Molecular Characterization of Cryptosporidium spp. in Wild Rats of Tehran, Iran Using 18s rRNA Gene and PCR_RFLP Method

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ABSTRACT

Background: Wild Rats have been implicated as potential reservoirs of Cryptosporidium spp., thereby responsible for transmission of the infection to humans by acting as natural sources of C. parvum, a zoonotic species. Recently, and possibly due to much closer and more frequent contacts to these animals, concerns have raised about spread of the disease among human environments.

Objectives: The purpose of this study is molecular characterization of Cryptosporidium in rats of Tehran.

Materials and Methods: Rats were hunted randomly from different zones of Tehran and their stool samples were studied by Polimerase Chain Reaction (PCR) method using 18s rRNA gene. Subsequently, positive (i.e. infected) samples were characterized and sequenced.

Results: Reported rates of Cryptosporidium infection among 77 rats by implementation of staining as well as nested PCR methods were %13 and 27.3 %, respectively. In order to recognize type of infective agent, all positive cases were tested by PCR – RFLP (Restriction Fragment Length Polymorphism) method by which C. parvum pattern was observed. The observed pattern in PCR-RFLP test was checked via sequence determination method in all positive isolations and the results were approved.

Conclusions: The results reveal that wild rats in Tehran are reservoirs of C. parvum that could be transmitted to humans. Also, it illuminates hygienic importance of freewild-rats eradication throughout the city.

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1. Background

Cryptosporidium is a species of protozoan parasites that may infect digestive system tegument cells in all vertebrates. Different subspecies of these parasites, found all around the world, may illustrate different infective patterns on their hosts; some of them infect many host species (rodents and ruminants) while some may infect fewer types of hosts and some may affect only a single type. (1). Cryptosporidium spp, grouped in phylum Apicomplexa, have many commonmorphological and life cycle specifications with Coccidia (2, 3). Cryptosporidiosis may involve different age groups with symptoms and signs of a diarrheal infection. These organisms reproduce and grow inside gastrointestinal enterocytes but out of cytoplasm. The disease is more severe in those who
are suffering from immune deficiencies, either acquired or congenital. Cryptosporidium hominis and C. parvum localize mostly in small intestine cells, however in some animals like rats and calves, the ileum, above cecum, is the place of choice for C. parvum. In severe immune deficient animals or humans, the parasite may also be found out of intestine. Other types such as C. muris, C. andersoni and C. serpentis prefer stomach mucus membrane, and C. bailey targets respiratory Tract, or cloaca.

2. Objectives

This study was performed to demonstrate molecular characterization of Cryptosporidium in rats of Tehran, animals that have been implicated as potential reservoirs for Cryptosporidium and possible resource for infection transmission to humans because they are naturally infected by C. parvum, a zoonotic species. Recently, some concerns about disease transmission developed, particularly in view of much closer and more frequent contacts with these animals in human environments.

3. Materials and Methods

In current study, 77 wild rats were hunted alive from different zones of Tehran, and transferred to the laboratory. They were anesthetized by chloroform and then killed. Collected stool samples were inserted in potassium dichromate 5 % and after performing DNA extraction by QIAGEN company kit (QIAmp DNA stool mini kit) (6), nested PCR method implemented on all 77 samples.

The samples were studied by two special primers for nested PCR technique. In order to study Cryptosporidium isolations, primers with 18s rRNA gene were used. The length of produced parts with these primers were 1325bp and 825bp (7-9).

First stage primers PCR:
- a. Forward (F1): 5’-TTCTAGAGCTAATACATGCG-3’
- b. Reverse (R1): 5’-CCCATTTCCTTCGAAACAGGA-3’

Second stage primers PCR:
- c. Forward (F2): 5’-GGAAGGGTTGTATTTATTAGATAAAG-3’
- d. Reverse (R2): 5’-CTCAAGGGTGCTGAAGGAGTA-3’

Because of high sensitivity of characterization to PCR, nested PCR method was selected for this purpose. This method consists of both primary and secondary PCR (9, 10).

Scheduled program for first stage of primary PCR by thermo cycler was:
- 94°C: 5 min
- 35 cycles of: 94 °C: 45", 58 °C: 45", and 72 °C:1 min
- 72 °C: 7 min
- 4 °C: soaking

Two enzymes, Ssp1 and Vsp1, were used to characterize genotype of the samples (Table 1).

For cutting product of second PCR , following protocol was used (11, 12):

- Mixture of 5 µL of second PCR, 1 µL of each enzyme, 2 µL of buffer of each enzyme was performed , followed by adding enough distilled water to make a 20 µL mixture. The mixture was put in 37°C water bath for 2 to 24 hours. Then whole product was put in gel.

4. Results

After first and second PCR (Figure 1), 21 out of 77 cases (%27.3) were reported positive with Cryptosporidium (830bp); nevertheless, infection rate of each city zone were specific to that area (Table 2).

![Nested PCR Test Results Positive and Negative Control Samples on Agarose Gel](Image)

Table 1. Enzyme Features

| Vsp1 (Asel) | Ssp1 |
|-------------|------|
| Recognition Sequence: | AATATT... |
| After cutting: | AATATT... |
| Reaction Conditions: | 37°C |
| Compatible ends: | Asel, PshBl |
| Thermal Inactivation: | 65°C for 20 minutes |

Table 2. Results of Nested PCR Method

| Tehran Zones | Number of Tested Samples | Results of Nested PCR Test (Positives), No.( %) |
|--------------|--------------------------|-----------------------------------------------|
| North        | 18                       | 3 (16.7)                                      |
| South        | 14                       | 5 (35.7)                                      |
| Center       | 12                       | 6 (50)                                        |
| East         | 17                       | 4 (23.5)                                      |
| West         | 16                       | 3 (18.7)                                      |

Left to right: 1: Sample 13.3 South of Tehran, 2: Sample 8.1 East of Tehran, 3: Positive control sample, 4: DNA marker 100bp, 5: Sample 13.1 West of Tehran, 6: Sample 2.3 North of Tehran, 7: Positive control sample, 8: DNA marker 100bp, 9: Sample 12.1 Center of Tehran
4.1. The Results of Enzyme Digestion and PCR-PFLP for 18s rRNA Gene

For type identification and genotype determination, Vsp1 and Ssp1 enzymes were used. Genotype of Cryptosporidium demonstrated 3 cutting with Vsp1 enzyme in locations 104, 115, and 629 of which 104 and 629 were visible after electrophoresis on agarose gel. With Ssp1 enzyme, cutting occurred in locations 11, 12, 108, 267, and 450 of which108, 267, and450 were visible after electrophoresis on agarose gel (Figure 2). All 21 samples were positive in nested PCR test and were digested by Vsp1 and Ssp1 enzymes. All collected samples had RFLP results the same as Cryptosporidium parvum. This observation revealed that all Tehran rats are infected by Cryptosporidium parvum.

4.2. Results of Sequence Determination

Following PCR-RFLP procedure on the positive samples by which Cryptosporidium type in Tehran rats were identified (Figure 3) and in order to approve such finding, sequence determination was applied on some samples. To perform this, DNA was extracted by Bioneer kits from the gel. The results are as follows (Figure 4 and Figure 5):

4.3. Registered (HQ651731) in Gene Bank (NCBI)

Similarities and differences of gene sequences in the samples were compared with registered Cryptosporidium genotype in Gene Bank. The result revealed that the genotypes of infective agents recognized in PCR-RFLP technique were exactly the same as for registered C. parvum genotype.

Figure 3. RFLP Products of Vsp1 and Ssp1 Enzymes in Positive Samples of West, East, North and South Zones of Tehran

1: Sample 13.2 west of Tehran under Vsp1 enzyme activity; 2: Sample 13.2 west of Tehran under Ssp1 enzyme activity; 3: Sample 8.1 east of Tehran under Vsp1 enzyme activity; 4: Sample 8.1 east of Tehran under Ssp1 enzyme activity; 5: DNA marker 100bp; 6: Sample 28.1 north of Tehran under Vsp1 enzyme activity; 7: Sample 28.1 north of Tehran under Ssp1 enzyme activity; 8: Sample 32.1 south of Tehran under Ssp1 enzyme activity; 9: Sample 32.1 south of Tehran under Vsp1 enzyme activity

Figure 4. Nucleotide Sequence 824bp, Product of Nested PCR Isolation Number 22.1 in Center of Tehran; Registered (HQ651732) in Gene Bank (NCBI)

Results of analysis revealed that isolation number 22.1 matched 100 % with C. parvum isolated from human and cow in Iran (Registration number in Gene Bank DQ656352.1 and DQ656355.1, respectively) as well as with
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Figure 6. Phylogenetic Analysis Results of Center and East Zones of Tehran

C. parvum sub-type Sakha 212 isolated from cow milk in Egypt (Ab 513881.1). It was matched 99 % with C.parvum isolated from human in Iran (DQ 656354.1). Isolation number 8.3 from East of Tehran matched 99 % with C.parvum isolated from human and cattle in Iran (DQ656352.1 and DQ656354.1, respectively) as well as matched 98 % with C. wrairi isolated from guinea pig (Ui1440.1).

4.4. Phylogenetic Analysis

Phylogenetic analysis was performed between sequenced isolation in this study and respective sequences of genotypes registered in Gen Bank.

In this analysis, results of sequenced isolations were arranged initially by Bio-Edit and Chromas, followed by CLUSTAL X program implementation developed for alignment and drawing primary pilot tree. Subsequent-
vum and C. hominis in human and lamb with Real-time PCR method. The results showed that not only human, who was considered as the only creature which can be infected by C. parvum, but also animals can play a role in this issue (15). Hajdušek et al. (2004) studied PCR identification of Cryptosporidium in human and animals. In their study, they used COWP and 18s rRNA genes; for type recognition and sequence determination. The study revealed that in Czech Republic the main and potential sources of this infection transmitted to humans are animals like cattle, horse and deer (16). Pirestani et al. (2008) studied the molecular determination of C. Parvum in human and cattle with 18s rRNA gene in Shahriar region. The study revealed that C. parvum isolated from cattle were prevailing zoonotic genotype associated with infection transmission to humans in that region (17).

Due to lack of studies about rats and Cryptosporidium infections in Iran, current study focused on Tehran rats to recognize the infection by staining and molecular methods. By using staining method, prevalence of recognized parasite was 13%; it was 27.3% when nested PCR method was performed. Higher sensitivity to Cryptosporidium detection might be considered as a reason for higher prevalence figure reported by second method. In both methods, prevalence of the infection was higher in center of Tehran than in other zones. Considering life style in Tehran in which animals are not at risk, possibility of Cryptosporidium transmission to humans by rats increases.

Nested PCR and PCR-RFLP methods, and Ssp1 and Vsp1 limiting enzymes on positive samples revealed that par

vum type was infective agent in all isolations. The possibility of C. Parvum transmission to humans in city zones increases when the zoonotic aspect of this infection is considered.

In sequence determination and its respective result analysis it was revealed that the C. parvum genotype in Tehran rats matched 99 to 100 % with the C. parvum iso

lates of human and cattle.

By using PCR-RFLP methods, and Ssp1 and Vsp1 limiting enzymes, sequence determination and analysis of the results revealed that C. parvum in Tehran rats matched 99 to 100 % with C. parvum isolates in human and cattle. The result of this study unveils the relationship between wild rats, humans and domesticated animals life cycle.

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