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Comparative Restriction Analysis of the Genomes of the Blastocystis spp Strains.

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ABSTRACT

The discussion about the clinical significance of Blastocystis spp. and its pathogenic potential is still ongoing. In recent decades, high rates of blastocyst insemination have been identified in various population groups. It is known that, being in the intestine, blastocysts participate in the formation of the microbiome of the ecotope. Disrupting the balance of microorganisms, these pathogens contribute to the creation of favorable conditions for the development of pathological processes. However, the mechanisms that reduce the protective forces of the macroorganism and contribute to the development and flow of the epidemiological process require deeper knowledge in addressing this issue. In the presented work a relatively simple way of determining a blastocyst with different degree of virulence using modern restriction analysis of the protozoa genome has been proposed. In the genome of blastocyst, a genetically determined number of recognition sites for a certain type of restriction enzymes was found, which led to the formation of a strictly defined number of DNA fragments of a fixed size in the study groups of protozoans. The proposed combination of endonucleases (Hae III, Pst I and Hind III) allowed to carry out species identification of strains of Blastocystis spp. differing in virulence indices, and also to judge about presence of different phenotypic groups of the protozoans in the studied biotope.

Key words: parasitic invasions, blastocystosis, Blastocystis spp., genetic analysis, restriction enzymes: BamH I, Hae III, Hind III, Pst I, Ecor I, virulence

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INTRODUCTION

Current scientific trends are based on the biological identification of different organisms on the basis of the unique sequence of DNA nucleotides. Fundamental analysis of such genomic data implies consideration of genomic problems from the point of view of fundamental processes, i.e. the nucleosomal organization of DNA, and makes it possible to optimize epidemiological diagnostics in terms of monitoring the progress of the epidemiological process [1, 2, 3].

Polymerase chain reaction makes it possible to detect a microbe in the studied material (water, products, patient's material) by the presence of microbial DNA in it without releasing the latter into a pure culture. A modern genetic method is the restriction analysis of bacterial DNA, which is widely used in molecular-biological studies and is one of the most important tools in the study of the microbial genome [4, 5]. By comparing the DNA restriction maps isolated from different strains, it is possible to determine their genetic affinity, to identify their belonging to a particular species or genus, and to detect the areas subjected to mutations.

The widespread use of restriction enzymes in genetic engineering research has led to growing interest among researchers in the properties of these enzymes and the methods for obtaining them, and has made it possible to develop specific markers based on the fragmentary analysis of DNA [6].

Analysis of modern literature has shown that protozoic invasion caused by parasitism in the large intestine of the human being by the simplest *Blastocystis spp*., occupies one of the leading positions in the structure of all intestinal protozoans. With all this, blastocystic lesion has not recently been considered as an etiological factor in human pathological conditions and was assessed as a harmless transient state. However, in recent years it has been established that *Blastocystis spp.* as a pathogenic microorganism directly or indirectly participates in the formation of intestinal biotope, contributes to the creation of favorable conditions for the development of pathological processes in the host organism [7].

According to the literature, the high incidence of Blastocystis spp. is noted among patients with chronic diseases of the digestive system (gastritis, stomach ulcer, duodenal ulcer, duodenal ulcer, irritable bowel syndrome) accompanied by diarrheal syndrome for up to one month and the possible schizogony of blastocyst in tissue infection, which is confirmed by the discovery of schizons in the cloned organs: in the intestine, in the urogenital mucosa, in the lymphoid tissue of the almondalin [8, 9].

Taking into account the wide spread of acute and chronic diseases of the gastrointestinal tract, including inflammatory bowel disease, the study of the epidemiology of blastocystic invasion is an urgent scientific and practical task of our time.

In recent years, the etiological significance of blastocyst in the development of infection has been widely studied using standard biological methods of investigation, which often allow us to assess only the potential role of the protozoan in the development of the infection [10]. There is no complete picture in the literature that allows us to assess the degree of virulence of the blastocysts studied with the use of modern medical and genetic research methods, and there is no information about the presence of pathogenicity genes of Blastocystis spp., isolated in people with diseases of the gastrointestinal tract, based on

the analysis of restriction fragments of DNA of the protozoan.

In connection with the above stated objective of the work was to study the possibility of using the method of restriction fragment length polymorphism (PDLP-analysis) to diagnose strains of blastocysts isolated in people with various diseases of the digestive system.

Experimental methods

Surech CEM was used to produce isolated cultures of Blastocystis spp.

DNA extraction was carried out in accordance with the instructions for the use of a set of reagents for the extraction of DNA approved by order of Roszdravnadzor (Federal Service for Supervision in Healthcare) on June 30, 2008 No. 5008-Пр/08, registration certificate of the Ministry of Health of the Russian Federation No. ФСР 2008/02938.

As a result of RFLP analysis, various methods of DNA extraction were used: phenol-chloroform extraction (Guanidinium Thiocyanate-Phenol-Chloroform Extraction), FTA-cards, where nucleic acids are isolated directly on a special paper impregnated with a mixture of reagents binding DNA, sorbent method with the use as silicate carriers (Silica, Silica Matrices). As the experiment has shown, the greatest yield of DNA was observed when using phenolic-chloroform extraction method.

Phenolic chloroform extraction for DNA extraction of blastocyst was carried out using standard kits "VectorDNA extraction", CJSC "Vector-Best" (Koltsovo village, Novosibirsk region, Russia). For the experiment, 78 strains of Blavtosudtish spp. were selected isolated in persons with diseases of the digestive system.

Restrictive endonucleases (restriction enzymes) were used in this study: Sma I, HpA II, Ecor I, Hae III, Hind III. produced in R&D Company SibEnzym. The reaction was stopped by adding 5 mcl of a inhibitor removal buffer containing 0.1 M EDTA, 0.05 % bromophenol blue and 40 % sucrose. Electrophoretic separation of restriction products of amplified DNA was carried out in 2 % agarose (Sigma) in tris-acetate buffer with ethidium bromide (0.5 mg/l) at 120 V within 4 hours. To determine the length of DNA fragments, DNA molecular weight markers (100bp + 1.5 Kb DNA markers, R&D Company SibEnzym) were used.

To determine the length of DNA fragments, we used DNA molecular weight markers (M1 - molecular weight marker - 100-10.000 bp, M2 - molecular weight marker - 300-10.000 bp, R&D Company SibEnzym). The restriction lengths were determined using the Gel Pro Analyzer software, version 4.0.00.00.001. The percentage of fragment lengths identity was calculated for each pair of microorganisms by comparing the restriction enzyme separately for each restriction. When comparing the lengths of the restriction samples, DNA fragments were considered identical, with a length difference of no more than 5%.

Statistical data processing was carried out with the help of "Statistica for Windows" program.

Research findings and discussion

350 patients with various gastrointestinal diseases were examined. It was shown that 127 of them were infected with the protozoan Blastocystis spp. The highest number of people with blastocystic infestation was registered in the group with duodenal ulcer disease - 78 people (61.41%). Therefore, it was decided to take this group of people for further research.

The carried out researches have shown that from 78 clinical blastocyst isolates isolated in gastroenterological patients only 60 (73,07±2,3%) possessed virulence. Indicators of virulence (LD50/lg) of these strains ranged from 1.7±0.2 to 5.4±0.1.

Therefore, all the strains of blastocyst studied were divided into four groups: the first group consisted of strains of blastocyst with highly virulent properties (15 strains). LD50/lg ranged from 5.0±0.3 to 6.4±0.5. The second group consisted of the strains of the protozoan with moderately virulent signs, LD50 /lg 3.5±0.2-4.9±0.3 (28 strains). The third group is represented by weakly virulent properties of 10 strains (12.92±1.4%). Blastocysts with the value of LD50 /lg 1.2±0.4-3.4±0.2 were classified as a group of avirulent strains (Table 1).

Table 1: Value of LD50 (lg) of Blastocystis spp. clinical isolates

Strains groups	Number of virulent strains of Blastocystis spp.		LD_{50} /lg Value
	abs.	%	
Highly virulent	15	27.43±2.1	5.0±0.3–6.4±0.5
Moderately virulent	28	43.14±5.3	3.5±0.2–4.9±0.3
Low virulent	10	12.92±1.7	
Avirulent	7	16.51±1.4	–
Total	60	100	–

The next stage of the work was the study of the molecular and genetic bases of the genome of the protozoan blastocyst with the use of the modern approach based on the study of the restriction fragment length polymorphism (RFLP – restriction fragment length polymorphism), with the help of which it is possible to determine the adjacent genes and to identify the strains of Blastocystis spp. by different phenotypic groups, and in our case also to compare the obtained fragments of protozoan DNA with the indices of their virulence. All this makes it possible to

compare the data obtained in different groups of the protozoans under study, identified in individuals with duodenal diseases, and to provide an opportunity for a more detailed clarification of the picture of the etiological significance of blastocysts in the pathology of diseases of the gastrointestinal tract.

As a result of the experiments performed using various restriction enzymes on extracted DNA of avirulent, low virulent, moderately virulent, and highly virulent blastocyst strains isolated from the clinical material of the patients, it was found out that the use of restriction enzymes BamH I - GATCC/CCTAG G on the lanes of 1% agarose gel was accompanied by bands stained with ethidium bromide and DNA of about 10 000 bps. (Fig.1).

Short fragments of chromosomal blastocyst DNA of 100-10.000 bp were not observed during the experiment.

The recognition site for restriction enzyme Hae III - GG CC/CC GG is shown in Fig. 2. The following results were obtained in the reaction with extracted total blastocyst DNA with different degrees of virulence: DNA fragments of about 850 and 10 000 bp were clearly visible on the agarose gel lanes after electrophoresis in avirulent blastocysts; in the rest of the blastocyst DNA lanes with different degrees of virulence, except for these fragments, bands with sizes from 1500 to 10 000 bp were observed.

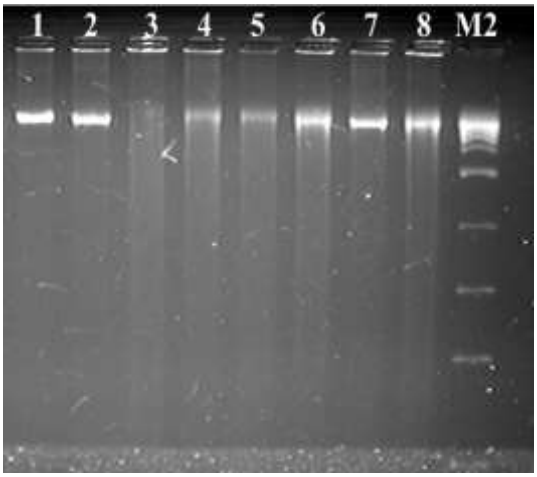

Fig. 1. Electrophoregram of restriction fragments of DNA of blastocyst strains using BamH I restriction enzyme. Blastocyst strains: 1, 2 - avirulent, 3, 4 - low virulent, 5, 6 - moderately virulent, 7, 8 - highly-virulent. M2-marker of molecular weight is 300-10000 bp.

Therefore, this restriction enzyme allows differentiation of avirulent and virulent blastocyst strains without revealing the severity of virulent properties.

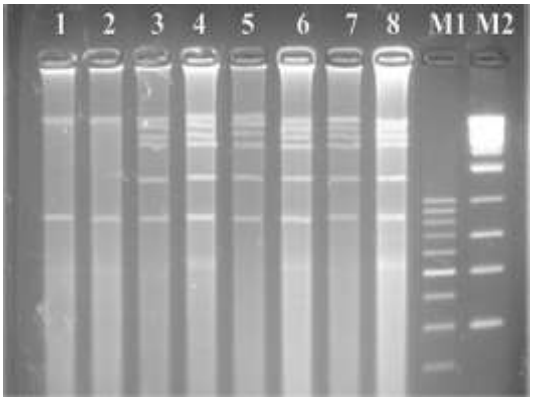

Fig. 2. Electrophoregram of restriction fragments of DNA of blastocyst strains using Hae III restriction enzyme. Blastocyst strains: 1, 2 - avirulent; 3, 4 - low virulent; 5, 6 - moderately virulent; 7, 8 - highly virulent. M1-marker of molecular weight - 100-1000 bp, M2-marker of molecular weight - 300-10000 bp.

Therefore, this restriction allows differentiation of avirulent and virulent blastocyst strains without revealing the severity of virulent properties.

When using restriction enzyme Hind III - A AGCTT/TTCGA A, 1% agarose gel lanes had bands of 10.000 or more bp, stained with ethidium bromide (Fig. 3).

In addition, a band of 700 bp was observed on the DNA lanes of low virulent blastocyst strains; two bands of 380 and 600 bp were detected on the DNA agarose lanes of high-virulent blastocyst strains. Consequently, Hind III restriction enzyme allows to reveal low and highly vivirulent protozoans.

The following results were obtained during the experiments of restriction (cleavage with the help of endonucleases) of the isolated DNA of the strains of blastocyst restrictase Pst I - CTGCA G/G ACGTC: on the lanes of the agarose gel of the strains of blastocyst with different degree of virulence there were observed bright blurred bands (shmers) with the size of 2000 to 10.000 (and even more on separate lanes) bp (Fig. 4).

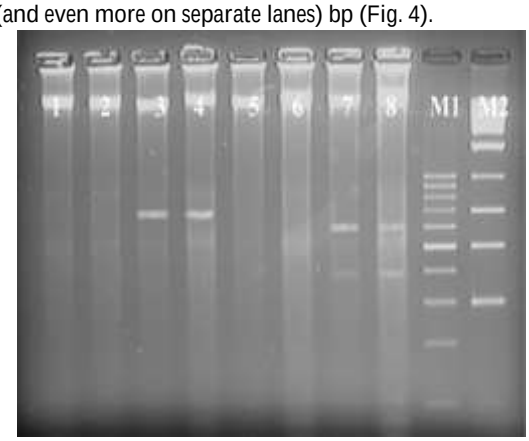

Fig. 3. Electrophoregram of restriction fragments of DNA of blastocyst strains using Hind III restriction enzyme. Blastocyst strains: 1, 2 - avirulent; 3, 4 - low virulent; 5, 6 - moderately-virulent; 7, 8 - highly virulent. M1-marker of molecular weight - 100-1000 bp, M2-marker of molecular weight - 300-10000 bp.

However, fragments of about 900 bp in size were observed on the chromosomal DNA tracks of moderately virulent blastocysts. Other strains of blastocyst have no such bands. Thus, Pst I restriction enzyme revealed only moderately virulent strains of Blastocystis spp.

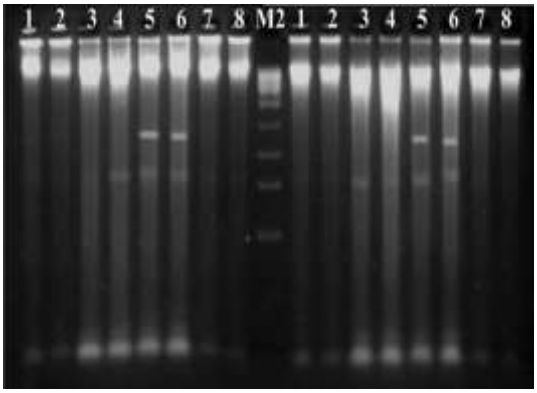

Fig. 4. Electrophoregram of restriction fragments of DNA of blastocyst strains using restriction enzyme Pst I. Blastocyst strains: 1, 2 - avirulent; 3, 4 - low virulent; 5, 6 - moderately virulent; 7, 8 - highly virulent. M2-marker of molecular weight - 300-10000 bp.

When using restrictionase EcoR I with the G▼AATTC/CTTAA▲G recognition site, smear bands were observed on the lanes of blastocyst strains of 1% agarose gel with different degrees of virulence on the lanes of the entire length of the lanes, indicating that multiple DNA fragments of different lengths overlap each other (Fig. 5).

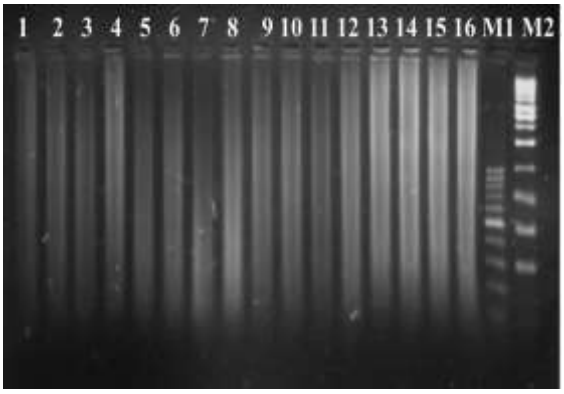

Fig. 5. Electrophoregram of restriction fragments of DNA of blastocyst strains using restriction EcoR I. Blastocyst strains: 1-4 - avirulent; 5-8 - low-virulent; 9-12 - moderately virulent; 13-16 - highly virulent. M1-marker of molecular weight - 100-1000 bp, M2-marker of molecular weight - 300-10000 bp.

Thus, we can conclude that all strains of blastocyst in the DNA structure of all strains of blastocyst are present in multiple GAATTC/CTTAAG sites, which indicates the impossibility of detecting the degree of virulence of blastocysts using EcoR I restriction enzyme.

Table 2 presents the results of virulence determination of blastocyst strains obtained by restriction DNA analysis of protozoans using restriction endonucleases (restriction enzymes): EcoR I, BamH I, Hae III, Hind III, Pst I.

Table 2: Value of DNA blastocyst fragments with different degree of virulence (bp)

Blastocyst strains	Value of DNA fragments in the action of restriction enzymes:				
	BamH I	Hae III	Hind III	Pst I	EcoR I
Avirulent	10 000	850. 10 000	10 000 and more	From 2000 to 10 000	Solid schmer
Low virulent	10 000	from 550 to 10 000	700, 10 000 and more	from 2000 to 10 000	Solid schmer
Moderately virulent	10 000	from 550 to 10 000	10 000 and more	from 2000 to 10 000, 900	Solid schmer
Highly virulent	10 000	from 550 to 10 000	380, 600, 10 000 and more	from 2000 to 10 000	Solid schmer

In this paper a method for determining the virulence of Blastocystis spp. has been proposed based on the analysis of restriction fragment length polymorphism (RFLP) of PCR-product with a length of 10.000 pairs of nucleotides obtained by isolating DNA of protozoans using five restriction endonucleases EcoR I, BamH I, Hae III, Hind III, Pst I.

CONCLUSIONS

Thus, restriction analysis showed that the use of restriction enzymes BamH I, EcoR I, Pst I does not allow typing blastocysts with varying degrees of virulence.

The use of restriction enzyme Hae III allows differentiation of avirulent and virulent blastocysts based on the absence or presence of DNA fragments ranging in size from 1500 to 10000 bp.

Blastocyst with moderate virulence was typed based on DNA fragments of about 1000 b.p. with the help of restriction enzyme Pst I.

In an experiment using restriction enzyme Hind III, we have typed a blastocyst with moderate virulence based on 700 bp DNA fragments as well as highly virulent strains of blastocyst based on 380 and 600 bp DNA fragments.

The method of restriction fragment length polymorphism (RFLP) used in the work allowed to reveal intraspecific polymorphism of protozoan polymorphism according to virulence indexes, and also revealed possibilities for broader understanding of the etiological role of blastocyst in pathogenesis of diseases of persons with duodenal ulcer. The results obtained show that the proposed combination of restriction endonucleases (Hae III, Pst I and Hind III) to determine the virulence of Blastocystis spp. can serve as a fairly simple and universal way to identify the protozoans, depending on the degree of severity of virulent properties. Besides, the restriction analysis method is much more sensitive and allows to study not only potentially pathogenic strains of blastocyst, which are revealed by biological methods of research, but also to reveal the whole spectrum of genes in the blastocyst genome possessing pathogenicity properties, which in general can tell about the presence of different phenotypic groups of protozoans in the biotope under study.

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