

Short Communication / Kısa Bilimsel Çalışma

PCR detection of *Mycobacterium genavense* DNA in fecal samples of caged birds

Orkun BABACAN^{1,2,a,✉}, Bülent BAŞ^{2,b}, Barış SAREYYÜPOĞLU^{2,c}

¹Balıkesir University, Kepsut Vocational School, Department of Veterinary, Kepsut, Balıkesir; ²Ankara University, Faculty of Veterinary Medicine, Department of Microbiology, Dışkapı, Ankara, Turkey
^aORCID: 0000-0003-0258-1825; ^bORCID: 0000-0001-9992-8738; ^cORCID: 0000-0002-2212 2610.

✉Corresponding author: orkun.babacan@balikesir.edu.tr
Received date: 07.08.2019- Accepted date: 04.11.2019

Abstract: In this study, pathogenic mycobacteria were investigated in fecal samples of caged birds by PCR. A total of 47 feces samples collected from 4 different aviaries in Ankara. DNA extraction from fecal samples was performed with a commercial kit using spin column technology. PCR was performed with designed primers respectively amplifying 274 base pairs (bp), 128 bp, 102 bp and 219 bp nucleotide sequences of specific genes (16SrRNA, IS1245, IS901 and hypothetical 21kDa protein gene) of *Mycobacterium* spp., *Mycobacterium avium* complex (MAC), *Mycobacterium avium* subsp. *avium* and *Mycobacterium genavense*, respectively. Five samples were positive and harbored the sequence for the *Mycobacterium* spp., of 4 of these 5 samples was identified as *M. genavense* by PCR. As a conclusion of this study, which is the first announcement of the detection of *M. genavense* DNA in fecal samples of caged birds in Turkey, PCR was seen to be a rapid, sensitive, and a reliable method in detection of avian mycobacteriosis.

Keywords: Bird, feces, mycobacteriosis, *Mycobacterium genavense*

Kafes kuşu dışkı örneklerinde *Mycobacterium genavense* DNA'sının PZR ile saptanması

Özet: Bu çalışmada, kafes kuşlarının dışkı örneklerinde PCR ile patojenik mikobakterilerin varlığı incelendi. Çalışmanın materyalini Ankara'da 4 farklı kuşhaneden toplanan 47 dışkı örneği oluşturdu. Dışkı örneklerinden DNA ekstraksiyonu, spin kolon teknolojisi kullanılarak ticari DNA ekstraksiyon kiti ile yapıldı. PCR, *Mycobacterium* spp., *Mycobacterium avium* complex (MAC), *Mycobacterium avium* subsp. *avium* ve *Mycobacterium genavense*'nin spesifik genlerinin (16SrRNA, IS1245, IS901 ve hipotetik 21kDa protein geni) sırasıyla 274 baz çiftini (bp), 128 bp, 102 bp ve 219 bp nükleotit sekanslarını çoğaltan tasarlanmış primerlerle gerçekleştirildi. PZR sonucunda, 5 dışkı örneği *Mycobacterium* spp. için pozitif bulundu. Örneklerin 4'ü PZR'da *M. genavense* olarak tanımlandı. Türkiye'deki kafes kuşlarının dışkı örneklerinde *M. genavense* DNA'sının saptanmasına yönelik ilk duyuru olan bu çalışmanın sonucu olarak, PZR'nin kanatlı mikobakteriyozisi tespitinde hızlı, duyarlı ve güvenilir bir yöntem olduğu görülmüştür.

Anahtar sözcükler: Dışkı, kuş, mikobakteriyozis, *Mycobacterium genavense*

Avian tuberculosis is a chronic wasting disease in cages, exotic, zoo and wild birds in the world. In recent years, for exotic birds, *Mycobacterium genavense* has been reported as agent of tuberculosis (2,12,16,17,18,20,22). *M. genavense*, in bird species, especially avian Passeriformes and Psittaciformes, described as the most frequent etiologic agent of avian tuberculosis (4,14). *M. genavense* was first described on AIDS patients in 1990 (17). In people with weakened immune systems, children and other animal species including the dog could cause the infection (1,3,7,8,11,14,20). The disease occurs sporadically and can also be seen in intensive breeding companies (6).

Mycobacteria can exist for months in the environment. The main sources of infection are infected animals, contaminated soil and water (12,20). *M. genavense* is transmitted through the digestive system by fecal-oral route (10). Feces of infected birds are one of the important source for infection. In intensive breeding, stool contains a large number of bacteria for bird to bird contact (9,16,20). People may inhale the bacteria as a result of direct contact with infected birds (9,10).

Mycobacteriosis generally occurs in adult birds more than young birds. Avian tuberculosis causes direct and indirect economic losses (19).

M. genavense shows similar clinical and histopathological lesions of *Mycobacterium avium* complex (14,15,17). The deaths occur after several months because it is a chronic infection. Infected birds usually are greater than one year of age (12). Also, they are weakened with a weight loss (5,12,15,20). Although some infected birds, which may exhibit a normal appearance and behavior, could be found dead. The most common route of transmission is by fecal-oral route. Because of that, lesions can be seen in the digestive tract (20).

M. genavense is a fastidious and slow growing bacterium (5). For this reason, it is very difficult to culture this bacteria (11,17,20). Because of this, the diagnosis based on Ziehl-Neelsen staining of asidoresistance bacteria from infected samples (2,17). Since *M. genavense* is a fastidious bacterium, PCR is a rapid and accurate method for detecting the small amount of DNA from clinical samples (2,9,15,19,20). Results are obtained within hours (19). Fresh tissues and feces can be used for the diagnosis of infection in PCR method (20). Because of the risk of zoonotic infection between individuals with a weakened immune system and their animals, rapid diagnosis is very important to prevent serious infection (11).

In this study, presence of *M. genavense* DNA was investigated by PCR from cage bird feces samples.

This study was carried out in Ankara University Faculty of Veterinary Medicine Department of Microbiology. A total of 47 feces samples were collected from four different aviaries in Ankara region, Turkey. There were canaries, parrots, budgerigars, pigeon, pheasant and dove birds in aviaries. The number of birds in cages ranged from 1-23. From the first aviary 10 feces samples were taken. 12, 13 and 12 feces samples were taken from the second, third and fourth aviaries, respectively. Feces samples were collected from cages floor and put into the sterile containers. The amount of each feces samples were approximately 25 grams. Symptoms were evaluated

following clinical examinations and anamnesis was obtained from the owners. Respiratory system symptoms of the birds were changing from slight to heavy. They showed conjunctivitis and diarrhoea, some birds without any symptoms.

PCR was used for the investigation DNA of *Mycobacterium* spp., *Mycobacterium avium* complex (MAC), *M. avium* subsp. *avium* and *M. genavense* from birds feces. DNA extractions were performed with the QIAamp DNA Stool Mini Kit (Qiagen), which works by spin column technology, from birds feces.

The primers used in this study, were designed by Barış Sareyyüpoğlu with Primer3 (21). PCR was performed with designed primers respectively amplifying 274 base pairs (bp), 128 bp, 102 bp and 219 bp nucleotide sequences of specific genes (16SrRNA, ISI245, IS901 and hypothetical 21kDa protein genes) of *Mycobacterium* spp., MAC, *M. avium* subsp. *avium* and *Mycobacterium genavense*, respectively. (Table 1). The PCR were performed for all target genes, in a total reaction volume of 25 µl, containing 2.5 µl 10x PCR buffer, 3 µl 25 mM magnesium chloride, 250 µM of each deoxynucleotide triphosphate, 1.25 U Taq DNA Polymerase, 20 pmol of each primer and 25 ng of template DNA. The reaction conditions for the *M. genavense* specific PCR are 35 cycles of denaturation at 94°C for one minute, annealing at 54°C for one minute and extension at 72°C for one minute, followed by a final extension step at 72°C for 7 minutes. The amplified products were detected by staining with 10 mg/ml ethidium bromide after electrophoresis at 80 V for two hours in 2% agarose gels. Results were screened from agarose gel by molecular imaging system (Gene Genius, Syngene, England).

M. genavense DNA used as positive control in PCR tests, was supplied by Enrico Tortoli from Regional Reference Centre for Mycobacteria, Florence, Italy. Also *M. avium* subsp. *avium* strain (German Collection of Microorganisms and Cell Cultures-DSMZ; DSM NO:44156) used as a positive control in PCR tests.

Table 1. Primers, target genes, sequences and product sizes

Primers	Name of primer	Target gene	Product size (bp)	Primer sequence
<i>Mycobacterium</i> spp.	Mycol1F	16SrRNA	274	TGGGTACTAGGTGTGGGTTTCC
	Mycol1R			TTAACCCAACATCTCACGACAC
MAC	MAC1F	ISI245	128	TGGCCGGCTCGGTACTCGTT
	MAC1R			GGCTGTGGGGGCAATGGTTT
<i>M. avium</i> subsp. <i>avium</i>	Masa1F	IS901	102	CTCGATGCTCACCGCCATCTT
	Masa1R			ATTTCGCCCCGAGTGCACATAG
<i>M. genavense</i>	Mgen1F	hypothetical 21kDa protein genes	219	TGACTGGTCGTTTGAGATGAAT
	Mgen1R			GATCGGAGGCAGTTCAATGTAG

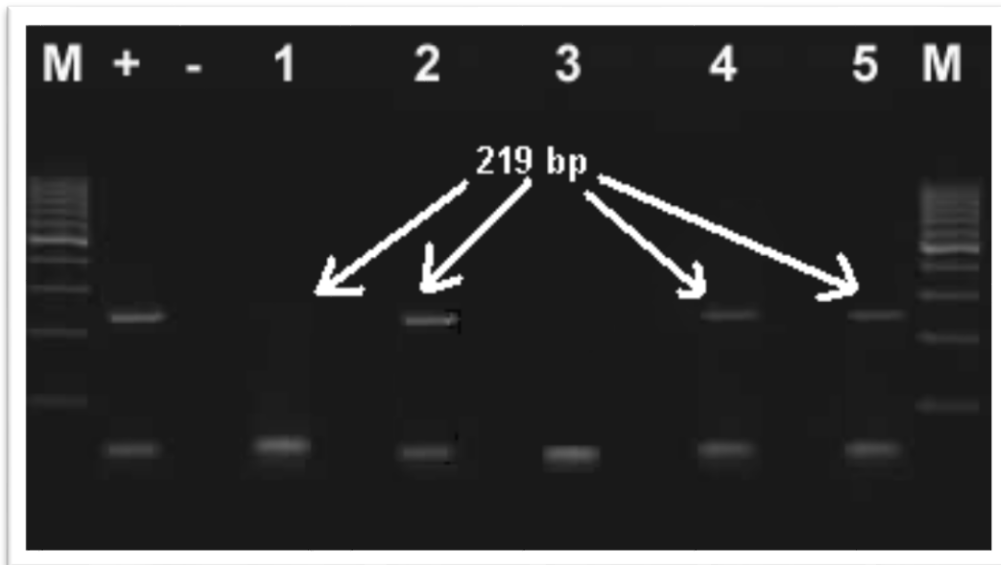


Figure 1. PCR results for *Mycobacterium genavense*.
M: marker (100bp) +: positive control -: negative control 1-5: feces samples.

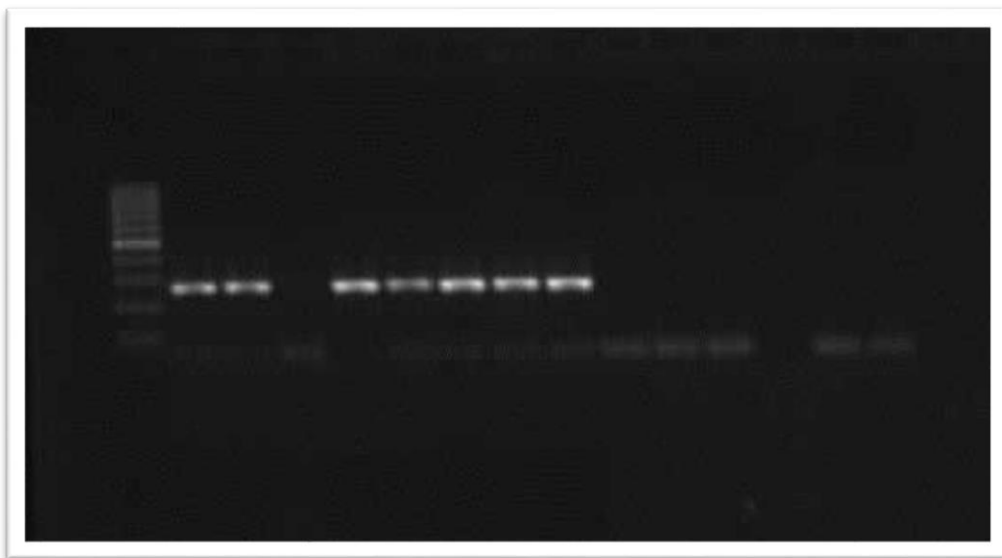


Figure 2. PCR results for *Mycobacterium* spp. (274bp)
M: marker (100bp) Line 1-2: positive controls, Line 3: negative control, Line 4-8: positive feces samples for *Mycobacterium* spp.

As a result of the molecular analysis of all samples, a total of 5 samples were found positive in terms of the sequence for the *Mycobacterium* spp. (Figure 2). Four of these 5 samples, positive for the *Mycobacterium* genus, were identified as *M. genavense* DNA by PCR (Figure 1).

In Turkey, *M. genavense* DNA was found for the first time in cage bird's feces with PCR. In birds, the primary source of infection is contaminated environment for mycobacterium infections. Feces of infected birds play an important role for the spread of infection among birds (20). The results of this study showed that feces are not only important for spreading of the infection but also detection of the mycobacteriosis. The important role of

fecal contamination in avian tuberculosis has been seen once more with this study.

Importantly, all *M. genavense* positive specimens were detected in cages of the same aviary. This result can indicate that the contamination by horizontal route from bird to bird.

In several studies, PCR has been used to diagnose mycobacterial infections from poultry organ samples. *M. genavense* is difficult to cultivate unlike other Mycobacteria species. PCR is a rapid and reliable method for the diagnosis of *M. genavense* infections (19,20). Tell et al. (19) reported that they found *M. genavense* in 67% of poultry organ samples. The *M. genavense* primers,

which were used in this study, were designed from the hypothetical 21kDa protein gene. Ledwon et al. (14) designed the primers from the same gene for the diagnosis of *M. genavense* from the organ samples of budgerigars and identified positive samples. Patino et al. (13) reported 3 (8 %) *M. genavense* DNA in free living birds' feces using *16s rRNA* gene specific primers of *Mycobacterium genavense*. Addition, Schimitz et al. (16) were used probe targeting the hypothetical 21kDa protein gene in their real-time PCR protocol. According to of all these studies, it was seen that hypothetical 21kDa protein gene could be used for the diagnosis of *M. genavense* in PCR.

Mycobacterium genavense-specific primers, tested for the first time in this study in Turkey, can be used in PCR tests performed with clinical materials other than feces (liver, spleen, bone marrow, tracheal swab, air sacs, lungs, intestines) especially in patients with suspected tuberculosis in cage birds. The results of this study showed that fecal screening can be performed especially for cage birds in terms of *M. genavense* for zoonotic risks.

Acknowledgements

The authors thanks to Dr. Enrico Tortoli (Regional Reference Center for Mycobacteria, Florence, Italy) who supplied *M. genavense* DNA which is used in this study as a positive control in PCR. This study is presented as an oral presentation in IX. Veterinary Microbiology Congress with International Participation, North Cyprus Turkish Republic, 5-7 October 2010.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. Aranaz A, Liebana E, Mateos A, et al (1997): *Laboratory diagnosis of avian mycobacteriosis*. *Semin Avian Exotic Pet Med*, **6**, 9-17.
2. Bougiouklis P, Brelleu G, Fragkiadaki E, et al (2005): *Outbreak of avian mycobacteriosis in a flock of two-year-old domestic pigeons (Columba livia f. domestica)*. *Avian Dis*, **49**, 442- 445.
3. Böttger EC, Hirschel B, Coyle MB (1993): *Mycobacterium genavense* sp. nov. *Int J Syst Bacteriol*, **43**, 841- 843.
4. Hoop RK, Böttger EC, Ossent P, et al (1993): *Mycobacteriosis due to mycobacterium genavense in six pet birds*. *J Clin Microbiol*, **31**, 990- 993.
5. Hoop RK, Böttger EC, Pfyffer GE (1996): *Etiological Agents of Mycobacterioses in Pet Birds between 1986 and 1995*. *J Clin Microbiol*, **34**, 991- 992.
6. Ikonomopoulos J, Fragkiadaki E, Liandris E, et al (2009): *Estimation of the spread of pathogenic mycobacteria in organic broiler farms by the polymerase chain reaction*. *Vet Microbiol*, **133**, 278- 282.
7. Kiehn TE, Hofer H, Bottger EC, et al (1996): *Mycobacterium genavense infections in pet animals*. *J Clin Microbiol*, **34**, 1840- 1842.
8. Ledwon A, Szeleszczuk P, Malicka E, et al (2009): *Mycobacteriosis caused by Mycobacterium genavense in Lineolated Parakeets (Bolborhynchus lineola). A case report*. *Bull Vet Inst Pulawy*, **53**, 209-212.
9. Lennox AM. (2007): *Mycobacteriosis in companion psittacine birds: a review*. *J Avian Med Surg*, **21**, 181- 187.
10. Manarolla G, Liandris E, Pisoni G, et al (2009): *Avian mycobacteriosis in companion birds: 20-year survey*. *Vet Microbiol*, **133**, 323- 327.
11. Mendenhall MK, Ford SL, Emerson CL, et al (2000): *Detection and differentiation of Mycobacterium avium and Mycobacterium genavense by polymerase chain reaction and restriction enzyme digestion analysis*. *J Vet Diagn Invest*, **12**, 57- 60.
12. OIE Terrestrial Manuel (2008): *Avian tuberculosis*. Available at <https://www.oie.int/doc/ged/D7710.PDF>. (Accessed January 10, 2019)
13. Patino LCW, Monge O, Suzan G, et al (2018): *Molecular Detection of Mycobacterium avium avium and Mycobacterium genavense in feces of free-living scarlet macaw (Ara macao) in Costa Rica*. *J Vildl Dis*, **54**, 357-361.
14. Portales F, Realini L, Bauwens L, et al (1996): *Mycobacteriosis caused by Mycobacterium genavense in birds kept in a zoo: 11- year survey*. *J Clin Microbiol*, **34**, 319- 323.
15. Ramis A, Ferrer L, Aranaz A, et al (1996): *Mycobacterium genavense infections in canaries*. *Avian Dis*, **40**, 246- 251.
16. Schmitz A, Korbelt R, Thiel S, et al (2018): *High prevalence of Mycobacterium genavense within flocks of pet birds*. *Vet Microbiol*, **218**, 40-44.
17. Shitaye JL, Halouzka R, Svobodova J, et al (2010): *First isolation of Mycobacterium genavense in a blue headed parrot (Pionus menstruus) imported from Surinam (South America) to the Czech Republic: a case report*. *Vet Med-Czech*, **55**, 339-347.
18. Sutherland M, Courtman N, Sacks P, et al (2018): *Severe leukemoid response associated with Mycobacterium genavense infection in a pet budgerigar (Melopsittacus undulatus)*. *J Exot Pet Med*, **27**, 11-16.
19. Tell LA, Leutenegger CM, Larsen RS, et al (2003): *Real-time polymerase chain reaction testing for the detection of Mycobacterium genavense and Mycobacterium avium complex species in avian samples*. *Avian Dis*, **47**, 1406- 1415.
20. Tell LA, Woods L, Cromie RL (2001): *Mycobacteriosis in birds*. *Rev sci tech Off int Epiz*, **20**, 180-203.
21. Untergasser A, Cutcutache I, Koressaar T, et al (2012): *Primer3- new capabilities and interfaces*. *Nucleic Acids Res*, **40**, e115.
22. Witte CL, Hungerford LL, Papendick R, et al (2008): *Investigation of characteristics and factors associated with avian mycobacteriosis in zoo birds*. *J Vet Diagn Invest*, **20**, 186-196.