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Predominance of *Cryptococcus neoformans* Var. *grubii* in Ahvaz, Molecular Identification and Evaluation of Virulence Factors

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Abstract

Background: *Cryptococcus neoformans* is an encapsulated yeast pathogen with worldwide distribution, and the highest incidence of cryptococcosis was attributed to *C. neoformans* (var. *grubii*. The pathogenicity of *Cryptococcus* species is associated with several factors, including capsule and melanin production, growth at 37 °C, and secretion of extracellular enzymes.
Objectives: The present study aimed to isolate and identify *Cryptococcus* species from pigeon guano in Ahvaz, Iran and investigate important virulence factors in the isolates.
Methods: Seventy-three isolates of *C. neoformans* var. *grubii* were identified based on classical and molecular microbiology methods. Capsule size was measured by the grow yeasts in the presence of 5% CO_2. Specific media demonstrated the activity of extracellular enzymes (phospholipase, hemolysin, proteinase, esterase, urease, catalase, and gelatinase). Besides, melanin production was evaluated by the niger seed agar medium.
Results: Two hundred and seventeen samples were examined for the presence of *Cryptococcus* over 165 days in Ahvaz. All tested isolates were contained capsules with variable sizes under 5% CO_2 concentration. Moreover, 100% of isolates were produced extracellular enzymes (urease, hemolysin, and catalase), whereas no proteinase and gelatinase activities were observed among isolates. Furthermore, most isolates had phospholipase (93.1%) and esterase activities (86.3%). Also, melanin was produced by all of the isolates.
Conclusions: Although two methods were used for recovery of *Cryptococcus*, only *Cryptococcus* was isolated from pigeon guano, and swabs from the cage walls were negative. *Cryptococcus neoformans* var. *grubii* was the only species from pigeon droppings from Ahvaz with more pathogenic factors. Owing to the high pathogenicity of the isolates, the frequency of the disease is expected to be higher.

Keywords: *Cryptococcus neoformans*, Virulence Factors, Capsule, Enzymes, Melanin

1. Background

Cryptococcus neoformans is an encapsulated yeast pathogen with worldwide distribution, and pigeon droppings are considered an important ecological niche of this pathogen yeast (1-4). Patients with impaired immune systems such as HIV patients, corticosteroid users, and organ transplantation have a higher risk for developing cryptococcosis (5, 6). Inhalation of environmental, infectious propagules or direct contact with etiologic agents can cause cryptococcosis in humans (1). About 70 *Cryptococcus* species have been identified (7); however, the highest incidence of cryptococcosis was attributed to *C. neoformans* (var. *grubii* and var. *neoformans*) and *C. gattii* (8, 9). *Cryptococcus neoformans* var. *grubii* has a worldwide distribution and alone causes about 80% of cryptococcal infections (6). Whereas *C. neoformans* var. *neoformans* is more common in Europe, and *C. gattii* is common in tropical and subtropical regions (5).

The pathogenicity of *Cryptococcus* species is associated with two factors, host factors and organism virulence factors. Organism virulence factors consist of capsule and melanin production, growth at 37°C, and secretion of extracellular enzymes (proteinase, phospholipase, esterase, and urease) (4, 10, 11). Virulence factors are an essential part of microbial pathogenesis. Some of these factors, such as extracellular enzyme production, are common among several species (4). On the other hand, some factors (e.g., capsule formation) are specific to one species (12, 13).

Extracellular enzymes cause host tissue damage and provide optimal conditions for microorganism adhesion

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to tissues (1, 14). Proteases and phospholipases degrade proteins and glycerophospholipids, respectively, as well as help to colonization and tissue invasion (1, 4, 15). Esterase activity has been associated with virulence factors in *Cryptococcus* (1); however, its role in tissue invasion remains still unclear. Urease is one of the most necessary enzymes for escaping *Cryptococcus* from the lungs to the blood-brain barrier (1). Furthermore, *C. neoformans* induces antioxidant activation enzyme (catalase). Catalase converts hydrogen peroxide to water and molecular oxygen and facilitates the growth and survival of *Cryptococcus* in macrophages (16).

The capsule has a key role in *C. neoformans* virulence; thus, capsule-free strains have low or without pathogenicity (10, 11, 13). Melanin is a critical pathogenic factor in *C. neoformans* that is produced by the phenoloxidase enzyme (17, 18). The function of melanin involves the protection of the fungal cell against oxidative degradation, increases the resistance of fungal cells in tissue and environment, immune modulation, and inflammatory response, and probably increased resistance to amphotericin B drug (1, 19, 20).

2. Objectives

Despite the development of molecular methods, very limited studies have been conducted on the diversity of *Cryptococcus* species in Iran. Most clinical available reports in Iran are as case reports (21-23), and environmental epidemiological studies are limited to formal identification. The present study aimed to isolate and identify *Cryptococcus* species from pigeon guano based on molecular methods in Ahvaz, Iran and investigate the important virulence factors in the isolated species.

3. Methods

3.1. Fungal Isolates and Growth Conditions

One hundred and forty-one dried pigeon droppings samples and 76 samples of pigeon cages were collected from 10 different areas in Ahvaz, southwest of Iran. Samples were collected from private houses and pet-shops in sterile packets and transferred to the medical mycology laboratory affiliated to Ahvaz Jundishapur University of Medical Sciences. Approximately 5-10 g of each pigeon dropping was transferred to a sterile tube containing 50 ml of sterile distilled water with several antibiotics, containing penicillin 1,200 U (1 g/L) (Pharmco Jabir Hayyan, Iran), gentamicin (20 μg/L) (Pharmco Jabir Hayyan, Iran) and chloramphenicol (1 g/L) (Bio Basic, Canada). The suspension was mixed for 5 minutes by a vortex (Heidolph, Germany) and allowed to sediment for 1 h, then, 0.1 mL of supernatant was streaked on a niger seed agar (NSA) plate (24).

Samples from cages were collected by rubbing the sterile and moist cotton swabs on the cage walls and immediately plated onto the NSA plates. The cultures were incubated at 32°C and monitored for mucoid and dark-brown colonies for up to two weeks. Then, the Indian ink smears of suspected colonies to *Cryptococcus* were prepared, and the presence of capsules confirmed *Cryptococcus* species. Brown colonies were streaked on the new NSA to obtain single and pure colonies. Pure colonies were subcultured on Sabouraud dextrose agar (SDA) (Pronadisa, Spain) slant tubes and stored at room temperature (25-29°C) until use. In this study, a sequenced *C. neoformans* var. *grubii* isolate (Accession no = KY216187.1) was gifted by prof. Pakshir (Shiraz University of Medical Sciences, Iran) was used as a positive control for all experiments.

3.2. Identification of Cryptococcus Isolates

3.2.1. Classical Identification

All isolates were initially identified as *Cryptococcus* spp. by morphological and microcopy features and biochemical tests, including the Indian ink test, thermotolerance at 37°C, melanin synthesis on NSA medium, and urease production (Figure 1) (24, 25).

3.2.2. Molecular Identification

For DNA extraction, each isolate was sub-cultured on SDA and incubated at 28°C for two days. A loopful of fresh yeasts was transferred into a sterile microtube containing 300 ml of lysis buffer and 50 mg glass bead (Sigma-Aldrich, USA) and put at -20°C for 24 h. Microtube contents were homogenized by a SpeedMill PLUS Homogenizer (Analytikjena, Germany) and then were extracted using phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, Germany) (26). The two sets of universal primers, including ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), were used to amplify the ITS rDNA region (12). The 500 bp bands were observed after the migration of the PCR product by electrophoresis on agarose gel 1.5% (Cinagen, Iran). The PCR products were purified, then sequenced by the Sanger method with primers ITS1 (forward primer), data aligned by MEGA 6 software, and blasted in the NCBI database. The similarity was 100% for 57 (78.1%) isolates whereas 16 (21.9%) isolates had < 99% similarity. All sequenced data were deposited in NCBI GenBank, and accession numbers were obtained.

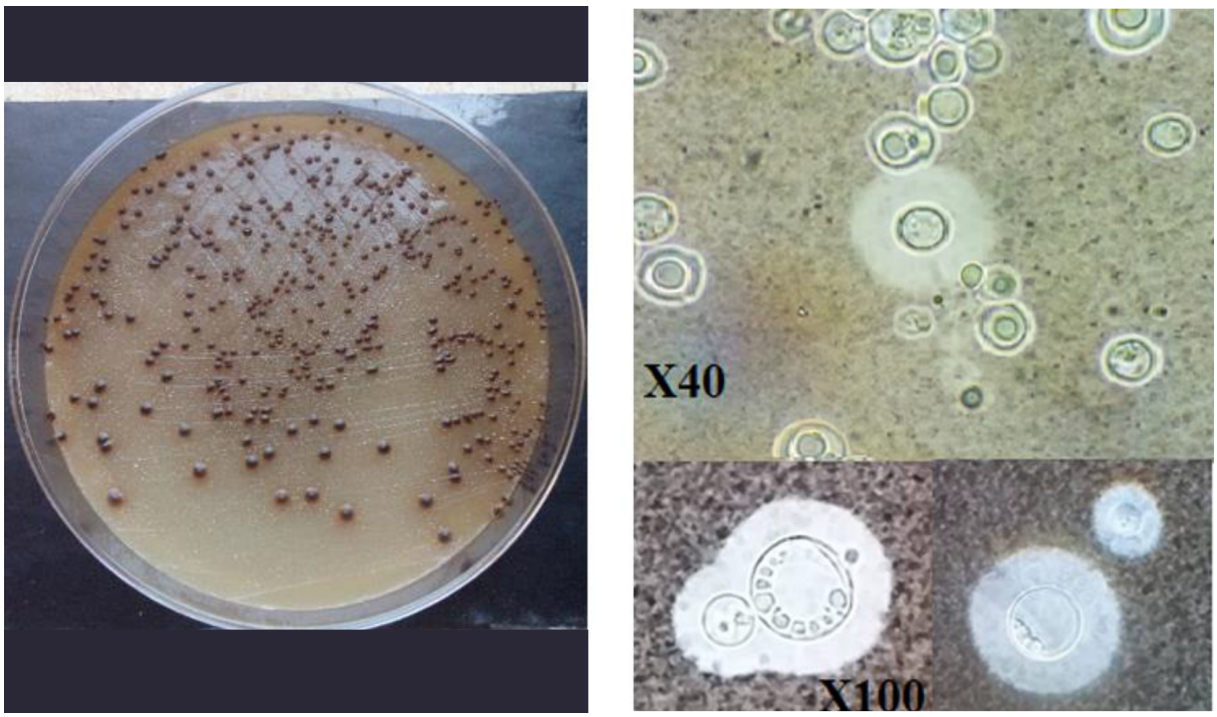

Figure 1. Isolation of *Cryptococcus* from pigeon dropping; small, mucoid, and brownish colonies of *C. neoformans* (Left), *C. neoformans* with a large capsule in Indian Ink (Right).

3.3. Identification of Pathogenic Factors of Cryptococcus Isolates

3.3.1. Capsule Size Determination

A loopful of fresh cultures was inoculated onto diluted Sabouraud dextrose broth (1:10) (BioLife, Italia) (pH = 7.3) and incubated at 37°C under CO_2 condition for 72h (13). Then, 10 μL of a cell suspension was mixed with 10 μL of India ink. A light microscope randomly photographed at least five different fields of each slide. The size of the capsules was scored as large, medium, and small.

3.4. Melanin Production

Thirty microliters of 0.5 McFarland cryptococcal cell suspension were inoculated on NSA and incubated at 30°C for six days (4, 27). Melanin production was evaluated by direct visualization of colony color and scored based on colony color intensity from 1+ to 4+ (4).

3.5. Different Temperature Tolerance

A loopful of each isolate was cultured on both NSA and SDA plates and incubated at 4°C, 30°C, 37°C, 42°C, and 45°C for one month, 48h, 48h, 48h, and 24h respectively. Then, plates were evaluated every day for temperatures 30°C, 37°C, 42°C, and 45°C and 3-4 days for 4°C. Finally, the plates that were previously incubated at 4°C, 42°C, 45°C, and 30°C for five days and then were considered for growth again.

3.6. Phospholipase Activity

Secretory phospholipase activity was determined by egg yolk agar medium, according to Price et al. method (28). Egg yolk agar plates were cultivated by isolates and incubated at 30°C for 15 days in triplicate. Enzyme activity was manifested as a dense zone of precipitation around the colonies, and its level was calculated as precipitation zones (Pz) (27, 29). Pz value is considered by dividing colony diameter by the colony plus precipitation zone. Pz calculated as: Pz value = 1 (negative); Pz value = 0.7-0.99 (weak); Pz value = 0.5-0.69 (mild); and Pz value < 0.5 (strong).

3.7. Proteinase Activity

The proteinase activity of *Cryptococcus* isolates was assayed based on Aoki et al. methods (30). In brief, 0.1 g KH_2PO_4 (Merck, Germany), 0.05 g $MgSO_4$, 7 ml H_2O (Merck, Germany), 0.01 g yeast extract (Merck, Germany), 1 g glucose (Merck, Germany), and 2 g agar (Mirmedia, Iran) were dissolved in 90 mL distilled water. After sterilization, 10 mL of 0.2% sterilized bovine serum albumin (BSA) (Merck, Germany) was added. The plates were inoculated with isolates and triplicate plates incubated at 30°C and 37°C for three weeks. Positive proteinase activity is defined as a clearance zone around the inoculum site. The proteinase activity index (Pz) was calculated as described above.

3.8. Hemolytic Activity

Determination of hemolysin activity was performed by SDA plates containing 3% (W/V) glucose (Merck, Germany) and 7 mL fresh sheep blood (Bahar Afshan, Iran) (V/V%) described by Luo et al. (31). The plates were inoculated with isolates, and triplicate plates were incubated at 30°C for five days. A translucent halo zone around the colonies indicates the positive hemolytic activity of each isolate. The hemolytic activity index was calculated as described above.

3.9. Esterase Activity

The esterase activity of isolates was evaluated using the described method by Slifkin et al. (32). In brief, the medium contained 10 g peptone (Merck, Germany), 5g NaCl (Mojallali Chemical Laboratories, Iran), 0.1 g $CaCl_2$ (Merck, Germany), 15g agar (Mirmedia, Iran), and 5 mLTween 80 (Merck, Germany) and 1,000 mL distilled water. The esterase medium plates were inoculated with isolates in triplicate and incubated at 30°C for 15 days. The esterase activity index was calculated as described above.

3.10. Urease Activity

Urease activity of isolates was determined by the urea agar base (Merck, Germany) supplemented by 50 ml/L urea 40%. The medium was inoculated with a 0.5 McFarland standard suspension of each isolate and incubated at 37°C for one week. The coloration of urea agar was graded from 1+ to 4+, which indicated the intensity of urea hydrolysis (12).

3.11. Catalase Activity

The catalase activity of isolates was detected using an overnight culture. A loopful of a small colony was suspended in an H_2O_2 drop on a clean microscope slide and monitored to generate Oxygen bubbles.

3.12. Gelatin Hydrolysis

The production of gelatinase was assayed by gelatin agar medium contained 8 g gelatin and 23 g nutrient agar (BioMerieux, France) in 1,000 mL of distilled water. The gelatin agar plates were inoculated with isolates and incubated at 30°C for four weeks, according to Kanemitsu et al. (33). The presence of a hyaline halo around the inoculated area indicates gelatinase activity.

4. Results

Of 217 samples from pigeon droppings (141 samples) and pigeon cages (76 samples), only 43 (30.5%) pigeon dropping samples were positive for *Cryptococcus*. Moreover, all cages samples were negative for *Cryptococcus*. In the present study, 73 *Cryptococcus* strains were isolated from samples, and based on the sequencing of the ITS rDNA regions, all of the 73 isolates were detected as *C. neoformans* var. *grubii* (44 accession numbers: from LC535977 to LC536020; 22 accession numbers: from LC537132 to LC537153; 7 accession numbers: from LC545841 to LC545847). It was found that 30 (41.1%) isolates produced large capsules as well as medium and small capsules, followed by 72 (98.6%) medium capsule size and 66 (90.4%) small capsule size.

All *C. neoformans* isolates had robust growth at 30°C and 37°C, whereas no growth was seen at 45°C. Moreover, 52 (69.9%) of the isolates could grow at 4°C, including moderate grow 36 (49.3%), and weak grow 15 (20.6%). When cultures were transferred from 4°C to 30°C conditions, most isolates (86.3%) had well growth, followed by moderate growth (12.3%), and weak growth (1.4%). Furthermore, the ability to grow at 42°C was only found at 14 (19.2%) isolates. Although the melanin intensity and urease activity varied among isolates, 100% of isolates were positive for melanin pigmentation and urease activity (Table 1).

Table 1. Melanin Production and Urease Activity in 73 Isolates of *Cryptococcus neoformans* var. *grubii*

Activity	Melanin production, No. (%)	Urease activity, No. (%)
Very high (4+)	6 (8.2)	29 (39.7)
High (3+)	24 (32.9)	26 (35.6)
Medium (2+)	24 (32.9)	13 (17.8)
Low (1+)	19 (26)	5 (6.8)
Total	73 (100)	73 (100)

Interestingly, 100%, 93.1%, and 86.3% of isolates produced hemolysin, phospholipase, and esterase extracellular enzymes with different pz index, respectively. Also, all isolates were capable of utilizing hydrogen peroxide. However, all isolates lack extracellular proteinase and gelatinase activity (Figure 2).

5. Discussion

Cryptococcus neoformans has a worldwide distribution, and it is also a common cause of cryptococcosis among patients suffering from AIDS (2, 5, 13, 34). However, few

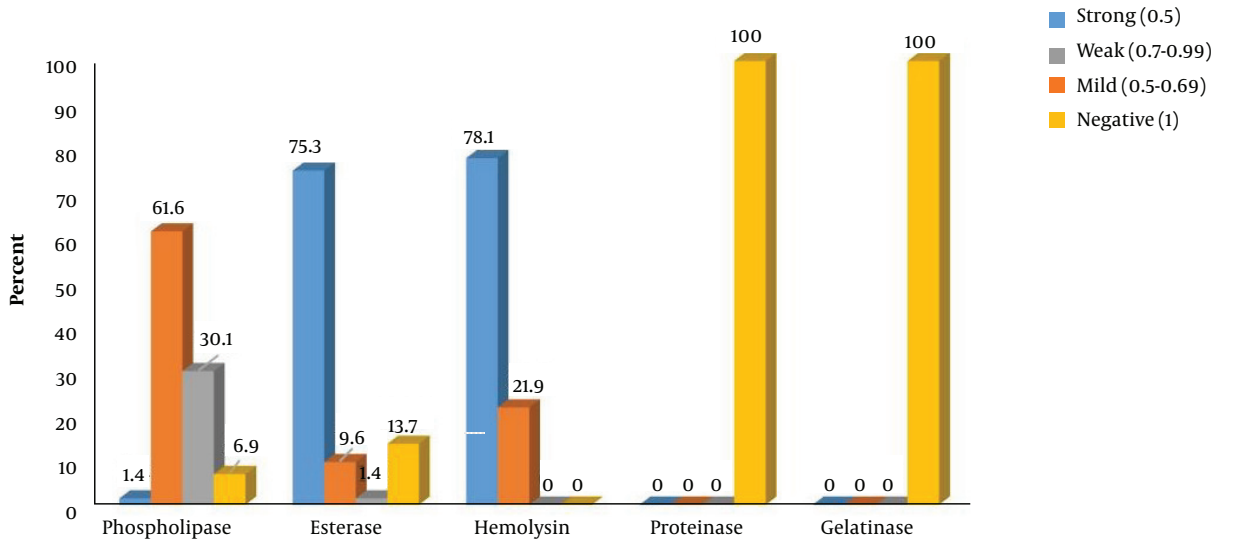

Figure 2. Extracellular enzyme activities of 73 isolates of *Cryptococcus neoformans* var. *grubii*.

studies have demonstrated the epidemiology of *Cryptococcus* species and their pathogenic factors in Iran. Moreover, some rare species of *Cryptococcus* have been isolated in some clinical forms (35). Although some clinical cases of cryptococcosis and a few environmental studies were reported in Iran, *C. neoformans* var. *grubii* was only detected by Badali et al., and Pakshir et al., using molecular methods (21-24, 36-38). Cryptococcosis was reported from different parts of the world, including India (39), Italy (40), Brazil (41), and China (7, 42). Besides, *C. neoformans* var. *grubii* was the most common variety in clinical and environmental samples (7, 39-43).

The present study is the first epidemiological and molecular identification of *Cryptococcus* species isolated from pigeon droppings in Ahvaz, southeast Iran. In the present study, 43 (30.5%) pigeon dropping samples were positive for *Cryptococcus*, and 73 isolates of *C. neoformans* var. *grubii* were isolated from samples. Afshari et al. have shown a lower occurrence (5%) of *C. neoformans* in pigeon excreta in Mazandaran, northern Iran, with the most common species *C. neoformans* var. *grubii* (90%) followed by *C. neoformans* var. *neoformans* (10%) (5). Although Kamari et al. isolated 12 *Cryptococcus* species from pigeon nest and *Eucalyptus* tree samples in Isfahan, only 1 (8.3%) *C. neoformans* var. *grubii* was detected (3). Similar to the current study, the frequency of positive cultures for *C. neoformans* from pigeon droppings was 34%, based on Zarrin et al. report from Ahvaz (44), and they were only identified based on morphological tests. The main source of *C. neoformans* is pigeon droppings; however, it was also recovered from several trees, including *Eucalyptus camaldulensis*, *Ceratonia siliqua*, *Olea europaea*, and *Pinus* spp. (45).

Extracellular enzymes have important key roles in the pathogenesis of *C. neoformans*. These enzymes decompose living host tissues and help invade organs (1, 29). However, the pattern of enzyme secretion varies in different species/strains with different sources (12, 29). Pini et al. believe that there is a significant difference in phospholipase activity between clinical and environmental isolates of *C. neoformans* (4). In contrast, differences were not found between clinical and environmental isolates of *C. neoformans* among the evaluated extracellular enzymes in virulence factors in the Andrade-Silva et al. study (41). None of our isolates could secrete proteinase or gelatinase enzymes, while all isolates exhibited a variable rate of hemolysin, catalase, and urease activities. Moreover, the majority of isolates were positive for the presence of phospholipase (93.1%) and esterase (86.3%). Pedroso et al. evaluated the production of phospholipase and urease in *C. neoformans* var. *grubii* and found that 100% and 90% of isolates had phospholipase and urease activities, respectively (12). However, they reported that most isolates (54.5%) had proteinase activity.

The ability to form a polysaccharide capsule in *C. neoformans* is one of the most important virulence factors. In the host body, the capsule prevents phagocytes and protects against oxidative bursts (1, 46). Although higher levels of CO_2 stimulate increasing capsule size in *C. neoformans* strains, CO_2 levels have not the same effect on all strains of *C. neoformans* (13). In the present study, different

capsule sizes were observed during incubation at CO_2 and 37°C for three days. Large capsules were produced in 41.1% of isolates. Although all of the isolates (100%) were positive for melanin production on *G. abyssinica* medium, it was found that they differed in the intensity of melanin formation. The melanization in *Cryptococcus* species is mainly associated with adaptation to adverse environmental conditions and resistance to oxidative damage and antimicrobial compound in tissue cells (19, 47). As a result, melanization plays a vital role in the pathogenicity of *Cryptococcus* species (17, 18). Pini et al. have shown that 2.4% and 3.1% of clinical strains and environmental strains were negative for melanization, respectively (4).

Although *Cryptococcus* species can grow in routine media at 25 to 37°C, the ability to grow at 37°C is very important for its pathogenicity (12, 48). In the present study, the isolates' growth was well and similar at temperatures of 30°C and 37°C. Moreover, all incubated plates at 4°C for one month began to grow after incubation at 30°C. Only 19.2% of isolates retained their ability to grow at 42°C and the rest of them (80.8%) were unable to grow at 42°C even after transfer to 30°C. However, all incubated isolates at 45°C for 24 h were unable to grow after transfer to 30°C. *Cryptococcus neoformans* can produce different heat shock protein (hsp) types (examples, hsp 60, 70, and 80) that protect the organism in mammalian hosts (48).

5.1. Conclusions

Although two methods were used for recovery of *Cryptococcus*, only *Cryptococcus* was isolated from pigeon guano, and swabs from the cage walls were negative. *Cryptococcus neoformans* var. *grubii* was the only species from pigeon droppings from Ahvaz with more pathogenic factors. Owing to the high pathogenicity of the isolates, the frequency of the disease is expected to be higher.

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Footnotes

Authors' Contribution: Study concept and design, Ali Zarei Mahmoudabadi, and Mahnaz Fatahinia; sampling, isolation, conducting the experiments, Maryam Moslem; data analysis, Maryam Moslem, and Neda Kiasat; drafting of the manuscript, Maryam Moslem; critical editing, Ali Zarei Mahmoudabadi.

Conflict of Interests: The authors declare that they have no potential conflicts of interest.

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