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Diagnosis of poultry mycoplasmosis by cultural isolation and PCR

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ABSTRACT

The present investigation was designed for diagnosis of poultry mycoplasmosis by cultural isolation and PCR assay. Clinical specimens (159: 47 lungs, 21 trachea, 91 choanal cleft swabs) of poultry were simultaneously subjected to cultural isolation of *Mycoplasma* spp., PCR for detection of mycoplasmosis and for isolation of *E. coli*. Isolation of *Mycoplsma* spp. was carried out using pleuropneumonia like organism (PPLO) medium. Identification of genus *Mycoplasma* and differentiation from *Acholeplasma* and *Ureaplasma* was done by conventional methods. A total of 15 isolates were identified as *Mycoplasma* spp. with isolation rate of 9.43%. In MG (*Mycoplasma gallisepticum*) and MS (*Mycoplasma synoviae*) species specific 16S rRNA PCR assay, all 15 isolates were confirmed as MG species. Direct detection of mycoplasmosis in 159 clinical specimens by PCR, targeting MG and MS species-specific 16S rRNA gene, revealed 108 (67.92%) positive specimens. Out of 108, 105 (66.04%) and 3 (1.86%) were positive for MG and MS respectively. *E. coli* was found to be major pathogen associated with poultry mycoplasmosis in 28 MG-PCR positive cases out of 42 *E. coli* isolates recovered. Comparative results of PCR and cultural isolation showed that 16S rRNA MG and MS species specific PCR is superior to cultural isolation for crucial, rapid, specific and sensitive detection of poultry mycoplasmosis directly in clinical specimens.

Key words: Cultural isolation, *E. coli, Mycoplasma gallisepticum*, *Mycoplasma synoviae*, PCR, Poultry mycoplasmosis

Poultry mycoplasmosis, an infectious disease of birds distributed worldwide and causing serious economic losses to the poultry industry, is caused by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). MG has been designated as notifiable disease by World Organization for Animal Health (OIE 2008). At field level, the diagnosis of disease is carried out with serological tests that lack specificity (Rauf *et al.* 2013). Cultural isolation and identification of *Mycoplasma* at species level is of crucial importance since it provides useful information on epidemiology of the disease and facilitates tracing the origins of pathogens, although it is time consuming and less sensitive (Kempf *et al.* 1993). Now-a-days, molecular techniques including various types of PCR assays targeting different genes are widely employed for detection of *Mycoplasma* (OIE 2008). In spite of being such an important disease, there is no such work carried out on this aspect of poultry mycoplasmosis in Maharashtra at our best knowledge. Therefore, present investigation was designed for detection of poultry mycoplasmosis by cultural isolation and PCR assay, since the accurate, rapid, sensitive and specific detection of poultry mycoplasmosis can help in prevention and control of disease and thereby reducing the economic losses.

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MATERIALS AND METHODS

Clinical specimens: Choanal cleft swabs, lungs and trachea.

Culture media: Pleuropneumonia like organism (PPLO) agar/broth base without crystal violet supplemented with pig serum 25%, penicillin G (200,000 IU/ml), glucose solution 5%, thallous acetate 1%, fresh yeast extract 10%, phenol red solution 0.1% (for broth medium only); for *Mycoplasma synoviae*, 1% solution each of NAD and cysteine hydrochloride was mixed in equal parts and 2 ml was added per 100 ml of medium.

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

Oligonucleotide primers: *Mycoplasma gallisepticum* MG-14F 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3', MG-13R 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3' (OIE, 2008). *Mycoplasma synoviae* MS–F, 5'-GAA-GCA-AAA-TAG-TGA-TAT-CA-3', MS–R 5'-GTC-GTC-TCC-GAA-GTT-AAC-AA-3' (Lauerman *et al.* 1993).

Collection of material

Success in recovery of *Mycoplasma* organisms after culturing specimens depends on careful collection of

Table 1. Details of specimens collected for investigation

Source	Sample code	Specimen	No. of samples	Total
Pune	PUN1 to PUN20	Lung	20	34
	PUN21 to PUN34	Trachea	14	
Mumbai	PAT2 and PAT3	Lung	02	03
	PAT1	Trachea	01	
Pen	PEN1 to PEN25	Lung	25	31
	PEN26 to PEN30	Trachea	06	
Pen	PENC1 to PENC4	Choanal cleft swabs	30	30
	PEND1 to PEND10			
	PENK1 to PENK16			
Palghar	PAL1 to PAL36	Choanal cleft swabs	36	36
Raigad	RAI1 to RAI20	Choanal cleft swabs	20	20
Sangli	SAN1 to SAN5	Choanal cleft swabs	05	05
Grand total				159

suitable specimens, proper transportation and accurate processing of clinical sample. In the present study, specimens were collected aseptically and placed in sterile leak-proof containers of appropriate size (OIE 2008). The specimens were packed in stout polystyrene foam containers and transported with care as early as possible.

Clinical specimens (159: 91 choanal cleft swabs, 47 lungs and 21 trachea) from different locations of Maharashtra from live and dead birds suspected for mycoplasma infection formed the material for study (Table 1). Samples collected in triplicates were simultaneously subjected for cultural isolation of *Mycoplasma* spp., direct PCR and for isolation of *E. coli*.

Isolation and identification of Mycoplasma *spp.* : Isolation of *Mycoplasma* spp. was carried out on pleuropneumonia like organism (PPLO) medium using suitable incubation conditions. Inoculated PPLO plates were incubated at 37° C temperature in presence of 5% CO_2 and humidity up to 21 days. Identification of recovered isolates as genus *Mycoplasma* and differentiation from *Acholeplasma* and *Ureaplasma* was done based on colony characteristics (morphology, micrometry, Diene's staining), Giemsa staining, 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 and MS species specific 16S rRNA gene (Lauerman *et al.* 1993, OIE 2008).

Molecular detection of mycoplasmosis from clinical specimens: All 159 specimens were simultaneously subjected to MG and MS species specific 16S rRNA PCR assays for detection of poultry mycoplasmosis directly in clinical specimens. DNA extraction and PCR assay was carried out as per OIE (2008).

Isolation and identification of E. coli: A total of 159 clinical specimens were subjected to isolation of *E. coli* on MacConkey's agar and identification was carried out based on morphology, colony characteristics, motility and biochemical tests (Quin *et al.* 1994).

RESULTS AND DISCUSSION

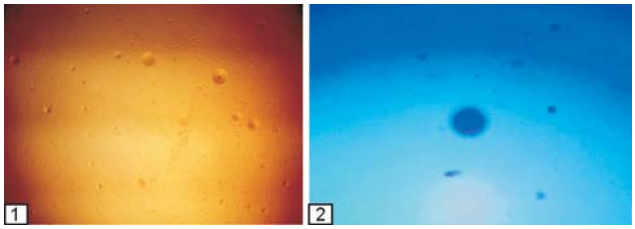
Isolation and identification of Mycoplasma *spp.*: In cultural isolation from 159 specimens, 15 isolates (9.43%) i.e. 12 from choanal cleft swabs and 3 from lungs were recovered on PPLO (Table 2). All 15 field isolates were identified and confirmed as *Mycoplasma* spp. by differentiating from *Acholeplasma* and *Ureaplasma* and based on results of conventional identification methods (Figs 1, 2).

Table 2. Details of *Mycoplasma* spp. isolates recovered

Isolate	Source	Specimen
PUN 6	Pune	Lung
PUN 14		
PUN 16		
PENK 7	Pen	Choanal cleft swab
PENK 10		
PEND 7		
PENC 3		
PAL 2	Palghar	
PAL 3		
PAL 10		
PAL 15		
PAL 23		
PAL 28		
PAL 30		
PAL 32		

Further, confirmation of conventionally identified 15 isolates by species specific 16S rRNA PCR assay revealed all isolates belonged to MG species with amplification product of 185 bp, as shown by *Mycoplasma gallisepticum* ATCC 19610. Whereas, none of the 15 field isolates of *Mycoplasma* spp. could yield MS specific amplification product of 207 bp except *Mycoplasma synoviae* ATCC 19610. The conventional procedures employed for identification of mycoplasma are time consuming. Therefore as an alternative to this method; PCR is used for identification of isolates at species level (Rauf *et al*. 2013).

Isolation and identification of organisms from clinical specimens has been considered as the "gold standard" for diagnosis of mycoplasmosis (OIE 2008). Though the procedure is grim, it is reliable and definitive since it



Figs 1–2. **1.** Fried egg colonies of *Mycoplasma* under stereo-zoom microscope. **2.** *Mycoplasma* colonies stained with Diene's stain.

provides direct evidence of presence of organism in the flock.

In the present investigation, isolation was successful only from 15 specimens (9.43%) out of 159 specimens processed. Several researchers (Nagalakshmi *et al.* 2013, Behbahan *et al*. 2008) throughout the world have reported isolation of *Mycoplasma* spp. from different clinical specimens with varying degrees of success (0 to 81.30%). Since a number of factors are likely to influence the successful isolation of *Mycoplasma* spp. from clinical specimens; especially the availability of appropriate material at the appropriate stage of the disease, season of sample collection, transportation conditions and processing time. Moreover, fastidious and fragile nature of organism, sensitivity to pH, overgrowth of other organism may account for its wide range of variable isolation rates and low sensitivity. Isolates recovered on PPLO agar were differentiated from genus *Acholeplasma* and *Ureaplasma* (Quinn *et al.* 2004) and confirmed as *Mycoplasma* spp. based on results of conventional methods viz. morphology, size and Diene's staining of colony, Giemsa staining, Digitonin sensitivity and modified urease test as applied and found reliable in conventional identification of *Mycoplasma* spp. by Mohamed (2004), Heleili *et al.* (2011) and Khalda *et al.* (2013).

Molecular detection of mycoplasmosis from clinical specimens

Direct molecular detection of mycoplasmosis in 159 clinical specimens using MG and MS specific 16S rRNA PCR revealed 108 (67.92%) positive specimens for mycoplasmosis. Out of 108 PCR positive specimens, MG specific amplification was observed in 105 (66.04%) and MS specific amplification in 3 (1.86%) specimens with the

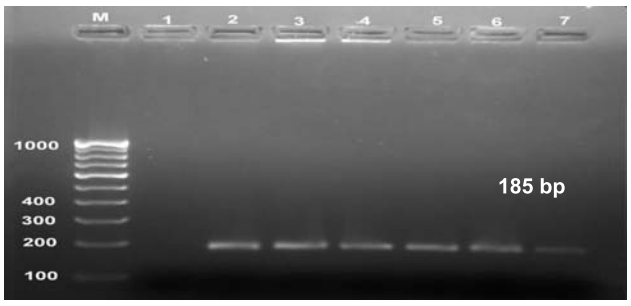


Fig. 3. *M. gallisepticum* species specific 16S rRNA PCR assay. Lane M, 100 bp DNA ladder; 1, Negative control; 2, MG (ATCC 25204); 3– 7, positive samples.

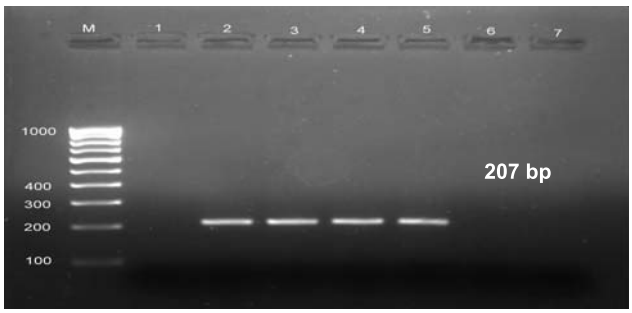


Fig.4. *M. synoviae* species specific 16S rRNA PCR assay. (Lane M, 100 bp DNA ladder; 1, negative control; 2, MS (ATCC 19610); 3–7, positive samples.

amplification product of 185 bp and 207 bp respectively (Figs 3, 4). All culturally MG positive specimens were also positive in MG specific PCR assay. Moreover, all MG PCR positive specimens were negative for MS and vice versa, indicating sensitivity and specificity of PCR assay in detecting and discriminating MG and MS infection directly in clinical specimens (Garcia *et al*. 1995, Kiss *et al*. 1997).

The comparative results of cultural isolation and direct 16S rRNA PCR assay of specimens showed 15 (9.43%) and 108 (67.92%) specimens respectively positive for mycoplasmosis. Cultural isolation and direct PCR yielded 9.43 and 66.03% MG positive cases respectively, whereas 1.89% specimens were found MS positive in PCR only. In both cultural and PCR methods, MG was the major pathogen involved in poultry mycoplasmosis (Behbahan *et al.* 2005, Domanska-Blicharz *et al.* 2008, Lysnyansky *et al.* 2005). In present study, PCR was found to be a superior technique for rapid, sensitive and specific detection of poultry mycoplasmosis directly in clinical specimens. The results of comparative efficacy of cultural isolation and PCR in detection of mycoplasmosis in present study are in accordance with Rauf *et al.* (2013) who reported isolation rate of 27.6% and PCR sensitivity as 68.94%.

Amongst 42 *E. coli* isolates recovered, 28 were found in association with MG-PCR positive cases, proving a major pathogen associated with poultry mycoplasmosis (Ongor *et al*. 2009, Nagalakshmi *et al.* 2013) and mycoplasmosis as reported to be multifactorial disease complex with other pathogens including *Escherichia coli* and *Haemophilus paragallinarum* (Jordan 1972, Kleven 1990, Naylor *et al*. 1992).

From the present research, it is concluded that species specific 16S rRNA PCR proved to be useful in discrimination and confirmation of field isolates of *Mycoplasma* spp. at species level. MG and MS species specific 16S rRNA PCR assay was sensitive and specific for diagnosis of poultry mycoplasmosis directly in clinical specimens with sensitivity rate of 67.92% in comparison with 9.43% sensitivity rate of cultural isolation. Comparative results of PCR and cultural isolation showed that PCR is superior to cultural isolation for crucial, rapid, specific and sensitive detection of poultry mycoplasmosis. MG was found to be prevalent than MS in respiratory form

of poultry mycoplasmosis, detected by both cultural isolation and PCR technique. *E. coli* was the major Gram negative bacteria in association with *Mycoplasma* spp. Further studies focusing on evaluation of PCR assays targeting various suitable genes on a larger numbers of field isolates will produce valuable data useful in molecular epidemiology for prevention and control of mycoplasma outbreaks.

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