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Identification of Some Potentially Pathogenic Bacteria in Cloacal Swap Samples of Caged Birds Using Partial 16S rRNA Sequence Analysis

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Caged birds can be reservoirs of various microorganisms in their gut microbiota some of which can be harmful to humans. Considering people's interests in keeping wild cage birds and the potential ability of wild cage birds to transmit bacteria to humans, the present study was conducted to investigate some bacterial agents in the microflora of caged birds by polymerase chain reaction (PCR) and sequence analysis. Altogether 68 samples from different birds were collected with sterile cotton swabs from different areas of Mazandaran province, Iran and evaluated for detection of bacteria by sequencing of partial 16SrRNA PCR product. In total, 11 samples were positive for PCR reaction using universal primers. PCR products of samples were sequenced and results were uploaded to NCBI with the accession numbers. Comparison of the sequences with other similar sequences showed the similarity of the sequences to *Corynebacterium*, *Tessaracoccus* and, *Christensenella* species. Results showed the possible role of caged birds in the circulation and transmission of some opportunistic pathogenic bacteria.

Key Words: *PCR, Caged birds, 16SrRNA sequencing*

Kısmi 16S rRNA Sekans Analizi Kullanılarak Kafesteki Kuşların Kloakal Örneklerindeki Bazı Potansiyel Patojen Bakterilerin Tanımlanması

Kafes kuşları, bağırsak mikrobiyotalarında insane sağlığını tehdit edebilecek çeşitli mikroorganizmaları taşıyabilmektedirler. İnsanların yabani kafes kuşlarını besleme konusundaki ilgileri ve yabani kafes kuşlarının bakterileri insanlara bulaştırma potansiyelleri gözönüne alındığında, bu çalışma kafes kuşlarının bağırsak florasındaki bazı bakyeriyel etkenlerin polimeraz zincir reaksiyonu (PCR) ve sekans analizi ile belirlemek için yapılmıştır. İran'ın Mazandaran eyaletinin farklı bölgelerinden steril svap ile toplam 68 farklı kuş örneği toplandı ve kısmi 16SrRNA PCR ürününün sekanslanmasıyla bakteriyel etkenler identifiye edildi. Toplamda 11 numune, evrensel primerlerin kullanıldığı PCR ile pozitif bulundu PCR ürünleri, NCBI'den sekans erişim numaraları alınarak sekanslandı. Dizilerin diğer benzer dizilerle karşılaştırılması sonucunda dizilerin *Corynebacterium*, *Tessaracoccus* ve *Christensenella* türleri ile benzerlik gösterdiği belirlendi. Sonuçlar, bazı fırsatçı patojen bakterilerin insanlara bluşmasında kafes kuşlarının rol oynayabileceğini gösterdi.

Anahtar Kelimeler: *PCR, Kafes kuşları, 16S rRNA dizilimi*

Introduction

The gastrointestinal microbiota of animals plays an important role in health, immune response, and defense against pathogens. This microbiota varies depending on the host and diet of animals and humans (1). Different microorganisms including microflora and other opportunistic transient pathogen bacteria live in the gastrointestinal tract of birds (2). The higher body temperature of the birds in comparison with other animals such as ruminants or human microflora makes their microbiota specific and unique. Various bacteria, fungi, and protozoa populations are living inside the gut of wildlife and domesticated birds and some of them may be spread into new hosts in human or other animal species at least as transient microflora (3). The presence of these bacteria in other hosts can lead to the incidence of some infectious diseases, immune reactions with malfunction disorders, and antibiotic resistance in new hosts (4-6). In recent decades, our relationship with birds has been increasing. More than poultry and other bird breeding farms all around the world, those were the origins of some significant viral diseases such as influenza; we kept and breed some birds as a game or ornamental birds. People trade the birds and birds are transferred from one country to another, with all contents in their bodies (7). These birds can transfer some bacteria to their owners and other humans. Identification of possible microorganisms particularly potential opportunistic bacteria existing in birds' gut microflora can change our point of view about the origin of some emerging pathogenic bacteria. So, the present study was conducted to investigate the some bacteria in cage using universal PCR primers and analysis of partial 16SrRNA sequence.

Materials and Methods

Research and Publication Ethics: The research was done in accordance to the ethical principles and the national norms and standards for conducting Medical Research in Iran and received the approval ID from Research Ethics Commitees of Amol University of Special Modern Technlogies: IR.AUSMT.REC.1401.001.

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Sample Collection: A total of 68 cloacal swabs were carefully collected from apparently healthy caged birds including pigeon (*Columba livia*) (n=44), chicken (*Gallus gallus*) (n=10), lovebird (*Agapornis roseicollis*) (n=8), and canary (*Serinus canaria*) (n=6) in Mazandaran province in Iran. Immediately after collection, each sample was inoculated into the sterile nutrient broth (NB) and kept in the ice box, and transported to the Bacteriology Laboratory of the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies.

DNA Extraction: Genomic DNA from cloacal samples was isolated using a stool DNA extraction kit (Bioneer, Daejeon, South Korea) according to the producer endorsements with some adjustments. Concisely, 100 mg of each sample was mixed with 20 µL proteinase K and 100 µL lysis buffer and incubated for 10 min at 55 °C. After centrifugation of the mixture at 13000 rpm, the supernatant was mixed with 200 µL binding solution in a new tube and incubated again for 10 min at 60 °C. After incubation, 100 µL isopropanol was added to the tube and then the liquid was transferred into the binding column and centrifuged for 1 min at 8000 rpm. This step was repeated using 500 µl for both washing buffers 1 and 2; then, DNA was precipitated using 100 µL elution buffer and centrifugation at 13000 rpm for 1 min. Extracted DNA was kept at -20 °C until using in PCR.

PCR Assay: Two universal oligonucleotide primers including forward primer with the sequence of `5-CGGTGGGTACTAGGTGTGGGTTTC -3` and reverse primer with the sequence of `5-CTGCGATTACTAGCGACTCCGACTTCA -3` previously described for *Mycobacterium* species were used to amplify the partial 16S rRNA locus (8). The PCR reaction mixtures consisted of 100 ng DNA template, 2.5 µl 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl2, 50 mM KCl, 20 mM (NH4)2SO4; Bioneer, Daejeon, South Korea), 0.2 mM dNTPs (Bioneer, Daejeon, South Korea), 1.5 U AmpliTaq DNA polymerase (Bioneer, Daejeon, South Korea), and 10 pmol each primer (Takapouzist, Tehran, Iran). The volume of the reaction mixture was completed to 25 µl using distilled deionized water. The thermal cycler (MJ mini, BioRad, USA) was adjusted under optimum conditions. Briefly, Initial denaturation at 94°C for 4 min, followed by 33 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 7 min. Amplified products, with 543 bp length, were separated by electrophoresis in 1.5% agarose gel electrophoresis stained with ethidium bromide (Cinacolon, Tehran, Iran). The 100 bp DNA ladder was used as a molecular size marker.

Sequencing and Phylogenetic Analysis: PCR products of the positive samples with the specific suspected bands for primers were subjected to sequencing (Takapuzist Co., Iran). The sequencing results were submitted, analyzed, and compared to the GenBank databases using the BLAST program maintained by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). In addition, multiple sequence alignments were made by the ClustalW method, using MEGA7 software and the phylogenetic tree was drawn using the Bootstrap method with 1000 replications (9).

Results

Out of 68 cloacal samples, 11 (16.1%) were positive for PCR reaction and produced a 543bp PCR fragment (Figure 1). PCR products of all 11 positive samples were subjected to sequencing and take the sequence accession numbers from NCBI respectively as follows: MG230316, MG203874, MG207961, MG208852, MG208062, and MG205784 for *Colombia livia* samples; MG206064, MG203881, and MG198762 for *Gallus gallus* samples; MG241109 for *Agapornis rosicollis* sample and MG206063 for *Serinus canaria* sample (Table 1).

Table 1. Possible microorganisms in bird fecal samples according to the NCBI BLAST software analysis of the PCR products sequences

NCBI accession number	Source of sample	Probable microorganism based on NCBI BLAST identity
MG230316	*Colombia livia*	*Corynebacterium* sp.
MG203874	*Colombia livia*	*Corynebacterium* sp.
MG207961	*Colombia livia*	*Corynebacterium* sp.
MG208852	*Colombia livia*	*Corynebacterium* sp.
MG208062	*Colombia livia*	*Corynebacterium* sp.
MG205784	*Colombia livia*	*Corynebacterium* sp.
MG206064	*Gallus gallus*	*Tessaracoccus* sp.
MG203881	*Gallus gallus*	*Christensenella* sp
MG198762	*Gallus gallus*	*Christensenella* sp.
MG241109	*Agapornis rosicollis*	*Weissella* sp.
MG206063	*Serinus canaria*	*Corynebacterium* sp.

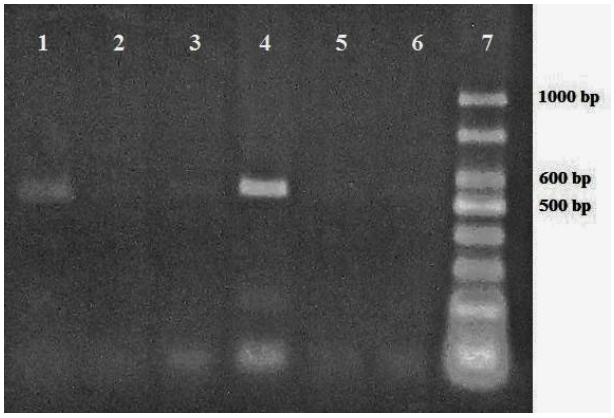

Figure 1. agarose gel electerophoresis of PCR product of the partial 16S rRNA universal primers. Lane 1 and 4: positive samples with 543 bp band; Lane 2, 3, 5: negative samples; Lane 6: negative control (without template DNA); Lane 7: 100 bp DNA ladder as molecular size marker

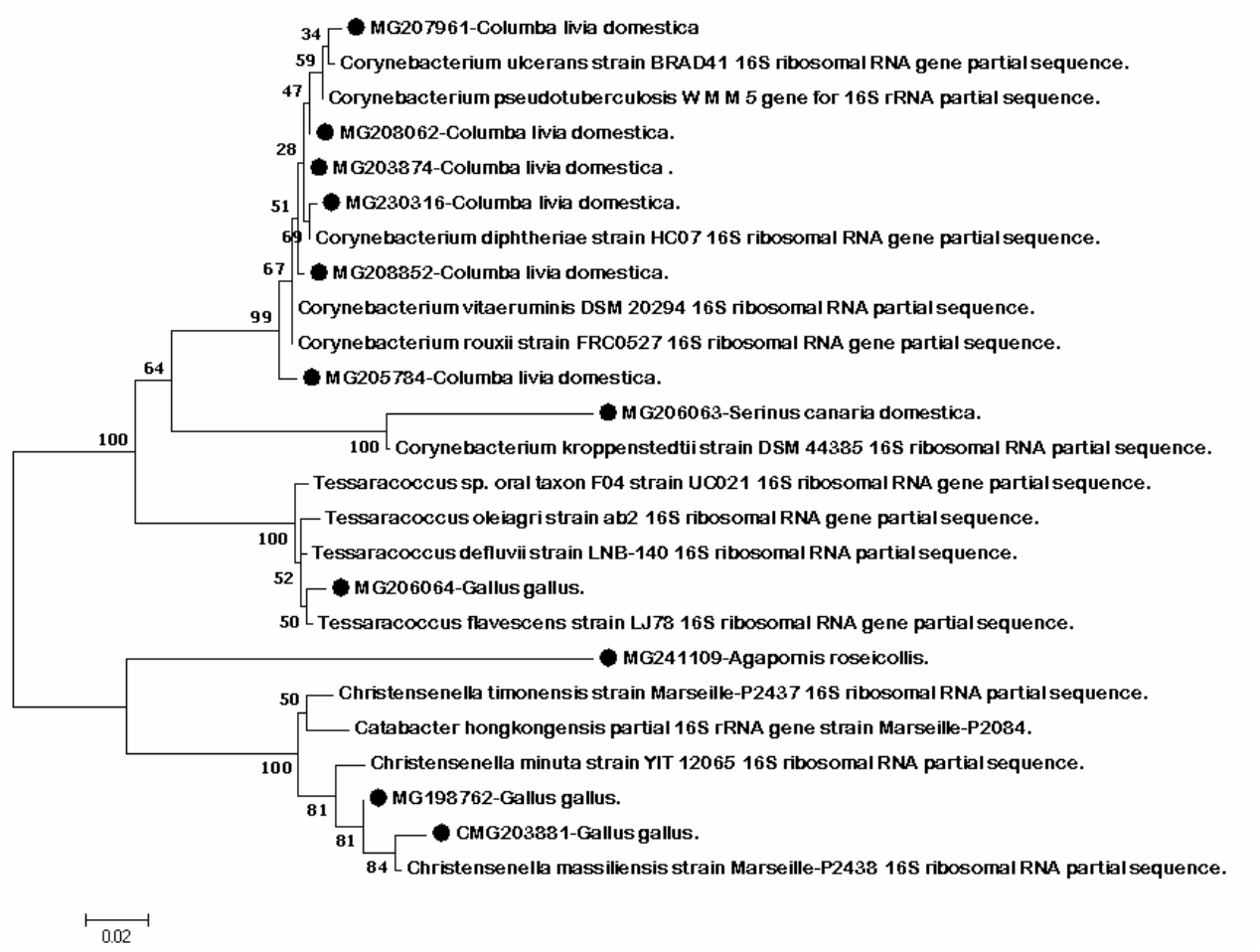

Figure 2. Phylogenetic tree drawn by MEGA7 software according to the similarity of the sequences of the present study and other database sequences

Sequence analysis using NCBI BLAST and comparison with GeneBank sequences by MEGA software revealed a phylogenetic tree (Figure 2). Comparison of the sequences with other similar sequences showed the similarity of the seven sequences to *Corynebacterium ulcerans*, *Corynebacterium pseudotuberclosis*, *Corynebacterium diphteriae*, *Corynebacterium rouxi*, and *Corynebacterium vitaruminins*. One sequence showed high similarity to *Tessaracoccus* sp. Two sequences showed similarity to *Christensenella* species (Figure 2).

Discussion

The gastrointestinal microbiota reveals not only the evolution of the mutualism life of microorganisms with their host but also reflects the relationship with foods and other possible hosts around it. Providing proper bacteria as microflora in the gastrointestinal tract of animals can be a significant approach to yielding more healthy life for both humans and animals (3, 10). Identification of these bacteria is commonly performed using 16S rRNA sequencing (11, 12). In the present study in opposition to other studies, we did not use routine 16S rRNA-specific primers which amplify the whole 1525 bp sequence of the DNA. Primers used in the present study are previously designed for the partial conservation sequence of the *Mycobacterium* genus and related organisms. So identification of the complete microbiota in accordance with other studies which cloned the 16S rRNA sequence into the vector plasmids (13, 14) was not done in the study.

Analysis of the sequences obtained from PCR products showed great similarity to some pathogenic, human-microbiota-related organisms or emerging bacteria. Particularly, similarity to *Corynebacterium* species was found in seven positive samples. As we know *Corynebacterium diphtheriae* is the cause of classical diphtheria, and *C. ulcerans* has been found to convey the gene that codes for producing the diphtheria toxin that can cause swelling of the pharynx in humans (15). Surprisingly in the present study sequences close to *Corynebacterium* species were associated with pigeon (*Columba livia*) samples.

The previous study showed that *Christensenella minuta* in the human gut has been associated with a decrease in body weight and adiposity of mice in the lab (16). In an assessment of a number of volunteers, people with higher levels of *Christensenella* in their guts were found to be more likely to have a lower body mass index (BMI) than those with low levels and the bacterium is better characterized in persons who are metabolically healthy (17, 18). However, there is an association with the potential pathogenic abilities of *C. minuta* in humans (19). In the present study sequences belonging to chicken (*Gallus gallus*) were related to *Christensenella* species. This issue can indicate the possibility of the presence of this opportunistic pathogen in breeding and native birds of each region.

Tessaracoccus is a Gram-positive, non-spore-forming, facultatively anaerobic, and non-motile bacterial genus from the family Propionibacteriaceae (20). One of the sequences obtained from the chicken sample was greatly similar to *Tessaracoccus flavescens*. Other species of this genus have recently been detected from other sources such as dark diving beetle (*Hydrophilus acuminatus*), human vaginal swabs, and the Antarctic environment (21-23). Definitely, in the present study, the exact determination of the species can be done after the isolation of bacterial isolates from these sources and with further studies.

Finally, the results of this study show that in addition to bacteria and other microorganisms present in the digestive system of birds under the title of microbiota, other bacteria as transient flora or accidental microorganisms can enter their digestive system as a potential reservoir to disperse and find a new host and environment.

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