

# OCCURRENCE, MOLECULAR IDENTIFICATION AND ANTIBIOTIC RESISTANCE PROFILING OF *MYCOPLASMA GALLISEPTICUM* AND *MYCOPLASMA SYNOVIAE* FROM CHRONIC RESPIRATORY DISEASE CASES IN POULTRY BIRDS AND FARM ENVIRONMENT

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**Abstract:** Avian mycoplasmosis is an important risk for commercial poultry production leading to enormous losses in terms of disease and productivity. The main causative agents are *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. To study the variable degree of resistance to commonly prescribed and used antibiotics in mycoplasmosis, a total of 115 samples including tissue specimen and swabs were collected from chronic respiratory disease (CRD) cases of broiler and layer birds and their contaminated farm environment. The samples were directly passaged into the Brain Heart Infusion broth (supplemented with 10 % horse serum, NAD, cysteine, penicillin and thallium acetate). Positive samples were transferred to Brain Heart Infusion agar (Difco) for the isolation of *Mycoplasma* spp. while negative samples were declared after the third passage. Of the samples, 61.5% were found positive for *Mycoplasma* spp., which were recovered mostly after second passage. Out of total culture positive cases, *Mycoplasma gallisepticum* (MG) was identified in 62% cases and *Mycoplasma synoviae* (MS) in 38%, as confirmed through Polymerase Chain Reaction (PCR) using specific primers. The MG and MS isolates showed variable degrees of sensitivity against the commercially available drug of choice, tylosin. The highest Minimum Inhibitory Concentration (MIC) of enrofloxacin ( $112.38 \pm 4.34 \mu\text{g/ml}$ ) was recorded against MG, followed by tetracyclin ( $91.58 \pm 4.66 \mu\text{l/ml}$ ), gentamicin ( $54.33 \pm 2.98 \mu\text{g/ml}$ ), spiro-mycin ( $52.23 \pm 3.99 \mu\text{g/ml}$ ) and tylosin ( $52.58 \pm 2.69 \mu\text{g/ml}$ ). The highest MIC for enrofloxacin ( $168.24 \pm 3.82 \mu\text{g/ml}$ ) was recorded against MS followed by tetracyclin ( $115.48 \pm 2.62 \mu\text{g/ml}$ ), spiro-mycin ( $95.96 \pm 2.17 \mu\text{g/ml}$ ), tylosin ( $84.84 \pm 2.56 \mu\text{g/ml}$ ) and gentamicin ( $46.4 \pm 2.18 \mu\text{g/ml}$ ). Multiplex PCR is a time tested tool for the molecular diagnosis and confirmation of *Mycoplasma* species.

**Key words:** avian mycoplasmosis; chronic respiratory distress; minimum inhibitory concentration; multiplex polymerase chain reaction

## Introduction

*Mycoplasma* infections continue to be an important cause of loss in poultry production. The economic consequences are important because of decreased egg production; and growth, and poor hatchability rates due to increased embryo mortality and account for 5-10% of early chick mortality

(1, 40). Mycoplasmosis results in reduced weight gain and feed conversion efficiency along with significant downgrading of carcasses at slaughter due to airsacculitis and arthritis lesions (2, 3). Mycoplasmosis is thus a major problem for the poultry industry; and the infections are commonly known as Chronic Respiratory Disease (CRD) of chickens and infectious sinusitis of turkeys (4).

*Mycoplasma gallisepticum* (MG) is an economically significant pathogen of poultry; and the World Organization of Animal Health (OIE), has declared

the disease caused by MG as notifiable (1). *Mycoplasma synoviae* (MS) infection most frequently occurs as sub-clinical upper respiratory problem, and causes air sac lesions when combined with Newcastle Disease, Infectious Bronchitis, or both (5, 6). Systemic *M. synoviae* infection results in synovitis, an acute to chronic disease of chickens and turkeys, involving the synovial membrane of joints and tendon sheaths (7, 8). In recent reports, *Mycoplasma synoviae* has also been diagnosed as a causative agent of an emerging issue of sharp decline in egg production with eggshell apex abnormalities in poultry (9, 41).

In view of the forthcoming World Trade Organization (WTO) recommendations, the establishment of *Mycoplasma*-free chickens, day old chicks and hatching eggs is needed; otherwise exports will be impossible (10). Control of *Mycoplasma* infections by vaccination is possible, and various measures are implemented in many countries and control and eradication programs, particularly for MG in breeding stock, are successful. Sometime, chemotherapeutic approaches become necessary to minimize *Mycoplasma* transmission in cases of outbreaks, as a complement to bio-security measures, in order to minimize economic losses as well as lateral and vertical transmission (8).

Many antimicrobial drug groups, such as macrolides, pleuromutilins, tetracyclines and fluoroquinolones, have been shown to possess inhibitory activity against various mycoplasmas in *in-vitro* studies (11). However, an increase in resistance of *Mycoplasma gallisepticum* against tetracyclines (12, 13), macrolides (14, 15) and quinolones (16) has been reported and treatment strategies have proved unsatisfactory (17).

The current study was aimed at the isolation molecular identification and antibiotic resistance profiling of MG and MS from commercial poultry birds (broilers and layers) and the farm settings/environment.

## Materials and methods

### *Sample collection*

A total of 115 samples were collected; most from diseased but alive broiler and layer birds showing signs of respiratory distress and other clinical signs were (n=94), and the rest from contaminated farm environments (n=21). Samples from birds included tissues such as trachea and lungs,

exudates from air sacs and oral swabs, as well as synovial fluids, while samples collected from farm environment were only swabs from building walls, water drinkers and feeders (18).

### *Isolation of Mycoplasma species*

The samples were directly inoculated into modified Brain heart infusion (BHI) broth (Difco TM Detroit, United States) as described previously (3), supplemented by 10% horse serum, NAD, cysteine chloride, penicillin, thallium acetate and phenol red. The samples were incubated at 37 °C for 24-72 hours. The negative samples (showing no growth) were further passaged while positive samples were transferred to Brain Heart Infusion agar (Difco) for the successful isolation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. The isolate identifications were confirmed by the biochemical tests; glucose fermentation, arginine hydrolysis, phosphatase activity, film and spot production, tetrazolium reduction and casein digestion (19, 20).

### *Molecular confirmation*

The DNA from the isolated *Mycoplasma* spp. was extracted and purified using Phenol Chloroform method (21). The isolated DNA was subjected to multiplex Polymerase Chain Reaction (PCR) using specific primer sequences (22). For MG, the lipoprotein gene was amplified using primer MG-F 5'-GGATCCCATCTCGACCACGAGAAAA-3' and MG-R 5'-CCTTCAATCAGTG: AGTAACTGATGA -3' and for MS, the 16SrRNA gene was targeted using primer MS-F 5'- GAA GCAAATAGTGATATCA- 3' and MS-R 5'- GTCGTCTCGAAGTTAACAA - 3'. The PCR was performed using 50 µl of master mix (Vivantis, USA) and 10 pM of each forward and reverse primer. The reaction protocol was optimized as: initial denaturation at 94 °C for five minutes, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 50 °C for 30 sec, and extension at 72 °C for 1 min, while final extension was done at 72 °C for 7 min in thermal cycler (PqLab, Germany). The amplified product was run on 2% agarose gel using 0.2 µg/ml ethidium bromide dye and visualized in gel documentation system (Dolphin Doc, USA) using a 100bp ladder (Vivantis, USA) as DNA marker (23).

### Determination of minimum inhibitory concentrations

The isolates were subjected to a broth micro-dilution technique to determine the Minimum Inhibitory Concentrations (MICs) as described previously (12, 24, 25). Five antibiotics were selected representing different antibiotic classes: enrofloxacin, tetracycline, gentamicin, spiroomicin, and tylosin. The assay was performed in 96-well micro-titration plates. Two-fold serial dilutions of the different antibiotics, were made in 50  $\mu$ l modified BHI broth. At the end, 150  $\mu$ l of broth containing organisms ( $10^4$ - $10^5$  CFU/ml) was added into each well. Two parallel controls were also run, including a control positive (Culture control) containing broth and test culture, and a control negative containing no antibiotic. The minimum concentration of antibiotic that prevented the color change of the medium was taken as the MIC (15, 26).

### Statistical analysis

Antimicrobial sensitivity of different types of isolates collected from different types of farms against different types of antibiotics makes a complex design of experiment. Descriptive statistics are presented as the mean of MIC values for different types of isolates for each antibiotic with standard deviation (Table 1). MIC values for both species against similar antibiotics were compared with one-way analysis of variance (ANOVA) and student t-test, with a significance level of 0.05.

In addition, data for each *Mycoplasma* spp. were analyzed separately with one-way ANOVA at a significance level of 0.01. All data analyses were done by using Minitab® version 16 (Minitab Inc.).

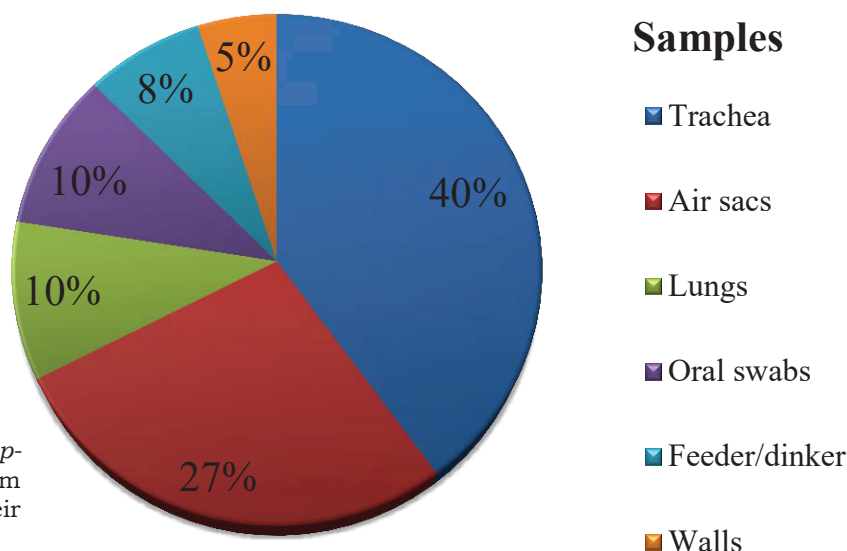
## Results

### Isolation of mycoplasma

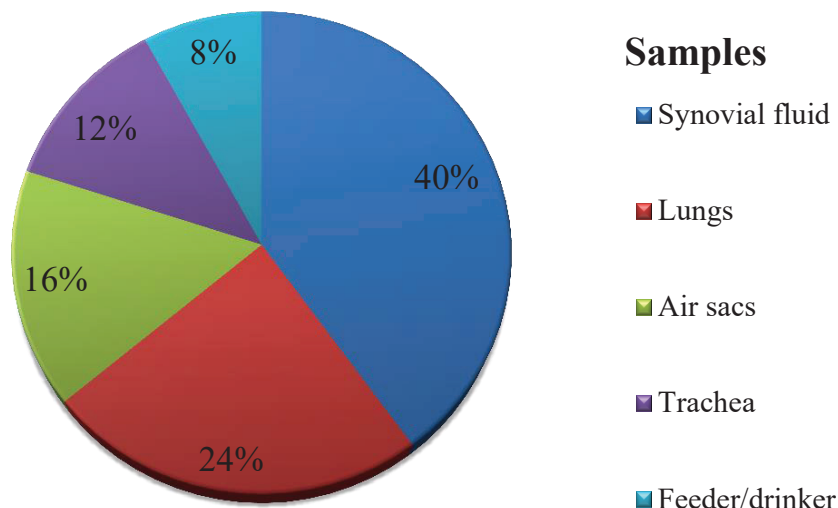
Of the 115 samples collected from chronic respiratory disease cases of broiler and layer birds, 61.5% were positive for *Mycoplasma* spp. Of these positive cultures, 62% were positive for *Mycoplasma gallisepticum* (MG) and 38% for *Mycoplasma synoviae* (MS). MG was isolated from tracheae (40%), air sacs (27%), lungs (10%), oral swabs (10%), feeders /drinkers (8%), and walls (5%) (Figure 1). MS was isolated from synovial fluid (40%), lungs (24%), air sacs (16%), tracheae (12%), and feeders / drinkers (8%) (Figure 2).

Maximum recovery of MG from clinically ill birds was observed from tracheas (42 %) and air sacs (29%), followed by lungs (8%) and oral swabs (8%), whereas recovery from the contaminated farm environment was recorded as 8% from feeder / drinker samples and 4% from wall samples. In the case of *Mycoplasma synoviae*, highest recovery was obtained from synovial fluid (40%) followed by lungs (27%), air sacs (13%), tracheae (13%) and feeder / drinker samples (7%).

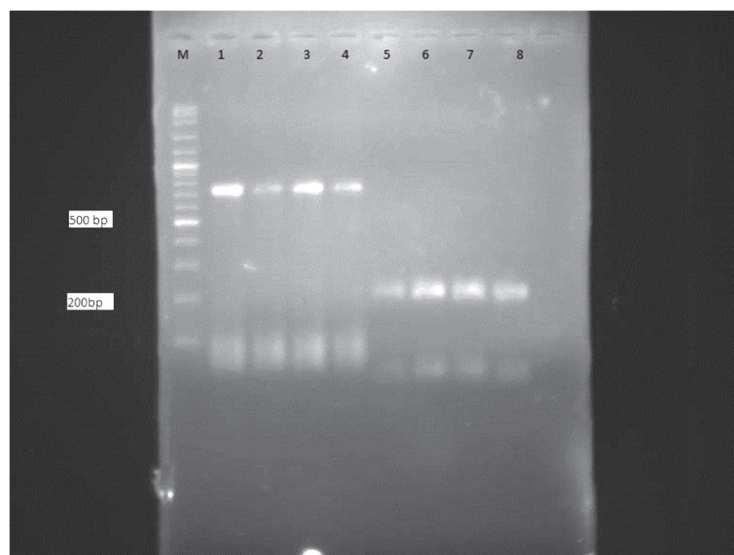
The isolates were confirmed through PCR. *Mycoplasma gallisepticum* yielded 720bp of PCR product, while 207 bp band size was observed in case of *Mycoplasma synoviae* (Figure 3).



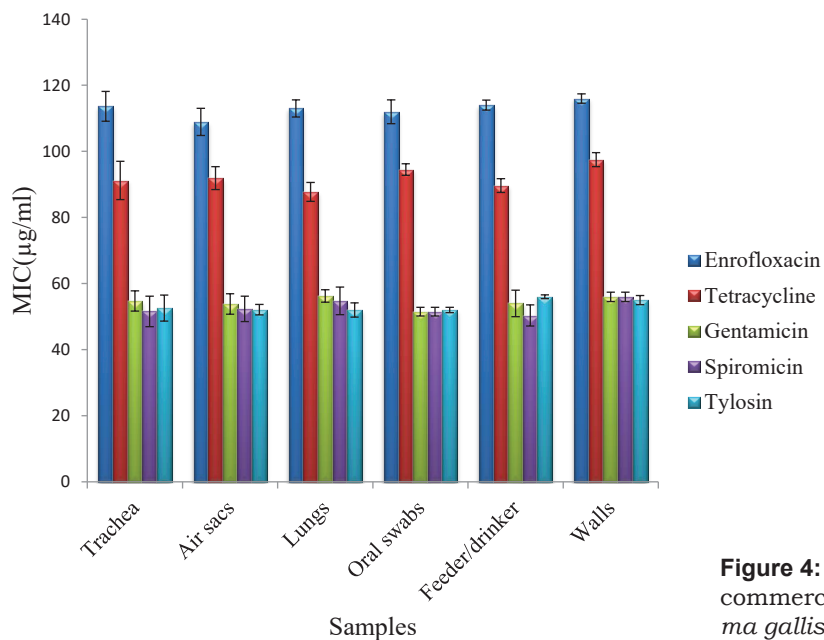
**Figure 1:** Isolation of *Mycoplasma gallisepticum* from different samples collected from broiler and layered birds as well as their farm environment



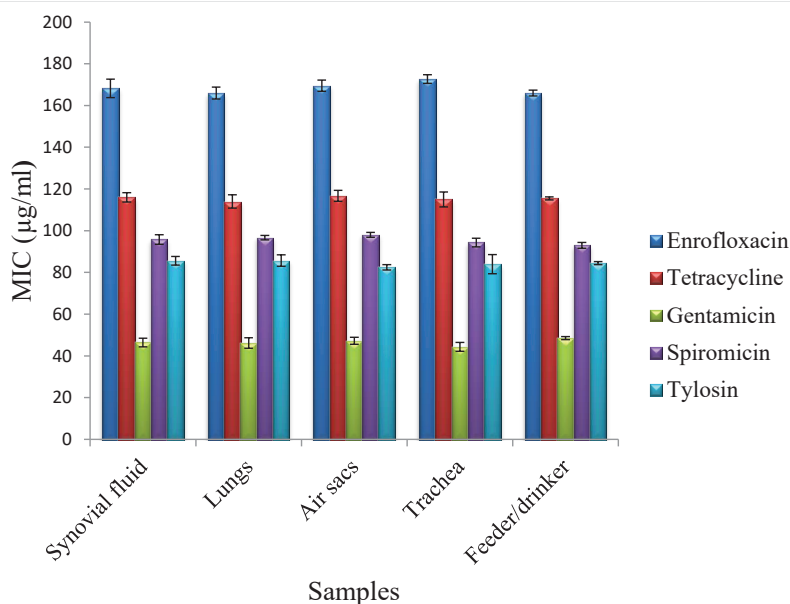
**Figure 2:** Isolation of *Mycoplasma synoviae* from broiler and layered birds



**Figure 3:** PCR-based confirmation of isolates. Lane M: Marker 100bp, Lane 1, 2, 3 and 4: positive samples for *Mycoplasma gallisepticum*, Lane 5, 6, 7 and 8: positive samples for *Mycoplasma synoviae*



**Figure 4:** Minimum Inhibitory Concentration (MIC) of commercially available antibiotics against *Mycoplasma gallisepticum* isolated from different samples



**Figure 5:** Minimum Inhibitory Concentration (MIC) of commercially available antibiotics against *Mycoplasma synoviae* isolated from different samples

### Antibiotic sensitivity profile

The isolates of *Mycoplasma gallisepticum* showed a variable degree of resistance against the antibiotics tested with significantly higher MIC<sub>50</sub> of enrofloxacin as compared to the other antibiotics tested (Table 1). Enrofloxacin was followed by tetracycline, gentamicin, spiromycin and tylosin.

In the case of *Mycoplasma synoviae* (MS), significantly higher MIC was also recorded against enrofloxacin. This was followed by tetracycline followed by, spiromycin, tylosin and gentamicin (Table 1).

One way analysis of variance (ANOVA) showed statistical differences ( $P < 0.05$ ) in the MIC of enrofloxacin, tetracycline, gentamicin, spiromycin

and tylosin against MG and MS (Table 1). Statistical analysis with student t-tests ( $P < 0.05$ ) showed that enrofloxacin, tetracycline, spiromycin and tylosin have greater MIC against MS, while gentamicin has greater MIC against MG.

One-way analysis of variance (ANOVA) showed no significant difference ( $P < 0.01$ ) in MIC of enrofloxacin against different isolates of MG. Tetracycline, gentamicin, spiromycin and tylosin also showed the same pattern for different isolates of MG (Figure 4). There were no significant differences ( $P < 0.01$ ) in the MIC of enrofloxacin against different isolates of MS. Tetracycline, gentamicin, spiromycin and tylosin also showed the same pattern for all isolates of MS.

**Table 1:** Minimum Inhibitory Concentration (MIC) of commercially available antibiotics against *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, shown as (Mean±SD) µg/ml

Antibiotics	<i>Mycoplasma gallisepticum</i> (n=40)	<i>Mycoplasma synoviae</i> (n=25)	P-value
Enrofloxacin	112.38±4.34	168.24 ±3.82	0.00
Tetracycline	91.58±4.66	115.48±2.62	0.00
Gentamicin	54.33±2.98	46.4±2.18	0.00
Spiromycin	52.23±3.99	95.96 ±2.17	0.00
Tylosin	52.58±2.69	84.84±2.56	0.00

Means are significantly different ( $P < 0.05$ )

## Discussion

The disease caused by *Mycoplasma gallisepticum* and *Mycoplasma synoviae* is known as Chronic Respiratory Distress (CRD), which causes heavy economic losses in the poultry industry by decreasing production and increasing the cost of production via medication expenses (10).

A multiplex PCR has been optimized for successful detection of five of the respiratory tract pathogens including *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Newcastle Disease virus, Infectious Bronchitis virus and Avian Influenza virus (23, 27, 28, 29). Several studies have shown successful detection of individual pathogens, including a multiplex PCR for the detection of *Mycoplasma* spp. (30) and multiplex RT-PCR for respiratory tract viruses (6).

A PCR assay for detection of the variable hemagglutinin gene (*vlhA* gene) of *M. synoviae* (31, 32) has proved to be a useful tool in the detection and typing of different MS strains. Comparing *vlhA*-PCR with *16SrRNA*-PCR (31) has also shown that in the very early stage of infection the *16SrRNA* procedure is more sensitive than the *vlhA* method (27).

The development of multiplex PCR for the detection of different pathogenic species of avian mycoplasma in a sample. The above approach is reliable, sensitive and specific and help to reduce the losses due to mycoplasma infections by early and accurate diagnosis of avian mycoplasma species (22, 24, 25).

The aim of this study was to assess the *in vitro* antimicrobial susceptibilities of *M. gallisepticum* and *M. synoviae* isolates from broilers, layer birds and the farm premises. In Pakistan, the control strategy for avian mycoplasmosis is largely based on the use of effective antibiotics, which helps in controlling the severity of clinical disease and prevents substantial economic losses. However, it is worth mentioning that administration of antibiotics depends on the dosage and duration of administration; which may fail to completely eradicate these pathogens from the infected flocks.

*Mycoplasma gallisepticum* and *Mycoplasma synoviae* have shown sensitivity *in vitro* to several antimicrobials including, tetracyclines, macrolides, lincosamides, fluoro-quinolones and others (14). PCR confirmed isolates of *M. gallisepticum* and *M. synoviae* were tested using enrofloxacin, tetracycline, gentamicin, spiroomicin and tylosin.

The isolates of *Mycoplasma gallisepticum* showed a variable degree of resistance against the antibiotics tested, with a significantly high MIC<sub>50</sub> of Enrofloxacin as compared to other antibiotics tested followed by tetracycline, gentamicin, spiroomicin and tylosin. This is similar to the findings reported by Behbahan and co-workers (33) and the findings of (16, 34, 36, 37). On the other hand, a previous study of MG isolates from Israel had increased resistance to enrofloxacin (26, 35).

In the case of *Mycoplasma synoviae* (MS), significantly high MIC against enrofloxacin, followed by tetracycline, spiroomicin, tylosin and gentamicin (46.4±2.18 µg/ml). These findings were similar to those of (25, 38, 39).

The results of the current study elucidate the significant losses incurred by poultry birds due to high morbidity and mortality, along with production losses. The multiplex PCR is an efficient, reliable and reproducible tool for the rapid confirmation and diagnosis of avian mycoplasmosis. The severity of the problem is of utmost importance and needs to be further investigated using classical diagnostic methods, along with latest molecular techniques for assessment of the disease scenario. There is a huge need to update the therapeutic regimen owing to increased antibiotic resistance from time to time.

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## POJAVNOST, MOLEKULARNA IDENTIFIKACIJA IN UGOTAVLJANJE ODPORNOSTI NA ANTIBIOTIKE MIKOBAKTERIJ *MYCOPLASMA GALLISEPTICUM* IN *MYCOPLASMA SYNOVIAE* IZOLIRANIH IZ KOKOŠI S KRONIČNIMI DIHALNIMI BOLENJI IN IZ NJIHOVEGA BIVALNEGA OKOLJA

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**Povzetek:** Ptičja mikoplazmoza je resno težava v perutninski proizvodnji, ki vodi v velike izgube zaradi obolevanja perutnine in posledično povzroča ekonomske izgube. Glavni povzročitelji mikoplazmoz so *Mycoplasma gallisepticum* in *Mycoplasma synoviae*. Za preučevanje spremenljive stopnje odpornosti na običajno predpisane in uporabljene antibiotike pri mikoplazmozi je bilo odvzetih skupno 115 vzorcev, vključno z vzorci tkiva in brisom, pitovnih piščancev, nesnic s kroničnimi boleznimi dihal (CRD) in iz njihovega bivalnega okolja. Vzorci so bili preneseni v tekoče gojišče BHI (iz angl. Brain Heart Infusion), z dodatkom 10 % konjskega seruma, NAD, cisteina, penicilina in talijevega acetata. Pozitivne vzorce smo prenesli v agar BHI (Difco) za izolacijo *Mycoplasma spp.* Vzorci so bili določeni kot negativni po tretji pasaži. Med vzorci je bilo 61,5 % pozitivnih na prisotnost *Mycoplasma spp.*, ki smo jih večinoma ugotovili po drugi pasaži. Od vseh pozitivnih primerov je bila ugotovljena *Mycoplasma gallisepticum* (MG) v 62 % primerov, *Mycoplasma synoviae* (MS) pa v 38 %, kar je bilo potrjeno z verižno reakcijo s polimerazo (PCR) z uporabo specifičnih primerjev. Izolati MG in MS so pokazali spremenljivo stopnjo občutljivosti na komercialno dostopno zdravilo tilozin. Minimalna zaviralna koncentracija (MIC) pri MG je bila najvišja pri enrofloksacinu ( $112,38 \pm 4,34 \mu\text{g/ml}$ ), sledili pa so tetraciklin ( $91,58 \pm 4,66 \mu\text{l/ml}$ ), gentamicin ( $54,33 \pm 2,98 \mu\text{g/ml}$ ), spiromicin ( $52,23 \pm 3,99 \mu\text{g/ml}$ ) in tilozin ( $52,58 \pm 2,69 \mu\text{g/ml}$ ). Najvišjo MIC proti MS smo ravno takougotovili pri enrofloksacinu ( $168,24 \pm 3,82 \mu\text{g/ml}$ ), ki so mu sledili tetraciklin ( $115,48 \pm 2,62 \mu\text{g/ml}$ ), spiromicin ( $95,96 \pm 2,17 \mu\text{g/ml}$ ), tilozin ( $84,84 \pm 2,56 \mu\text{g/ml}$ ) in gentamicin ( $46,4 \pm 2,18 \mu\text{g/ml}$ ).

**Ključne besede:** ptičja mikoplazmoza; kronična bolezen dihal; minimalna zaviralna koncentracija; mnogokranta PCR reakcija