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Rapid serum agglutination, cultural isolation and PCR for detection of *M. gallisepticum* and *M. synoviae* infection in poultry

S R BUKTE[1] and R S GANDGE[2]

Bombay Veterinary College, Parel, Mumbai 400 012 India

ABSTRACT

Poultry mycoplasmosis, is an infectious disease of birds distributed worldwide causing serious economic loss to the poultry industry. Diagnosis of poultry mycoplasmosis is considered to difficult task due to different aspects of etiological agent from conventional bacteria. Therefore, the present study was aimed to detect mycoplasma infection in poultry using serological, cultural and molecular techniques. The specimens included were, sera samples and choanal swabs (150 each) collected from 150 birds housed in semi arid and costal area of Maharashtra (India). *Mycoplasma gallisepticum* (MG) was more prevalent than *Mycoplasma synoviae* (MS) in mycoplasmosis detected by rapid serum agglutination test (RSA), cultural isolation and direct PCR of samples. Nucleotide sequences of three representative MG isolates with NCBI accession nos. KY467400, KY467401 and KY467403 did not show any variations in their sequences analysed and matched with published strains of MG. RSA, cultural isolation and direct PCR yielded 93 (62%), 24 (16%) and 85 (56.67%) positive cases respectively. The sensitivity of PCR and RSA was 95.83% and 83.33% respectively. Thus, RSA and PCR were better than cultural isolation in diagnosis of poultry mycoplasmosis, therefore these methods can be used for screening the flocks for detection of mycoplasma infection depending on availability of specimens and facility.

Key words: Cultural isolation, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, PCR, RSA

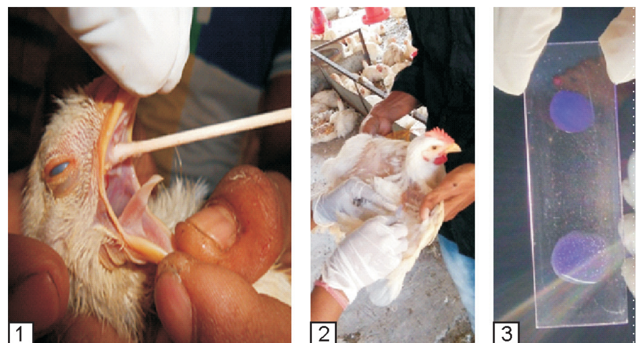
Avian mycoplasmosis, an important infectious disease of commercial birds affects wide variety of birds including commercial poultry. Infection in poultry mainly caused by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are of a major concern (Sarfaraz *et al.* 2017, Umar *et al.* 2017). The economic loss to poultry industry is due to decreased growth rate and loss in egg production (Lockaby *et al.* 1998, Kleven and Ferguson-Noel 2008). In spite of being such an important disease; there are very few laboratories, which have diagnostic facility with regular services for detection of poultry mycoplasmosis. Therefore, present study will help to select suitable method for diagnosis or screening poultry mycoplasmosis. Further, the results of present study on prevalence of poultry mycoplasmosis will help to know current scenario of disease in the Maharashtra.

MATERIALS AND METHODS

Reference strains: Mycoplasma gallisepticum (ATCC-19610) and *Mycoplasma synoviae* (ATCC-25204).

Oligonucleotide primers: Mycoplasma gallisepticum mgc2 2F-5'-CGC-AAT-TTG-GTC-CTA-ATC-CCC-AAC-A-3', mgc2 2R-5'-TAA-ACC-CAC-CTC-CAG-CTT-TAT-TTC-C-3' (Lysnyansky *et al.* 2005). *Mycoplasma synoviae* MS–F, 5'-GAA-GCA-AAA-TAG-TGA-TAT-CA-3', MS–R, 5'-GTC-GTC-TCC-GAA-GTT-AAC-AA-3' (World Organization of Animal Health, 2008), supplied by Sigma Aldrich.

Collection of specimens: A total of 300 samples (150 choanal swabs + 150 sera samples) from 150 birds suspected for mycoplasma infection were collected from different farms located in semi arid and costal area of Maharashtra (India) (Figs 1-2). The specimens were collected aseptically (World Organization of Animal Health, 2008) and placed in sterile leak-proof containers and transported to the laboratory as early as possible for processing. The clinical samples were processed for detection of *Mycoplasma* infection employing conventional (RSA and cultural isolation) and molecular methods using species specific PCR assays, targeting mgc2 gene of *M. gallisepticum* and 16S rRNA gene of *M. synoviae*.



Figs 1–3. **1.** Collection of choanal swabs; **2.** Collection of blood from wing vein; **3.** Rapid serum agglutination test

Present address: [1]Veterinary officer (srbukte@gmail.com), Tineta Pharma Pvt. Ltd., Mumbai. [2]Professor (rajashrigandge @yahoo.co.in), Department of Microbiology.

Rapid serum agglutination test (RSA): Antibodies against MG and MS were detected in mycoplasmosis suspected poultry sera samples by Rapid Serum Agglutination Test (RSA) using commercial MG and MS antigens from x–OvO Limited, IZS Venezie, Italy. The test was carried out at room temperature (20–25°C) within 24 h of collection of sera samples. The RSA test was carried out as per method described by Arefin *et al.* (2011). Positive reaction was indicated by agglutination within 2 min. Known positive and negative control sera samples were included in the test.

Cultural isolation and identification of Mycoplasma *spp:* Isolation of *M. gallisepticum* was carried out using Pleuropneumonia Like Organism (PPLO) agar/broth base without crystal violet (HiMedia) supplemented with Pig serum (25%), Penicillin G (200,000 IU/ml), Glucose solution (5%), Thallous acetate (1%) (Sigma Aldrich), Fresh yeast extract (10%), Phenol red solution (0.1%) (for broth medium only); whereas for *M. synoviae* additionally, 1% solution each of nicotinamide-adenine dinucleotide (NAD) and cysteine hydrochloride was mixed in equal parts and 2 ml of this preparation was added per 100 ml of medium as mentioned above for MG. The inoculated culture media were incubated at 37°C in presence of 5% CO_2 and humidity (World Organization of Animal Health, 2008). Identification of recovered isolates at genus level was carried out by differentiation from *Acholeplasma* and *Ureaplasma* based on colony characteristics, morphology, digitonin sensitivity and modified urease test (Quin *et al.* 1994). Further identification of *Mycoplasma* isolates at species level was carried out by PCR targeting MG specific mgc2 (Lysnyansky *et al.* 2005) and MS species 16S rRNA gene (World Organization of Animal Health, 2008).

Detection of Mycoplasma *spp. in clinical specimens by PCR:* All 150 choanal swabs were subjected to MG (mgc2) and MS (16S rRNA) species specific PCR assays for detection of poultry mycoplasmosis directly in clinical sample. DNA extraction and PCR assay carried out as per the protocol described by Lysnyansky *et al.* (2005) and World Organization of Animal Health (2008) for MG and MS respectively.

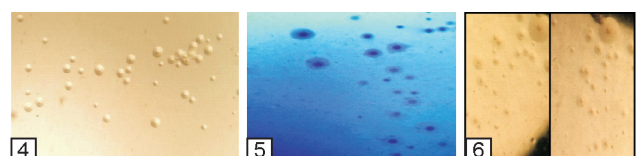
Gene-targeted sequencing (GTS) of M. gallisepticum*:* The sequencing of PCR products of mgc2 gene of isolates was carried out with ABI Big Dye Terminator Kit version 3.1 using automated genetic analyser at Bioserve Biotechnologies (I) Pvt Ltd, Hyderabad, India. The chromatogram were visualised with finch TV application and forward and reverse sequences assembled manually. The sequences were subjected to BLAST analysis with GenBank using BLASTn algorithm. The sequences were submitted to NCBI GenBank database through online submission system Bankit and accession numbers were obtained.

RESULTS AND DISCUSSION

Poultry industry is one of the most organized and vibrant sectors among agriculture and allied sectors of India with current investment of ₹ 200 billion with 15 to 20% annual growth rate (Anonymous 2011). It's a source of employment for about 1.5 million people. Every family in rural areas and every fifth family in urban areas are associated with poultry production in one way or the other (Rashid *et al.* 2009). It is essential to perform rapid, accurate, precise and sensitive diagnosis of mycoplasmosis for effective control of the disease/ outbreaks and maintain the proper production level and profit rates. Therefore, present study was aimed for detection of mycoplasma infection in poultry by serological, cultural and molecular techniques.

Rapid serum agglutination (RSA) test: A total of 150 sera samples were subjected to rapid slide agglutination test using commercial MG and MS antigens from x–OvO Limited, IZS Venezie, Italy (Fig. 3). Serological evidence of *Mycoplasma* antibodies was detected in 62% (48.67% MG and 13.33% MS) cases of birds showing respiratory distress. Out of 93 RSA positive birds, 22 (23.65%) and 41 (44.08%) were also positive in cultural isolation and PCR respectively. The nearer values of serological evidence of MG and MS are recorded in different parts of country by various authors. Mahfuzul *et al.* (2014), Ali *et al*. (2015) and Elbehiry *et al*. (2016) recorded 55.83%, 56.13% and 46.11% prevalence of MG antibodies respectively by RSA test in Bangladesh, whereas Luciano *et al*. (2011) and Elgnay and Azwai (2013) found 26.46% and 9.3% positive cases respectively in RSA for MS. The serological tests are long been used for diagnosis of mycoplasmosis since those are easy to perform, rapid and cheap. However, in RSA, there could be possibility of nonspecific agglutination and cross reactions of antibodies to some avian mycoplasma antigens, induced by injecting chickens with several commercial poultry disease vaccines (Yoder 1989, Ross *et al.* 1990); this possibility can also be employed for higher RSA positive cases in the present study as compared to PCR and isolation.

Cultural isolation and identification of Mycoplasma *spp.:* In cultural isolation from 150 specimens, 24 (16%) isolates were recovered on PPLO agar. All 24 field isolates were identified and confirmed as *Mycoplasma* spp. by differentiating from *Acholeplasma* and *Ureaplasma* based on results of conventional identification methods (Figs 4-6). Further, confirmation of conventionally identified 24 *Mycoplasma* isolates at species level by species specific mgc2 gene (MG) and 16S rRNA (MS) PCR assay revealed that, all isolates belonged to MG species with amplification product of 300 bp, as shown by *Mycoplasma gallisepticum* ATCC-19610**.** Whereas, none of the 24 field isolates of



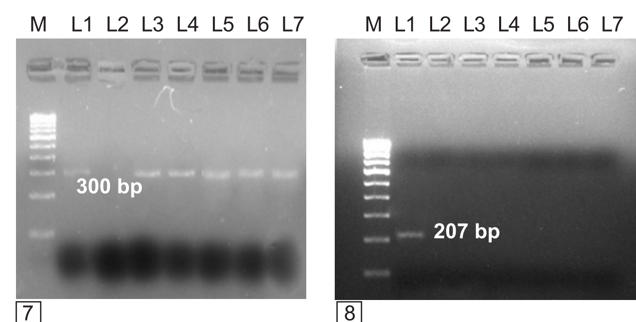
Figs 4–6. **4.** *Mycoplasma* colonies showing fried egg appearance under stereo zoom microscope **5-6.** *Mycoplasma* colonies: Diene's staining.

Mycoplasma spp. could yield MS specific amplification product of 207 bp except in positive control (*Mycoplasma synoviae* ATCC-25204) in PCR. Out of 24 birds positive in cultural isolation, 22 (91.67%) and 23 (95.83%) were positive in RSA and direct PCR respectively.

A number of factors are likely to influence the successful isolation of *Mycoplasma spp.* from clinical specimens, viz. the availability of appropriate material at the appropriate stage of the disease, season of sample collection, transportation conditions and processing time etc., therefore several researchers throughout the world have reported isolation of *Mycoplasma* spp. from different clinical specimens with varying degrees of success from 0% to 81.30% (Nagalakshmi *et al.* 2013, Behbahan *et al.* 2005). Moreover, fastidious and fragile nature of organism, sensitivity to pH, overgrowth of other organism may also account for its wide range of variable isolation rates and low sensitivity. The isolation rate of *Mycoplasma* spp., i.e. 8.18% and 9% was observed by Tiong *et al.* (1979) and Hanif and Najeeb (2007) respectively. Behbahan *et al*. (2005) investigated the cases of avian mycoplasmosis and could recover 100 (81.31%) *Mycoplasma* isolates from 123 tracheal and airsac samples, whereas isolation rate of 14.4% was reported by Reda and Abd El-Samie (2012). Khalda *et al.* (2013) recovered 7 (4.11%) isolates from 170 tracheal swabs, Nouzha *et al.* (2013) achieved 72.72% (7/18) isolation rate of *Mycoplasma* spp. using tracheal swab. Rauf *et al.* (2013) and Elbehiry *et al.* (2016) reported isolation rate of 27.6% and 37.01%; however, Nagalakshmi *et al.* (2013) could not succeed in isolation of *Mycoplasma* spp. The results of isolation rate (16%) of present study observed correlates with the rate reported by Reda and Abd El-Samie (2012).

Gene-targeted sequencing (GTS) of M. gallisepticum*:* A representative PCR products of mgc2 gene of MG isolates were sequenced (Bioserve Biotechnologies (I) Pvt Ltd, Hyderabad, India). BLAST analysis of obtained sequences revealed that the sequences matched to published mgc2 gene of different strains of MG. A total of three accession numbers, i.e. KY467400, KY467401 and KY467403 were obtained from NCBI for sequences submitted. The mgc2 gene, a fairly well conserved gene in MG, encodes a second cytadhesin protein which proved to be located at the tip organelle in MG and also known to play a role in the attachment process, adhering to mucosal membranes, hence initiate infection and also important in establishing chronic infection (Hnatow 1998). This gene can be used as a basic reference in molecular identification of MG isolates (Hnatow 1998, Winner 2000), therefore sequencing of 300 bp mgc2 gene PCR products of representative isolates was got done by Sangers method from Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad. In BLAST, the sequence showed more than 95% similarity with published *Mycoplasma gallisepticum* strain Putative Cytoadhesion Protein (mgc2) gene. GTS analysis of surface-protein genes is a sensitive and reproducible typing method and will allow rapid global comparisons between laboratories (Ferguson 2005). Shaker *et al.* (2015) performed partial sequencing of mgc2 gene of MG isolates (10) for detection of nucleotide variation and genetic diversity which indicated that the different MG isolates of Tehran province, Iran were not new or diverged MG strains.

Detection of Mycoplasma *spp. in clinical specimens by PCR:* One of the benefits of PCR over cultural isolation of avian *Mycoplasma* is that, the PCR is not dependent on viable or structurally intact cells and the presence of DNA in the tissues is sufficient to yield a positive result (Tomar *et al.* 2017) making it valuable tool in the diagnosis of *Mycoplasma* species, not only for its sensitivity but for its better specificity (Kempf *et al.* 1993). PCR has several features that simplify the diagnosis of mycoplasmosis which can be completed in one day, whereas cultural isolation of *Mycoplasma* species require one or more weeks for its growth and identification (Garcia *et al.* 2005) due to fastidious and slow-growing nature of organism. Their isolation is often impaired by the overgrowth of non-pathogenic mycoplasma species or other faster growing bacteria and fungi (Garcia *et al.* 1995) resulting in low sensitivity in diagnosis. Looking into this, in the present study, *Mycoplasma gallisepticum* specific mgc2 gene PCR and *Mycoplasma synoviae* specific 16S rRNA PCR assays were employed for detection of mycoplasma directly in clinical specimens. The single locus typing using mgc2 gene target for studying MG isolates by mgc2-PCR is the most specific and the most sensitive PCR for the detection of MG (Gracia 2005) whereas for *Mycoplasma synoviae*, 16S rRNA PCR is suitable (WOAH/OIE 2008). Out of 150 choanal swabs subjected for direct PCR, overall 85 (56.67%) samples were positive for mycoplasmosis. All 85 PCR positive samples yielded an amplification product of 300 bp specific for MG (Fig. 7) and, none of 150 samples were positive for MS (Fig. 8). Out of 85 (56.67%) specimens positive in direct PCR, 41 (48.23%) and 23 (27.05%) were also positive in RSA and cultural isolation respectively. Thus the PCR was more sensitive method giving 95.83% sensitivity in detecting mycoplasma infection directly in clinical specimens when compared with cultural isolation. The near range of sensitivity of PCR in detection of mycoplasmosis was observed by Bayatzadeh *et al.* (2011),



Figs 7–8. **7.** *M. gallisepticum* species specific mgc2 PCR assay of *Mycoplasma* spp. isolates. M, 100bp of DNA ladder; L1, ATCC control for *M. gallisepticum*; L2-MG negative sample; L3-L7, MG positive specimens **8.** *M. synoviae* species specific 16S rRNA PCR assay of clinical specimens.

Rauf *et al*. (2013) and Elbehiry *et al.* (2016) who reported sensitivity of PCR in 55.9%, 68.94% and 51.92% cases respectively. However, lower sensitivity of PCR than the present investigation was reported by Evans *et al.* (2009), Bagheri *et al.* (2011), Khalda *et al.* (2013) and Kaboli *et al*. (2013), i.e. 36.7%, 29.63%, 17.8% and 31.50% respectively. This may be due to the reason that, the various factors affect sensitivity of PCR, which includes quality and concentration of template DNA, specimen collection, preservation and processing method etc.

Investigation of total 150 birds using RSA, cultural isolation and direct PCR yielded 93 (62%), 24 (16%) and 85 (56.67%) positive cases respectively. PCR showed the highest sensitivity (95.83%) followed by RSA (83.33%) when compared with cultural isolation as gold standard. Similar sensitivity pattern of these three methods was reported by Arefin *et al.* (2011) who observed 12%, 9% and 16% specimens positive by RSA, culture isolation and PCR respectively although lower percentage of positive cases yielded in comparison of the present study. The results of comparative efficacy of cultural isolation and PCR in detection of mycoplasmosis in present study were in accordance with Rauf *et al.* (2013) who reported isolation rate of 27.6% and PCR sensitivity as 68.94%. One of our samples was PCR negative but yielded a positive culture. Several factors such as the presence of Taq DNA polymerase inhibitors or gene sequence variations lead to false negative PCR results (Joaquin *et al.* 2010). Sixty two (62) of our samples were PCR positive but yielded negative culture. Several factors such as deficiency in culture requirements, temperature during transportation etc. reduce the viability of MG and leads to false negative culture result (Pourbakhsh *et al.* 2010), however PCR can detect and identify the dead particles of organisms in the original samples from chickens swabs. The PCR is currently under consideration as a confirmation test in the OIE terrestrial manual. Feberwee *et al.* (2005) compared PCR, rapid plate agglutination, hemagglutination inhibition, ELISA and concluded that a certain level of false-positive results can be expected in about any serologic test. Thus, the results of higher % of RSA and positive cases of MS in the present study may be due to false positive results (Feberwee *et al.* 2005) or due to cross reaction of antibodies produced in vaccinated birds (Ross *et al.* 1990).

In conclusion, PCR is more rapid, sensitive, effective and inexpensive method than standard cultural method for diagnosis of poultry mycoplasmosis. Therefore, PCR can be alternative method for traditional culture towards the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in chicken. However, if facilities and expertise are not available; RSA is next option to PCR for screening the flock for detection of mycoplasma infection.

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