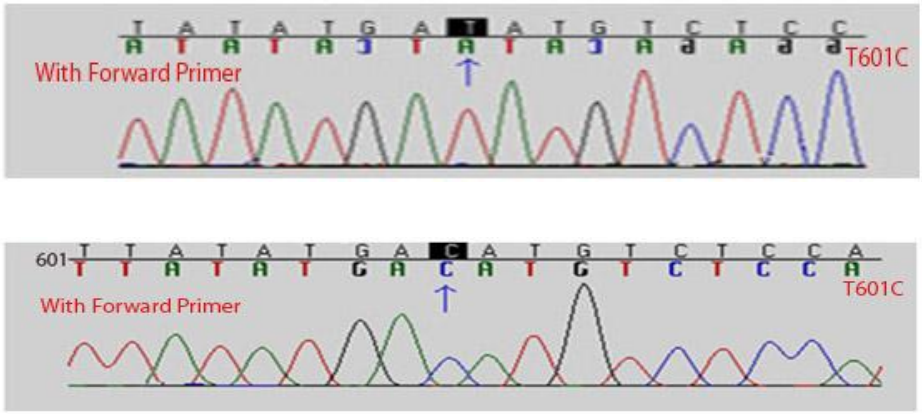

Fig. (2): The mutation in subtype 3 in T601C nucleotide in TC region converse ATA sequence to ATC with forward primer

The nucleotide sequencing showed that mutation occurred in the region of TC in the position of T661C and converse from ATA to ATC with reserve primer.

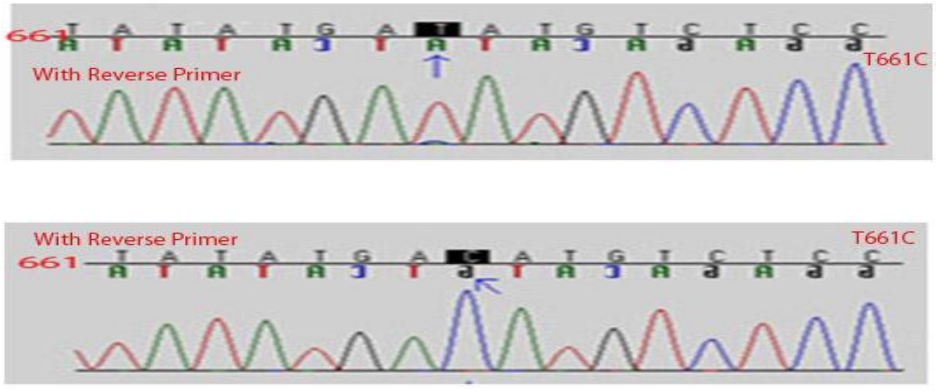

Fig. (3): The mutation in subtype 3 in T661C nucleotide in TC region converse ATA sequence to ATC with reserve primer

The current study has shown that there are several sub-types of B. hominis strains which have been identified by some definite enzymes which were (8) of sub type SB83 strain, (7) sub type SB155 strain and 5 of sub type SB227 strain [47]. The mutation of these species, which is determined by the sequencer of the genetic device of the Microgene Korea, Prove that there are mutations in subtype 3, in the region of TC in the position of T601C and

converse from ATA to ATC with forward primer and mutation occurred in the region of TC in the position of T661C and converse from ATA to ATC with reserve primer. These mutations may have occurred because of the synergy with the H. pylori, which have made these parasites more dangerous and virulent in the occurrence [48].

5. Conclusion

It is concluded that the rate of H. pylori (35.47%) was lower than B. hominis (38.66%). The rate of mixed infections of B. hominis and H. pylori were greater than each infection alone. Infection with B. hominis and H. pylori was common and was predominant in females than males. The rate of infection in IBS patients with B. hominis (51.95%) was higher than H. pylori (47.7%). The main clinical symptoms associated with both infection were flatulence, abdominal pain, diarrhea, nausea, weight loss, anemia and vomiting.

A genetic diversity would be a powerful tool for identification or classification of B. hominis. Cluster analysis of the ribo-print patterns showed 3 distinct genotypes out of 20 B. hominis which were (8) of sub type SB83 strain, (7) sub type SB155 strain and 5 of sub type SB227 strain

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