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First molecular identification and subtype distribution of *Blastocystis* sp. isolated from hooded crows (*Corvus cornix*) and pigeons (*Columba livia*) in Tehran Province, Iran

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ABSTRACT

Blastocystis is a common intestinal parasite among humans and animals such as non-human primates, pigs, cattle, birds, amphibians, and less frequently, rats, reptiles and insects. Since *Blastocystis* is a widely transmissible parasite between humans and mammals or birds, it is prominent to determine whether newly secluded non-human isolates are zoonotic. There are no comprehensive studies in Iran assessing the prevalence and molecular identification of *Blastocystis* infection in birds, especially in pigeons and crows. So, the aim of this study was to identify *Blastocystis* subtypes (STs) in crows and pigeons in Tehran province, Iran, using Nested PCR-RFLP and sequencing. Overall, 300 *Blastocystis* isolates from birds (156 pigeons and 144 crows) were subtyped by PCR, and the homology among isolates was then confirmed by RFLP analysis of the 18S rRNA gene. The prevalence of *Blastocystis* infection was detected 42.9% in pigeons and 44.4% in crows. All positive pigeons were owned by ST13 (100%). Among crows, 46 samples (71.8%) like pigeons were ST13, and 13 samples (20.3%) were ST14. Five samples (7.9%) remained unknown. This study was the first report of ST13 and ST14 of *Blastocystis* from birds. In the present study, our data revealed a high prevalence of *Blastocystis* sp. in pigeon's and crow's samples and the isolates from these birds were classified into two genetically distinct STs. Therefore, birds appear to be infected with various STs. It is important to determine the phylogenetic relationships between unknown STs from these birds and the multiple STs of *Blastocystis*.

1. Introduction

Blastocystis is a zoonotic parasitic protist that displays meant host specificity and lives in the intestinal tract of humans and a wide range of vertebrates such as mammals, birds, reptiles, fishes and even invertebrates like cockroaches [1–4]. It is transmitted among hosts by the fecal-oral route [1,2]. In addition, other routes of transmission such as water-borne, food-borne, and person-to-person have been pondered [2,4]. Despite the clinical importance of the parasite is under inspection, *Blastocystis* has been involved as a causative agent of diarrhea, abdominal pain, and irritable bowel syndrome in humans [5–7]. New studies cited *Blastocystis* sp. as an emerging pathogen [1,4,6]. *Blastocystis* has been contemplated morphologically polymorphic, and several new species names have been recommended for isolates from birds based on morphological characteristics [4,8].

Showing considerable genetic diversity, *Blastocystis* from humans and a variety of animals including other mammals, and birds comprises at least 17 subtypes (ST) based on analysis of SSU-rRNA genes [9–12]. Nearly, 90% of human isolates subtyped so far belong to ST1-ST4, with a majority of carriage assignable to ST3 in numerous countries globally [2,11]. Temperate host specificity as proved by comparison of subtype distribution demands that *Blastocystis* could be engaged in zoonotic transmission [13].

Epidemiological studies on *Blastocystis* infection in animals have shown a relatively high incidence in birds [14]. However, little genomic analysis of isolates from birds has been accomplished. At present, genomic analysis is the best tool for screening isolates of zoonotic *Blastocystis*.

Blastocystis seems to be omnipresent, with recent reported prevalence depicts of up to 100% [15,16]. Prevalence of infection varies widely from one region to another but in general it is higher in developing countries than developed countries [17]. In Iran the prevalence of

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human *Blastocystis* varies from 0.22% to 54.5%, which makes this organism the most frequently reported protist in stool [18]. Until now, very limited data is available about the distribution, host specificity, and genetic variation of *Blastocystis* found in non-human hosts, including birds. Avian STs were comprise of ST6, ST7, and ST8, because of their relative predominance in birds [19–22]. However, this supposition was based on the molecular typing of a still restricted number of avian isolates [20]. It is still obscure whether birds may function as reservoir of *Blastocystis* strains colonizing humans. Moreover, little is known about the distribution of STs in birds. It is implied that various STs have different host, geographical distribution, and routes of transmission. Therefore, subtyping *Blastocystis* is consequential for epidemiological studies because it assists to recognize possible sources and routes of transmission of a unique ST in a special region and this new data can help us to complete the knowledge about pathogenicity of *Blastocystis* and its scattering via direct contact and/or contamination of water and food supplies.

In Iran, there are no studies concerning prevalence of *Blastocystis* in birds, specifically crows and pigeons. Crows and pigeons live in close proximity to humans in open and semi-open environments, including farmland and cities. These birds have conformed themselves well to living conditions along with humans. Due to their interesting abilities, they are heeded by scientists. Crows and pigeons, like humans and primates, have abstract intelligence and they can understand concepts like space and time. Furthermore, they have the ability to recognize and memorize the faces of humans. They are All-eater, and this makes them also carriers of disease.

To date, there are several methods to detect *Blastocystis* [2,9,13]. Recently, detection of *Blastocystis* directly from stool via conventional PCR or real-time PCR has been reported [23,24]. The real-time assay allows subtyping of *Blastocystis* isolates by direct sequencing of quantitative PCR products. Because, the STs isolated from humans may have a different ribodeme pattern than birds, so we executed RFLP-PCR. Moreover, restriction enzymes used in this study are for the first time applied for subtyping of *Blastocystis* from crows and pigeons. Thus, the purpose of this study was to determine and characterize the STs of *Blastocystis* present in crows and pigeons of Tehran Province, Iran by Nested PCR-RFLP, sequencing, and assessing the ribodeme patterns.

2. Materials and methods

2.1. Sample collection and culture of Blastocystis isolates

This cross-sectional descriptive study was executed from January 2014 to June 2015. A total of 300 fecal samples were collected from wild population of 156 pigeons and 144 crows in Tehran province, Iran. They were tested for the presence of central-body form of *Blastocystis* by using saline wet mount method. Furthermore, samples were stained by Trichrome technique.

All samples were subjected to short-term in vitro culture by adding approximately 50 mg of fresh fecal sample into a sterile screw-cap tube containing 2-mL slant of coagulated hen's egg overlying with 1 mL of autoclaved Locke's solution together with starch and 4 mg/mL streptomycin. Following, the tube was incubated at 37 °C for 24–48 h and examined for the vacuolar form of *Blastocystis* using light microscopy at 400× magnification prior to DNA extraction.

2.2. DNA extraction

Total genomic DNA of each *Blastocystis* isolate was extracted directly from approximately 250 mg of bird's fecal samples using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, samples were weighed, homogenized in lysis buffer, and incubated at 95 °C for 5 min to ensure lysis of the protist. After centrifugation, the DNA in the supernatant was purified using a silica column supplied with the kit. At last, DNA was eluted in 100 µL of elution buffer to increase its concentration. The quantity and quality of the DNA was determined using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) and gel electrophoresis. The extracted DNA was stored at −20 °C until used.

2.3. Nested-PCR-RFLP

Amplification of a fragment of the 18S rRNA gene locus (1767-bp) was carried out using a two-step Nested-PCR approach. This fragment has been shown to be very specific to *Blastocystis*.

The primers F1: 5′ – GGA ATC CTC TTA GAG GGA CAC TAT ACA T – 3′; R1: 5′ – TTA CTA AAA TCC AAA GTG TTC ATC GGA C – 3′; F2: 5′ - CAT ACG CTC GTC TCA AAG ATT AAG CCA - 3′; and R2: 5′ - TAC GGA AAC CTT GTT ACG ACT TCA CC - 3′ were used for the first and second round Nested-PCR [25]. Nest-2 primers were designed for the first time in this study by AlleleID version 7.84. Briefly, the PCR conditions were composed of pre-denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 45 s, and extension at 72 °C for one minute, followed by final extension at 72 °C for 10 min. The secondary annealing temperature was 67 °C. Electrophoresis was performed by adding 5 µL of the PCR products and a size marker of a 100-bp ladder (Vivantis, Subang Jaya, Malaysia) to a 1.5% agarose gel and staining with 0.5 µg/mL ethidium bromide for 1 h at 90 V. Bands were observed by UV trans-illumination (Uvitec, Cambridge, UK).

RFLP of the secondary PCR product was carried out using *Rsa*I, *Bsp*143 and *Hinf*I restriction enzymes (NEB, Ipswich, MA, USA) for subtype identification. The reaction mixture contained 5 µL of the secondary product, 0.5 µL of *Rsa*I, *Bsp*143, or *Hinf*I (20 U), 2.2 µL of restriction buffer; this was incubated at 37 °C for 2 h, under conditions recommended by the manufacturer. The digestion products were separated on a 2% agarose gel and visualized after ethidium bromide staining. The restriction fragment patterns obtained for each ribodeme were analyzed.

2.4. Sequencing and phylogenetic analysis

For confirmation, the secondary PCR products were purified by using QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced on both ends through the sequencing service of Bioneer Laboratories (Bioneer, Daejeon, Korea). The sequences obtained were compared with all *Blastocystis* sp. homologous sequences available from the NCBI using the nBLAST program. The resulting sequences identified in the present study were edited and aligned with the BioEdit, version 7.2.5 [26]. Multiple alignments were performed with GenBank copies of *Blastocystis* species data from Iran and other countries. A phylogenetic tree was constructed with the Kimura 2-parameter option of the Maximum Likelihood (ML) method using MEGA-7 [27]. The reliability of the ML tree was assessed by the bootstrap method with 1000 replications.

2.5. Nucleotide sequence accession numbers

The partial sequences of the 18S rRNA genes of *Blastocystis* obtained in this study were deposited in the GenBank database under accession numbers MH489081-MH496651.

3. Results

3.1. Identified subtypes

In this study, a total of 300 fecal samples were collected from 156 pigeons and 144 crows and inspected for *Blastocystis* species. Overall, 64 fecal samples from crows (44.4%) and 67 fecal samples from pigeons (42.9%) were positive. All positive samples could be successfully DNA-amplified and sequenced. Nested-PCR was accomplished for

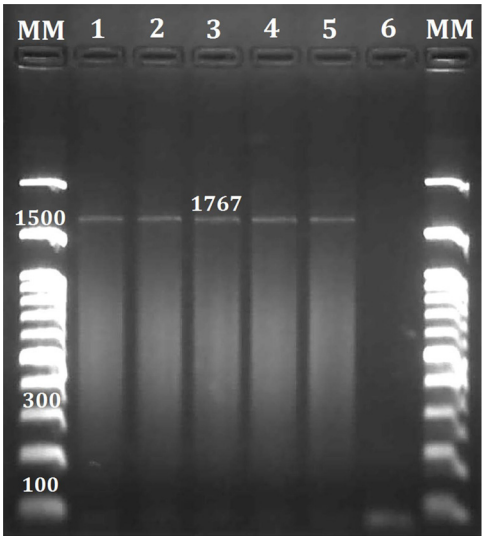

Fig. 1. PCR product electrophoresis of 18S rRNA gene of *Blastocystis* isolated on 1% agarose gel. MM: 100-bp molecular marker; lanes 1-4: *Blastocystis* isolates; lane 5: positive control; lane 6: negative control.

amplification of estimated size of 1767-bp band (Fig. 1). Ribodeme patterns of 131 isolates of *Blastocystis* were obtained by digestion with restriction endonucleases *Hinf*I (Fig. 2A), *Rsa*I (Fig. 2B), and *Bsp*143 (Fig. 2C). Of the 67 positive pigeon's samples, 100% were ST13. Of the 64 positive crow's samples, 71.8% were ST13, 20.3% were ST14, and 7.9% were untypeable. The dominant ST among pigeons and crows was ST13. ST14 was only isolated from the crows.

3.2. Molecular and phylogenetic analysis

Blastocystis-specific 18S rRNA gene was amplified and sequenced successfully for all positive samples. Comparison with sequences in the GenBank database revealed that obtained sequences had high similarity to the *Blastocystis* recorded in the GenBank (Table 1). Multiple alignment and analysis of intra-subtype variation based on the sequenced 18S rRNA gene fragment resulted in an intra-subtype genetic identity of 99%–100% for ST13 and ST14, respectively. Inter-subtype variation was also calculated and showed similarities of 88% for both ST13 and ST14.

Table 1
Sequencing results of *Blastocystis* isolates in this study.

Subtype detected	Host	Gene Access Number	Isolated
ST14	*Corvus cornix*	MH496651	COCO-ASG2
ST13	(*Columba livia*) and (*Corvus cornix*)	MH489081	COCO-ASG3

The partial sequences were submitted to GenBank under accession numbers MH496651-MH489081.

All two subtypes could be clearly distinguished using phylogenetic analysis. The phylogenetic relationships of the isolates were evaluated using the ML phylogram method (Fig. 3). In the ML method, the two ST13 and ST14 obtained in this study formed two clades with the published isolates in GenBank.

4. Discussion

Blastocystis sp. is the most common unicellular eukaryote found in human stool samples in a majority of epidemiological surveys conducted globally. Several virulence factors including cysteine proteases have involved in the pathogenesis of this parasite [28]. *Blastocystis* sp. infection would thus be associated with a variety of non-specific intestinal disorders, such as diarrhea and abdominal pain, and skin rash or urticarial [29]. A wide genetic diversity has been revealed within the genus *Blastocystis* based on the comparison of SSU rRNA gene sequences [30,31]. *Blastocystis* contains at least 17 lineages, which later became known as STs. In fact, 17 known STs have been characterized thus far among mammalian and avian isolates, each exhibiting enough genetic diversity to be classified as distinct species [12,32]. Between these STs, ST1-ST9 has been reported in human that ST1-ST4 is the most common. Usually, the most frequent reported ST of the whole world is ST3 [11,29,32–34]. Among ST1-ST9 except ST9, other STs are isolated from animals in addition to human. The ST5, ST8, and ST12 believed to be of animal origin, are more rarely found in the human population and their presence may be linked with low host specificity and zoonotic transmission of *Blastocystis* sp. [19]. ST10-ST17 are exclusively found in animals. ST10 and 15 are present among *Artiodactyls* and non-human primates, ST11 among *Proboscideans*, ST12 among *Artiodactyls* and

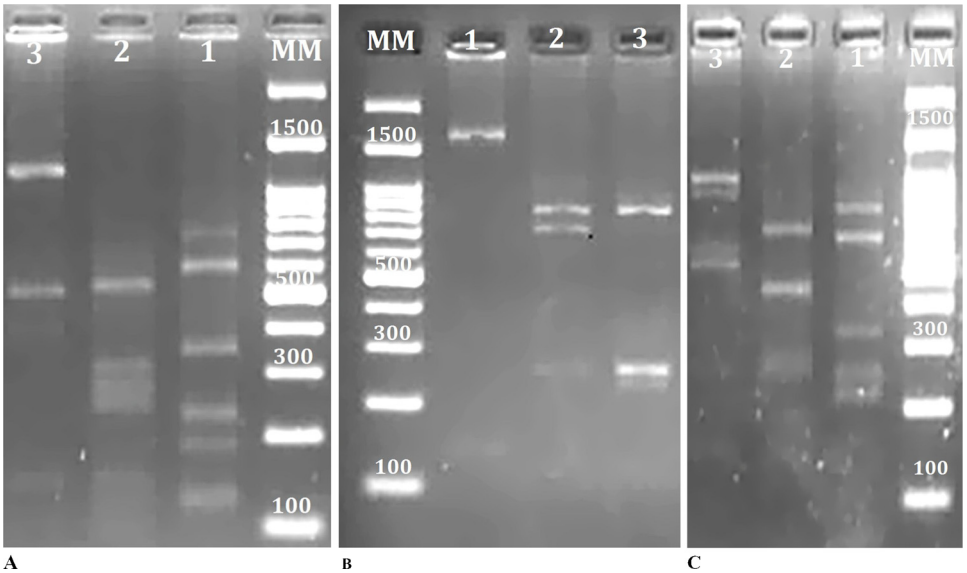

Fig. 2. A. Isolate COCO-ASG3 (Common prevalent subtype among pigeons and crows). 3: *Rsa*I; 2: *Bsp*143; 1: *Hinf*I; MM: molecular marker (100-bp). **B.** Unknown subtype. MM: molecular marker (100-bp); 1: *Rsa*I; 2: *Bsp*143; 3: *Hinf*I. **C.** Isolate COCO-ASG2: 3. *Rsa*I; 2. *Bsp*143; 1. *Hinf*I; MM: molecular marker (100-bp).

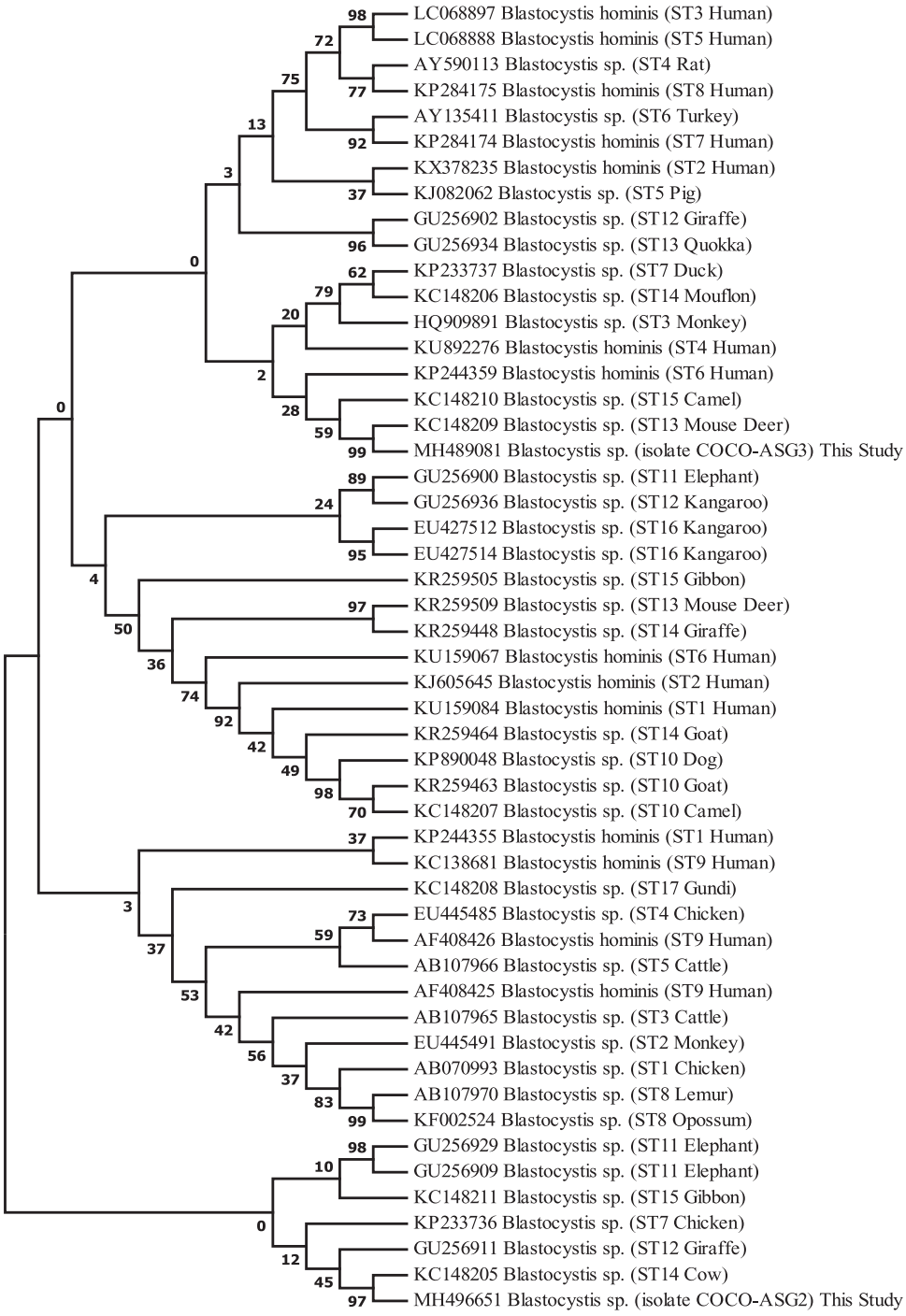

Fig. 3. Molecular phylogenetic analysis among various *Blastocystis* isolates to each other as inferred by Maximum Likelihood method based on 18S rRNA gene. The evolutionary history was inferred based on the Kimura 2-parameter model. Numbers on branches are percentage bootstrap values of 1000 replicates. The reference sequences accession numbers are inserted. Evolutionary analyses were conducted in MEGA-7.

marsupials, ST13 among non-human primates and marsupials, ST14 among *Artiodactyls*, ST16 among marsupials and ST17 among rodents [35]. Moreover, ST6 and ST7 were contemplated avian STs because of their relative superiority in birds [14,19]. This hypothesis was based on the molecular screening of a still restricted number of avian isolates since the largest scale survey to date only incorporated about fifty bird samples gathered in Colombia, for which only ST6 was detected [20]. It has been suggested that avian STs could be zoonotic based on the SSU rRNA gene sequence similarity between ST6 or ST7 isolates of birds and humans [30,31,35]. In this study, for the first time ST13 and ST14 were isolated from crows and pigeons.

This study aims to determine the prevalence and ST distribution of *Blastocystis* sp. in crows and pigeons by screening 300 fecal samples using molecular methods. To identify and characterize the different STs of *Blastocystis* in fecal samples, molecular methods based on PCR have increased dramatically. In this study, Nested-PCR was applied to detect *Blastocystis* infection, because it is rapid and more sensitive relative to other methods including Formalin-Ether, staining and culture methods. This method is also used in epidemiological studies for precise estimation of parasite prevalence in various regions [36]. Various genes have been used to determine the *Blastocystis* STs, but due to enough sequence variation and heterogeneity in the 18S rRNA gene sequences,

this gene is a suitable candidate for molecular epidemiological studies and can identified all STs of this parasite and distinguished from each other [30,31]. In this study, RFLP was used to compare STs, since it is proportionately fast, accurate and reliable for the typing of *Blastocystis* compared to other methods. In the aspect of simplicity and time saving, this method has its own advantages. RFLP can detect *Blastocystis* STs through their comparison with the reported restriction enzyme pattern or through direct sequencing. The latter provides a comparison among STs; and ultimately among the similar patterns. Crows and pigeons were selected in this study, because they are omnivores and live in proximity of humans; and more important there is no any comprehensive study on *Blastocystis* STs in birds of Iran, especially crows and pigeons.

In this study, the prevalence of *Blastocystis* infection was reported 42.9% in pigeons and 44.4% in crows. In previous studies performed in chickens in Brazil [8], Indonesia [37], Malaysia [38], and Australia [39], the prevalence of *Blastocystis* sp. was reported between 30%–95%, respectively. Abe et al., (2002); conducted a research with zoo animals and reported a great prevalence of *Blastocystis* infection around 80% in pheasant and 56% in ducks [40]. Delightfully, *Blastocystis* sp. was also often observed on chicken egg shells in a study conducted in an urban area in Colombia [20].

In this study, for the first time ST13 and ST14 were isolated from crows and pigeons in Tehran province, Iran. The results of PCR-RFLP using restriction enzymes and sequencing showed a high prevalence of ST13 in pigeons, and ST13 and ST14 in crows. These STs was earlier isolated from nonhuman primates, marsupials, and *Artiodactyls* (like cattle, and camels), but not from birds [41]. These STs are reported for the first time from crows and pigeons in this study. Sequencing, their alignment with the sequences recorded in the GenBank and the analysis of the phylogenetic tree, proves the presence of ST13 and ST14 in the birds of this region. In a previous study, it was shown that other STs could occasionally infect chickens, including ST1, ST2, ST4, and ST5 [19]. Earlier epidemiological studies demonstrated the presence of only ST6 in five bird species in Colombia [20], and in ostriches in Malaysia [35], and ST7 in domestic chickens [37].

The high prevalence of *Blastocystis* sp. identified in crows and pigeons induces a potential risk of zoonotic transmission of the parasite, especially in individuals handling pigeons. The results obtained from this study were in contrast to the results reported from Iran and other parts of the world, because these STs were earlier isolated from nonhuman primates, marsupials, and *Artiodactyls*. On account of migrating to different regions by pigeons and crows, it seems that contamination of these birds is due to the feeding of animal feces such as cattle and sheep. The prevalence of *Blastocystis* sp. was assessed among pigeons and crows using molecular method. Other studies had reported this prevalence very low among crows and pigeons using microscopic detection [42–44]. However, this study reported high prevalence of *Blastocystis* infection, using molecular tools. Since there was no similar study in Iran, specifically on crows, and despite of many reports on *Blastocystis* as being pathogenic, conducting such a study deemed necessary.

5. Conclusion

The present study represented the first molecular epidemiological study conducted on crows and pigeons, providing new insights into the prevalence and ST distribution of *Blastocystis* sp. in these animals and emphasizing the zoonotic potential of this parasite. Overall, the results of this study demonstrated that crows and pigeons were often infected by *Blastocystis* sp. and are thus natural hosts of the parasite. However, more comprehensive studies are needed to determine the exact relationship between these birds and other animals and the STs isolated from them. It is counseled to comprise PCR-RFLP technique in routine ST diagnosis and epidemiological investigations.

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