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Detection of mycoplasma species in turkeys by culture and polymerase chain reaction

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Summary

The purpose of the present study was to investigate the presence of pathogenic mycoplasma species in the turkey population of Turkey. Tracheal samples randomly collected from a total of 624 apparently healthy meat-type turkeys at a commercial abattoir located in the north of the country were examined by culture and genus- and species-specific polymerase chain reaction (PCR) assays for mycoplasma. In the direct plating onto solid specific media, mycoplasma growth was observed from 1.4% (9/624) of the samples, which were confirmed to belong to the *Mycoplasma* genus by genus-specific PCR. *Mycoplasma iowae* (MI) and *M. meleagridis* (MM) were identified by the species-specific PCR from eight and one of the samples, respectively. However, genus-specific PCR amplification was obtained from 2.6% (16/624) of the samples which produced turbidity in the liquid media. Interestingly, these positive samples were different from those obtained from solid agar and mycoplasma growth was not observed when the broth samples were inoculated onto solid media. In the species-specific PCR analysis of the broth samples, MI, MM and *M. gallisepticum* were identified from twelve, two and two samples, respectively. The inconsistency between the results obtained from liquid and solid media raises questions about the efficiency of isolation procedures for mycoplasma and this warrants further investigation.

Keywords

Mycoplasma – Polymerase chain reaction – Trachea – Turkey – Turkeys.

Introduction

Mycoplasmas differ from other bacteria in their very small size and total absence of a cell wall; these characteristics account for their 'fried egg' type of colonial morphology, complete resistance to antibiotics that affect cell wall synthesis, and their complex nutritional requirements. They tend to be host-specific: avian mycoplasmas are not generally known to infect mammalian or other species, but some non-avian mycoplasmas, such as *Mycoplasma bovis* (3, 17), can cross the species barrier and have been found in avian species.

The avian mycoplasma species that are pathogenic to commercial poultry, namely *M. gallisepticum* (MG) and *M. synoviae* (MS) in chickens and turkeys, and *M. meleagridis* (MM) and *M. iowae* (MI) in turkeys, have been associated with significant economic losses (6).

Although MM and MI, which are more host-specific to turkey and primarily transmitted vertically from the breeding flock, are less frequently encountered, they remain endemic in some western European countries (15, 21). They can cause reduced hatchability, embryo mortality, poor quality birds, reduced viability of young birds and disease of the air sacs (12). *Mycoplasma iowae* has more antigenic diversity, can survive longer in the environment and is relatively more resistant to many antimicrobials than the other avian mycoplasmas (1).

The chicken and turkey industries have invested heavily in the production of primary breeding stocks that are free from these pathogens, and control programmes have been established in many countries, often with government support. For example, in the United States of America (USA), control programmes in commercial breeding stocks are administered under the National Poultry Improvement Plan, which is run by state and federal departments of agriculture and industry representatives (2).

Rapid and reliable diagnostic methods are required to establish and maintain stocks that are free of avian pathogenic mycoplasmas or to eradicate the pathogen from a flock. The diagnosis of avian pathogenic mycoplasmas has typically been carried out by culture, serology and molecular-based assays (polymerase chain reaction [PCR], deoxyribonucleic acid [DNA] probe). These tests each have advantages and disadvantages. Traditional culture isolation of mycoplasmas is time-consuming and complicated, requiring two to three weeks to complete because of the slow-growing nature of some mycoplasmas such as MI. Birds infected with MI do not consistently produce a humoral response that can be detected by the conventional serological tests that are used with the other avian mycoplasma pathogens (18).

This abattoir-based study was carried out to investigate the presence of avian pathogenic mycoplasmas in commercial turkeys by conventional culture and genus- and species-specific PCR assays.

Materials and methods

Sample collection

The tracheal samples were randomly collected from a total of 624 turkeys from 23 different flocks at a commercial turkey abattoir located in the north of Turkey between February and April 2008. The turkeys were a white-feathered Californian breed with an average age of 15 weeks for females and 22 weeks for males. There were 322 samples from males from 12 different flocks and 302 samples from females from 11 different flocks, all located in Bolu province in the north of the country. Each flock had approximately 7,000 turkeys. The tracheal samples were stored in cool boxes and transported to the laboratory within 2 days.

Isolation of mycoplasma

The samples were aseptically opened and swabs taken from the trachea were cultured both by serial dilution in modified Hayflick medium (PPLO [pleuropneumonia-like organisms] broth without crystal violet [21 g/l], 20% de-complemented horse serum, 10% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate, 0.04% ampicillin) and by streaking onto solid agar (PPLO agar) of the same medium, simultaneously. The broth media was solidified by adding 1% agar noble (Difco, Detroit, USA). The samples were diluted by five consecutive 10-fold dilutions. The swabs were added to the first broth medium tube (5 ml) and were mixed thoroughly. Then, 300 µl broth was taken from each tube and mixed in a separate tube. The samples were incubated at 37°C in 5% CO_2 for seven to ten days. The broths were checked daily and the samples with growth, indicated by turbidity in the broth cultures, were sub-cultured onto agar plates from the last turbid tube. The plates were checked daily for the appearance of colonies. The isolated colonies were inoculated into the stock suspension (50% broth / 50% horse serum) and were kept at – 20°C. The presence of MS was specifically investigated by culturing samples in the above medium supplemented with 1% nicotinamide adenine dinucleotide.

Deoxyribonucleic acid extraction and polymerase chain reaction amplification

Positive cultures in liquid broth (as indicated by turbidity) were centrifuged at 12,000 *g* for 20 min. and the pellet was re-suspended in 300 µl of distilled water. Suspected mycoplasma colonies from solid agar (as indicated by a 'fried egg' appearance) were transferred into a microcentrifuge tube containing 300 µl distilled water. Both solid and broth suspensions were then treated with 300 µl TNES buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and proteinase K (200 µg/ml), and were kept at 56°C for 1 h. Suspensions were heated at 95°C for 10 min to inactivate proteinase K. The above method was also employed to extract DNA from tracheal samples directly.

Two different PCR procedures were applied. In the first procedure, DNA samples were tested using two different genus-specific PCRs for *Mycoplasma* species (4, 13) and in the second, they were tested using specific PCR assays for MG, MM, MI and MS (5, 10, 20). The PCRs were performed in a TC 512 Temperature Cycling System (Techne, Staffordshire, United Kingdom [UK]) in a reaction volume of 50 µl containing: 5 µl of 10 × PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 5 µl 25 mM $MgCl_2$, 250 µM each deoxynucleotide triphosphate, 1.25 U Taq DNA polymerase (MBI Fermentas, St Leon-Rot, Germany), 20 pmol each primer (Iontek, Turkey) and 25 ng template DNA. The amplified products were detected by staining with ethidium bromide (0.5 mg/ml) after electrophoresis at 80 V for 2 h (7 V/cm) in 1.5% agarose gels.

In addition, two randomly selected PCR products amplified with MI-specific primers were submitted to a sequencing company (Iontek, Istanbul) for partial DNA sequence analysis.

Reference strains of MG, MM, MI and MS (kindly gifted by Dr Stanley H. Kleven, University of Georgia, USA and Robin A.J. Nicholas, Veterinary Laboratories Agency, UK) were included as positive controls and distilled water and *Escherichia coli* were used as a negative control in all assays.

Statistical analysis

A chi square test was used to compare differences between the results and a probability of less than 0.05 was considered significant.

Results

Bacteriological findings

Bacterial growth, as indicated by 'fried egg' colonies in the agar plates, was observed from 9 (1.4%) of the 624 tracheal samples. All of the samples in which bacterial growth was observed were from males from two different flocks. Mycoplasma growth was not seen when 411 (210 from males and 201 from females) samples which produced turbidity in the broths were inoculated onto solid media. *Escherichia coli* and other bacteria were determined to grow in most of the liquid media.

Polymerase chain reaction findings

When the primers reported by Botes *et al.* (4) were employed, positive results were obtained with negative controls (*E. coli*), so the results of this genus-specific PCR were not considered. The nine solid agar-positive isolates were confirmed to belong to the *Mycoplasma* genus by the genus-specific PCR, which produced specific bands with the molecular size of approximately 1,013 base pairs (bp) when the primers developed by Lierz *et al.* (13) were used. In the genus-specific PCR analysis of broth cultures with turbidity (n = 411), amplification was obtained from an additional 16 samples (13 from males and 3 from females in five different flocks). These positive samples were different from those obtained from solid agar. When the PCR results were considered together, the overall proportion of mycoplasma identification was calculated as 4.0% (25/624). The positive results were obtained from 6.8% (22/322) of the male and 1.0% (3/302) of the female samples in a total of seven different flocks (Table I). The difference by gender was determined to be statistically significant (p = 0.004). When the positive results were analysed at flock level, mycoplasma agents were determined in 30.4% (7/23) of the turkey flocks. Within these seven flocks, the proportion of mycoplasma-positive samples was calculated to range from 3.3% to 26.3%, with an overall proportion of 13.8% (25/181) (Table II).

Table I
Detection of *Mycoplasma* species from tracheal samples of turkeys by polymerase chain reaction
Samples were taken from 624 turkeys in 23 different flocks: 322 from males and 302 from females

Type of PCR	No. of positive flocks (%)	No. of positive samples (%)		
		Male	Female	Total
Genus-specific	7(30.4)	22 (6.8) [a]	3 (1.0) [a]	25 (4.0)
MI-specific	7(30.4)	19 (5.9)	1 (0.3)	20 (3.2)
MM-specific	3(13.0)	2 (0.6)	1 (0.3)	3 (0.5)
MG-specific	2 (8.7)	1 (0.3)	1 (0.3)	2 (0.3)
MS-specific	–	–	–	–

PCR: polymerase chain reaction
MI: *Mycoplasma iowae*
MM: *M. meleagridis*
MG: *M. gallisepticum*
MS: *M. synoviae*
a) p = 0.004

Table II
Distribution of positive samples in the seven flocks in which *Mycoplasma* species were detected

Positive flocks	No. of samples collected [a]	No. of positive samples	Proportions [b] (%)
A	20	1	5.0
C	19	5	26.3
H	20	2	10.0
K	31	6	19.4
M	31	7	22.6
O	30	1	3.3
J	30	3	10.0
Total	**181**	**25**	**13.8**

a) Each flock had approximately 7,000 turkeys
b) p > 0.05

In the species-specific PCR analysis of positive samples obtained from solid media, MI was detected in eight samples in one flock and MM was detected in one sample in one flock. In one turkey flock both agents were determined. In the species-specific PCR analysis of positive samples obtained from liquid media, MI was identified in 12 samples (11 males and 1 female) in five flocks, MM was identified in two samples (1 male and 1 female) in two flocks and MG was identified in two samples (1 male and 1 female) in two flocks. Two flocks had a mixture of MI, MM and MG-positive samples. In total, MI was detected in 20 samples (3.2%) in seven flocks, MM was detected in three samples (0.5%) in three flocks and MG was detected in two samples (0.3%) in two flocks (Table I). None of the isolates was found to be positive for MS.

In spite of several trials, the authors could not succeed in the PCR amplification of mycoplasmas directly from tracheal samples.

Two DNA samples belonging to MI were investigated further by partial sequence analysis using the amplicons yielding a specific product in 16S ribosomal ribonucleic acid (rRNA) gene-specific primers (19). Polymerase chain reaction products were sequenced on forward strands using the ABI 310 Genetic Analysis System (Iontec, Istanbul, Turkey). Analysis of the sequencing results in the BLAST (Basic Logical Alignment Search Tool) showed that there was 99% homology between these isolates and the MI reference strain (sequence accession number EF447273.1).

Discussion and conclusion

There is a paucity of information on avian mycoplasmas in the turkey populations of Turkey (there have been a small number of studies conducted in chickens [8]). This study therefore provides quantitative information for the first time on the potential role of avian mycoplasmas in apparently healthy meat-type turkeys reared in flocks located in the north of the country. Mycoplasma infections in meat-type turkeys have been reported all over the world, including the USA and Israel, with relatively high prevalences of up to 30% in the north European countries (15, 21). Breeding mycoplasma-free parent turkeys in these countries is therefore costly. In the present study, an overall proportion of 4% positive samples was obtained (25/624). Approximately 30% of the flocks sampled (7/23) were determined to be infected, and nearly 14% of the samples taken from these infected flocks were positive for mycoplasma infections (25/181). These findings are important, as the turkey-breeding sector is on the rise in the country.

Although infections due to MM have been reported to be more frequent in turkey flocks in other studies, MI was identified to be the most common (in 20 of the 25 positive samples) in the current study. While this agent was found in all the infected flocks, MM was present in only three flocks and MG in only two. MI is known to be more resistant than the other mycoplasma species to antibiotics and other environmental factors. Management factors and age might have also been responsible for this difference.

Mycoplasma infections have been reported to occur in female turkeys more frequently as the agents are generally located in the genital tract of the animals (6). However, the disease was observed to be significantly higher in male turkeys in this study. The type of samples collected (tracheal) and the time of slaughter (approximately seven weeks later in males than in females) might have contributed to this difference. In addition, it has been reported that lateral transmission among male turkeys may be an important factor in the transmission of mycoplasma infections and this may be what occurred in this study (14).

Although culture is the gold standard in the diagnosis of mycoplasmas, both the culture media (Frey's, Hayflick, Friis, M-Ort) and the methods used currently for selective isolation of the agents (filtration, antibiotic supplement, dilution) lack reliability. Culture usually lacks sensitivity possibly because it fails to prevent the rapid growth of other bacteria despite the addition of various antibiotics (9). The fact that other bacteria, *E. coli* in particular, were isolated from most of the broth samples which showed turbidity in this study supports this hypothesis. Inconsistency between the results obtained from liquid and solid media raises questions about the efficiency of isolation procedures for mycoplasma. In some studies it was shown that false negative results and the lack of pure isolation could be overcome by applying filtration (0.45 µm) when sub-culturing from broth to agar (7, 22). The filtration method was not used in the current study owing to its high cost.

On the other hand, molecular-based methods have frequently been used in recent years with better results than those obtained with conventional culture (11). The findings of the present study are a case in point: PCR analysis of the 16 broth samples from which no growth was observed in the solid media produced positive results. It is not surprising, as specific PCR was not inhibited by the interference of other bacteria. The use of negative controls in all steps of the PCR assays, and the consistently negative results obtained from these controls, dismissed the possibility of false positives as well. In this study, it was found that the genus-specific primers developed by van Kuppeveld *et al.* (24) and modified by Botes *et al.* (4) gave positive results with *E. coli* as well. The fact that sequence analysis of some samples, which could not be amplified in the species-specific PCRs, produced 100% homology with *E. coli* supported this finding. This false positivity problem was overcome by using a primer pair, developed by Lierz *et al.* (13) which did not produce any PCR products with *E. coli* or other cell-walled bacteria.

It should be underlined that direct PCR detection of avian mycoplasmas from biological material is crucial in the rapid control of the infection in breeding stocks. In the current study, PCR trials to detect the agents in positive tracheal samples directly failed possibly because of the presence of inhibitors in materials, as classic extraction methods were applied. This can be overcome by using more robust DNA extraction methods. Moalic *et al.* (16) used internal DNA controls in their study in order to detect false negative results when investigating mycoplasma agents by direct PCR from biological materials, and reported that 40% of the samples included inhibitory substances. In another study, internal DNA controls were

used for each clinical sample to monitor false negative results (23).

Although a limited number of samples in a small area have been examined, the results of the current study indicate that mycoplasma infections are a serious consideration in turkey breeding in Turkey. The PCR assays used here facilitated identification of mycoplasma agents from even contaminated broth samples. However, it is essential to develop more robust PCR assays which can detect the agents from clinical materials directly with high sensitivity and specificity.

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Identification des espèces de *Mycoplasma* isolées chez des dindes par culture et amplification en chaîne par polymérase

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Résumé

Les auteurs présentent les résultats d'une étude conduite en Turquie afin de caractériser les espèces pathogéniques de *Mycoplasma* présentes dans la population de dindes de ce pays. Des écouvillons trachéaux prélevés de manière aléatoire sur 624 dindes de chair apparemment en bonne santé dans un abattoir commercial situé dans le nord du pays ont été analysés pour la recherche de mycoplasmes par culture et leur caractérisation au moyen d'épreuves d'amplification en chaîne par polymérase (PCR) spécifiques pour les différents genres et espèces de mycoplasmes. Le comptage direct en milieu solide spécifique a permis d'observer la croissance de mycoplasmes dans 1,4 % (9/624) des échantillons ; leur appartenance au genre *Mycoplasma* a été confirmée par une PCR spécifique du genre. Le recours à des PCR spécifiques de l'espèce a permis d'identifier *Mycoplasma iowae* (MI) dans huit échantillons et *M. meleagridis* (MM) dans le neuvième échantillon. Toutefois, une amplification génique par PCR spécifique du genre a été obtenue dans 2,6 % des échantillons (16/624) ayant présenté une turbidité du milieu liquide. Fait intéressant, ces échantillons positifs étaient différents de ceux obtenus en gélose solide, et ces mêmes bouillons inoculés en milieu solide n'ont pas permis d'obtenir la croissance de mycoplasmes. Les seize échantillons de bouillon ont été soumis à une procédure PCR spécifique de l'espèce, qui a permis d'identifier MI dans douze d'entre eux, MM dans deux autres et *M. gallisepticum* dans les deux derniers. Les résultats différents obtenus suivant le milieu, liquide ou solide, posent la question de l'efficacité des procédures d'isolement des mycoplasmes, question qui mérite d'être approfondie par de recherches plus poussées.

Mots-clés

Amplification en chaîne par polymérase – Dinde – Mycoplasme – Trachée – Turquie.

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Detección de especies de *Mycoplasma* en pavos mediante cultivo o por reacción en cadena de la polimerasa

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Resumen

Los autores describen un estudio encaminado a investigar la presencia de especies patógenas de *Mycoplasma* en la población de pavos de Turquía. A tal efecto, en un matadero industrial del norte del país se extrajeron de forma aleatoria muestras traqueales de un total de 624 pavos de engorde aparentemente sanos, muestras que después fueron cultivadas y también sometidas a una técnica de reacción en cadena de la polimerasa (PCR) para la caracterización del género y la especie de micoplasmas. Con siembra directa sobre medios sólidos específicos se observó crecimiento de micoplasmas en un 1,4% (9/624) de las muestras. Éstas se sometieron después a una PCR específica de género que confirmó la pertenencia de los microorganismos al género *Mycoplasma*. La aplicación de una PCR de caracterización de la especie sirvió para identificar a *Mycoplasma iowae* (MI) y *M. meleagridis* (MM) en ocho y una de esas muestras respectivamente. Sin embargo, tras una PCR específica de género, se obtuvo amplificación de genoma en un 2,6% (16/624) de las muestras que habían generado turbidez en medio líquido. Un dato interesante es que esas muestras positivas resultaron diferentes de las obtenidas en agar sólido y que no se observó crecimiento de micoplasmas al sembrar en medios sólidos las muestras procedentes del caldo de cultivo. Tras analizar por PCR específica de especie los microorganismos cultivados en caldo se identificaron MI, MM y *M. gallisepticum* en doce, dos y dos muestras respectivamente. La falta de concordancia entre los resultados obtenidos en medio sólido y en medio líquido plantea interrogantes acerca de la eficacia de los procedimientos de aislamiento de micoplasmas, cuestión que merece un estudio más detenido.

Palabras clave

Micoplasma – Pavo – Reacción en cadena de la polimerasa – Tráquea – Turquia.

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