Mycoplasma gallisepticum strains with identical random amplified polymorphic DNA (RAPD) patterns in chukar partridges (*Alectoris chukar*) and broilers: a case report

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ABSTRACT: We used the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique to discriminate the major emerging poultry pathogen, *Mycoplasma gallisepticum* (MG), in broiler and chukar partridge cases referred to the veterinary medicine teaching hospital. Amazingly, the chickens and partridges random amplified polymorphic DNA (RAPD) patterns were similar. This suggests the risk of a common source for the strains isolated from the different animals and illustrates the necessity of novel and improved control programs to prevent and restrict this significant disease which is prevalent among poultry species.

Keywords: RAPD; *Mycoplasma gallisepticum;* chukar partridge; broiler

Mycoplasma gallisepticum (MG) infection results in a chronic respiratory disease (CRD) of poultry that is characterised by inflammation of the infraorbital and the nostril, leading to conjunctivitis and swelling of the sinuses (Welchman 2008). Other signs include nasal exudate, snicking/sneezing, lacrimation and depression (Forrester et al. 2012). MG is often transmitted by direct contact between ill or natural carriers and susceptible animals (Marois et al. 2001). Apart from chickens, turkeys, quails, partridges, pheasants, pigeons and others are natural hosts of MG (Ley 2003). Serological examinations, culture, polymerase chain reaction (PCR) and immunohistochemical methods are used in the diagnosis of mycoplasma infections. Classical methods for identifying and detecting Mycoplasmas are laborious and time-consuming but molecular tools have improved the detection and identification of this microorganism. PCR can be performed on clinical samples with high sensitivity and fast turnaround time which making it the most frequently used test in the monitoring of MG infection (Garcia et al. 2005). The chukar partridge (*Alectoris chukar*), is a native game bird of the Middle-Eastern countries and consequently introduced to many other parts of the world. Nowadays, with the development of poultry and partridge breeding, complicated infections and diseases have been observed concurrently among partridge and broiler chickens in developing countries such as Iran. The ability to distinguish between MG strains is useful for analysis of the origin of contamination and determination of interactions among strains isolated from neighbouring flocks (Marois et al. 2001). Molecular analysis of MG field isolates can provide important epidemiologic information for determining the source of the infection. Intraspecies heterogeneity among isolates and strains of MG can be studied by random amplified polymorphic DNA (RAPD) analysis (Rawadi 1998). RAPD analysis is a powerful molecular technique used for epidemiological studies and is appropriate for identification and differentiation of various field isolates (Fan et al. 1995; Ley et al. 1997). In this paper, we report similarity in the RAPD pattern between MG isolates obtained from an infected broiler flock and a small partridge flock in Shiraz, southern Iran.

Case description

During January 2012, two chukar partridges (*Alectoris chu*kar) and some broilers from flocks with clinical respiratory signs were separately referred to the department of poultry diseases in the veterinary school of Shiraz University. These birds were not vaccinated against MG. Primary examinations showed respiratory signs including conjunctivitis, infectious sinusitis of infraorbital sinuses, bilateral conjunctival and periorbital swelling with mucopurulent drainage and reduction in weight. Tracheal swabs, trachea, lungs, and air sacs were used for isolation of Mycoplasma according to the methods of the OIE (OIE 2004) and specimens were referred to the microbiology lab of the Department of Pathobiology, School of Veterinary Medicine. Cultures were made from specimens using a specific and selective medium containing horse serum, yeast extract, thallium acetate and penicillin. The cultured samples were incubated at 37 °C for isolation of *Mycoplasma*. DNA extraction was performed from mucopurulent fluid of infectious sinuses as previously described (Fadl et al. 1995). Using specific primers, simple PCR and RAPD PCR reactions were done for identification of MG. Simple PCR amplifications for identifying *Mycoplasma-* and MG-specific bands were performed in a final volume of 25 µl, separately. The reaction mixtures consisted of 2 µl of the DNA template, 2.5 µl 10 × PCR buffer (75mM Tris-HCl, pH 9.0, 2mM MgCl$_2$, 50mM KCl, 20mM (NH$_4$)$_2$SO$_4$), (CinnaGen, Iran), 1 µl dNTPs (50µM), (CinnaGen, Iran), 1 µl (1 IU Ampli Taq DNA polymerase), (CinnaGen, Iran) and 1 µl (25 pmol) of the forward and reverse primers (CinnaGen, Iran) (Table 1); the volume of the reaction mixture was brought up to 25 µl using distilled deionized water. The thermal cycler (MJ mini, BioRad, USA) was programmed to the following settings: initial denaturation at 94 °C for 5 min, followed by 35 cycles
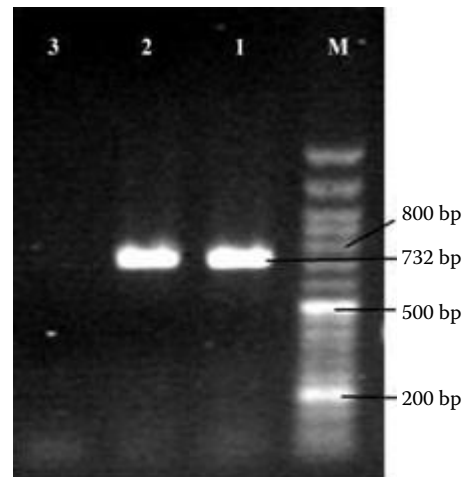

Figure 1. Detection of *Mycoplasma* genus in chicken and partridge samples. Lane 1 = chicken sample, lane 2 = partridge sample, lane 3 = negative control, lane M = 50 bp DNA marker

of denaturation at 94 °C for 1 min, annealing as shown in Table 1 for 1 min and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 10 min and the PCR products were stored in the thermal cycler at 4 °C until they were collected. RAPD-PCR reactions were performed for colonies which were raised on specific medium according to the program previously described by Gharaibeh et al. (2003). The RAPD primers for this reaction were previously described by Geary et al. (1994) (Table 1). Amplified products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. Visualization was performed using a UV transilluminator (BTS-20, Japan) and 50 bp and 100 bp DNA ladders were used as molecular size markers. From both seven broilers and two chukar partridges from two separate farms MG was isolated and identified using the culture method. Furthermore, molecular characterisation of MG using simple PCR reactions confirmed the presence of MG, showing PCR product bands, specific for genus and MG strains (Figure 1 and 2). The

Table 1. PCR primers used for MG detection

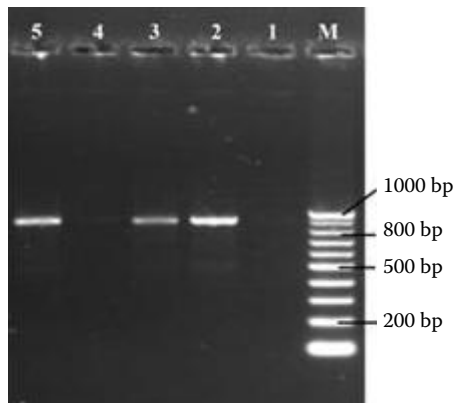| Primer's Name	Primer Sequences (5' to 3')	Annealing temperature (°C)	PCR product size	Type of primers
Upstream	GGATCCCATCTCGACCACGAGAAAA	54	732 bp	specific
Downstream	CCTTCAATCAGTGAGTAACTGATGA			
Forward	ACACCATGGGAGCTGGTAAT	47	888–938 bp (for MG)	universal
Reverse	CTTCATCGACTTTCAGACCCAAGGCAT			
Geary 1254	CCGCAGCCAA	40	variable	RAPD

Figure 2. Agarose gel electrophoresis of PCR products amplified by universal primers. Lane 1 = negative sample, lanes 2, 3 = chicken positive sample, lane 5 = partridge positive samples, lane M = 100 bp DNA marker

Random amplified polymorphism DNA (RAPD) pattern of DNA extracted from MG strains isolated from broilers and partridges were identical (Figure 3).

DISCUSSION AND CONCLUSIONS

Control of MG has been generally based on the eradication of this organism from poultry flocks. Nowadays, with the widespread development of the poultry industry in different areas of the world, poultry are now reared in relatively close milieus, sometimes with mixed avian species, mixed types of commercial poultry, or wild birds in their immediate environment. In such circumstances maintenance of infection-free flocks may be complicated or impossible, and the consequent re-emergence of *Mycoplasma* infection has necessitated a reassessment of the strategies used to manage *Mycoplasma* infections in the poultry industry. It seems that the first step in MG control programs is determination of the origin of the bacterium in various outbreaks. MG prevalence in chickens has been reported to be high in many countries with no control strategy or in countries before the implementation of a control strategy (Gharaibeh and Al Roussan 2008). In addition, recent investigations of different outbreaks of upper respiratory diseases in partridges and other game birds have indicated that MG is frequently involved (Luttrell et al. 1996; Bradbury et al. 2001; Welchman et al. 2002). Natural infection with MG has been reported in a variety of game birds, in some cases with marked clinical signs, however the pathogenicity of MG strains is partly depend-
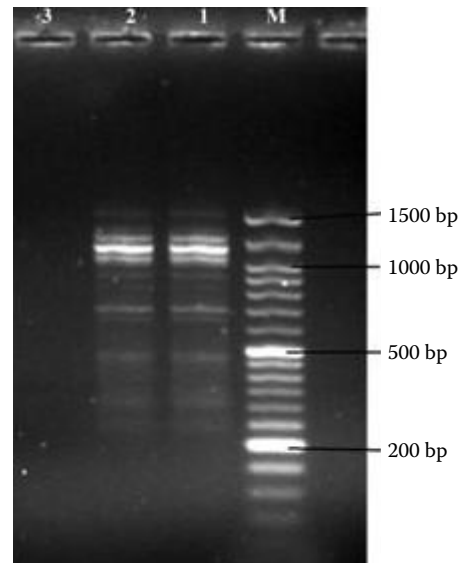

Figure 3. Electrophoresis analysis of RAPD patterns of MG isolates using Geary 1254 primers. Lane 1 = MG strain isolated from chukar partridges, lane 2 = strain isolated from chicken, lane 3 = negative control, lane M = 50 bp DNA marker

ent upon the host. Game birds, particularly partridges, appear to experience more severe disease than domestic fowl (Bencina et al. 2003; Vitula et al. 2011). Reliable methods for the discrimination of MG strains play an essential role in understanding the epidemiology and spread of the disease because they generate the information required for recognising and following new outbreaks. Genotyping methods also have a high degree of reproducibility, which is particularly important for the construction of reliable databases. Furthermore, genotyping methods are easy to interpret and can be performed rapidly (Naola et al. 2005). The RAPD method has been successfully utilised to identify vaccine strains (Kleven and Fan 1998; Turner and Kleven 1998), and for tracking epidemiologically related isolates in the field (Levisohn and Kleven 2000). The results of other studies have indicated that the RAPD method has a greater discriminatory power than other methods like the pulse field gel electrophoresis (PFGE) because some *Mycoplasma* strains could not be typed by PFGE (Marois et al. 2001). DNA polymorphisms among MG isolates revealed by RAPD shows genetic diversity between different poultry farms (Hosseini et al. 2006). On the other hand, MG isolates with the same band pattern indicates the possibility of a single source of infection or infection with closely related strains. In our results, MG isolates from partridges and

chickens had identical RAPD band patterns (Figure 3). Therefore, the outbreak of MG in these birds appears to have been caused by a single strain or very closely related strains of MG. Previous studies have clearly shown that MG isolates from songbirds had RAPD patterns identical to each other but different from other birds strains and isolates tested (Ley et al. 1997). Birds originating from infected flocks may introduce new agents into wild populations (Millan et al. 2004; Villanua et al. 2008). The present findings show that these MG strains are not host-species specific under natural conditions. This suggests the possibility of a single source for the strains isolated from both chukar partridges and chickens outbreak. In other study Ley et al. (1997) showed that the songbird MG RAPD band pattern was different from the patterns of isolates from chickens and turkeys. Comparison of isolates from poultry and songbirds provided evidence of shared MG strains. RAPD analyses of MG isolates from different sources including commercial poultry and wild or game birds will provide an exceptional opportunity to track this emerging disease. We are now applying RAPD analyses to additional MG isolates, both current and archival, from commercial poultry to search for MG strains shared by partridges and poultry or other birds. We envision that this will greatly facilitate the designing appropriate control programs. In conclusion, this report suggests that flocks and game birds have a high chance of contracting MG infection from the same origins.

REFERENCES

Bencina D, Mrzel I, Rojs OZ, Bidovec A., Dovc A (2003): Characterisation of Mycoplasma gallisepticum strains involved in respiratory disease in pheasants and peafowl. Veterinary Record 152, 230–234.

Bradbury JM, Yavari CA, Dare CM (2001): Mycoplasmas and respiratory disease in pheasants and partridges. Avian Pathology 30, 391–396.

Fadl AA, Nguyen AV, Khan MI (1995): Analysis of Salmonella enteritidis isolates by arbitrarily primed PCR. Journal of Clinical Microbiology 33, 987–989.

Fan HH, Kleven SH, Jackwood MW (1995): Application of polymerase chain reaction with arbitrary primers to strain identification of Mycoplasma gallisepticum. Avian Diseases 39, 729–735.

Forrester CA, Bradbury JM, Dare CM, Domangue RJ, Windsor H, Tasker JB, Mockett AP (2011): Mycoplasma gallisepticum in pheasants and the efficacy of tylvalosin to treat the disease, Avian Pathology 40, 581–586.

Garcia M, Ikuta N, Levisohn S, Kleven SH (2005): Evaluation and comparison of various PCR methods for detection of Mycoplasma gallisepticum infection in chickens. Avian Diseases 49, 125–132.

Geary SJ, Forsyth MH, Abul Soaud S, Wang G, Berg DE, Berg CM (1994): Mycoplasma gallisepticum strains differentiation by arbitrary primer PCR (RAPD) fingerprinting. Molecular and Cellular Probes 8, 311–316.

Gharaibeh S, Al Roussan D (2008): The use of molecular techniques in isolation and characterization of Mycoplasma gallisepticum from commercial chickens in Jordan. International Journal of Poultry Science 7 (1), 28–35.

Gharaibeh R, Saadoun I, Mahasneh A (2003): Genotypic and phenotypic characteristics of antibiotic-producing soil Streptomyces investigated by RAPD-PCR. Journal of Basic Microbiology 43, 18–27.

Hosseini H, Bozorgmehrifard MH, Peighambari SM, Pourbakhsh SA, Razzazian M (2006): Random amplified polymorphic DNA (RAPD) fingerprinting of Mycoplasma gallisepticum isolates from chickens. Archives of Razi Institute 61, 67–71.

Kleven SH, Fan H (1998): Pen trial studies on the use of live vaccines to displace virulent Mycoplasma gallisepticum in chickens. Avian Diseases 42, 300–306.

Lauerman LH, Chilina AR, Closser JA, Johanssen D (1995): Avian mycoplasma identification using polymerase chain reaction amplification and restriction fragment length polymorphism analysis. Avian Diseases 39, 804–811.

Levisohn S, Kleven SH (2000): Avian mycoplasmosis (Mycoplasma gallisepticum). Revue Scientifique et Technique 19, 425–442.

Ley DH (2003): Mycoplasma gallisepticum infection. In: Saif YM, Barnes HJ, Fadly AM, Glisson JR, McDougald LR, Swayne DE (eds.): Diseases of Poultry. 11th ed. Iowa State University Press, Ames. 122–144.

Ley DH, Berkhoff JE, Levisohn S (1997): Molecular Epidemiologic Investigations of Mycoplasma gallisepticum conjunctivitis in songbirds by random amplified polymorphic DNA analysis. Emerging Infectious Diseases 3, 375–380.

Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH (1996): Field investigation of Mycoplasma gallisepticum infections in house finches (Carpodacus mexicanus) from Maryland and Georgia. Avian Diseases 40, 335–341.

Marois C, Dufour-Gesbert F, Kempf I (2001): Comparison of pulsed-field gel electrophoresis with random

amplified polymorphic DNA for typing of Mycoplasma synoviae. Veterinary Microbiology 79, 1–9.

Millan J, Gortazar C, Villafuerte R (2004): A comparison of the helminth faunas of wild and farm-reared red-legged partridge. Journal of Wildlife Management 68, 701–707.

Naola MF, Hepp D, Sun S, Ikuta N, Levisohn S, Kleven SH, Garcia M (2005): Use of molecular diversity of Mycoplasma gallisepticum by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies. Microbiology 151, 1883–1893.

Nascimento ER, Yamamoto R, Herrick KR, Tait RC (1991): Polymerase chain reaction for detection of Mycoplasma gallisepticum. Avian Diseases 35, 62–69.

OIE (World Organisation for Animal Health) (2004): Avian mycoplasmosis (Mycoplasma gallisepticum). Chapter 2.7.3 in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 5th ed.

Rawadi GA (1998): Characterization of mycoplasmas by RAPD fingerprinting. Methods in Molecular Biology 104, 179–187.

Turner KS, Kleven SH (1998): Eradication of live F strain Mycoplasma gallisepticum vaccine using live ts-11 on a multiage commercial layer farm. Avian Diseases 42, 404–407.

Villanua D, Perez-Rodriguez L, Casas F, Alzaga V, Acevedo P, Vinuela J, Gortazar C (2008): Sanitary risks of red-legged partridge releases: introduction of parasites. Europian Journal of Wildlife Research 54, 199–204.

Vitula F, Peckova L, Bandouchova H, Pohanka M, Novotny L, Jira D, Kral J, Ondracek K, Osickova J, Zendulkova D, Rosenbergova K, Treml F, Pikula J (2011): Mycoplasma gallisepticum infection in the grey partridge Perdix perdix: outbreak description, histopathology, biochemistry and antioxidant parameters. BMC Veterinary Research 7, 34.

Welchman D (2008): Diseases in young pheasants. In Practice 30, 144–149.

Welchman DD, Bradbury JM, Cavanagh D, Aebischer NJ (2002): Infectious agents associated with respiratory disease in pheasants. Veterinary Record 150, 658–664.

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