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Development and Evaluation of Culture-Enhanced Tetra-PCR for Differential Diagnosis of *Mycoplasma gallisepticum* and *M. synoviae*

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ABSTRACT

Avian mycoplasmos is one of the economically significant disease in commercial poultry industry. This study aimed to develop and optimized a novel tPCR approach to detect *Mycoplasma gallispeticum*, *Mycoplasma synoviae* and nonpathogenic mycoplasmas at the same in a single PCR reaction collected from diseased chicken. Direct PCR from the clinical samples produced false negative results. Both culture and PCR were combined as culture-enhanced PCR approach. Three different DNA crude preparation methods were used and broth dilution method found simpler and most efficient for positive PCR. Primers were selected for 16s rRNA and it could detect up to 100 cfu and 250 cfu from MS and MG respectively in a samples. We have tested pure DNA of other mycoplasmas (5 species from avian origin and 3 other mycoplasmas species) but it produced only the genus specific band. The optimized ratio of tPCR primers were 1: 1: 10: 1. For Outer F, Inner R, and Inner F and Outer R, respectively. $MgCl_2$ concentration did not affect and added at 2.0 mM. Four different culture methods were compared for their efficiency for avian mycoplasmas culture. The B method (parts of trachea) were found most efficient for producing growth within 36 h while method A produced within 48 h nevertheless both had the same positivity (70%). While other two methods C (filtrate into broth) and D (filtrate onto agar) produced just 40% and 31%. Moreover, the C methods is the most time consuming method for growth production. The former two methods were superior in their productivity 30% and 39% over C and D methods, respectively. The tPCR approach could also be utilized for avian mcyoplasmas contamination on cell lines or suspect fertile eggs for virus vaccine production.

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Authors' Contribution
TAK and FM conceived, designed and executed the study and wrote the article. KF collected samples from field. UZ reviewed the article. AA supervised the work.

Key words
***Mycoplasma gallisepticum*, *Mycoplasma synoviae*, DNA, tPCR, Culture.**

INTRODUCTION

Avian mycoplasmosis is one of the most important bacterial respiratory diseases of the commercial poultry. There are 23 avian mycopalasma species known to prevail worldwide. Among them *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are mainly responsible for considerable economic losses. Both species exhibit variable clinical manifestations, asymptomatic to severe (Kleven, 2008). MG mainly affects respiratory and reproductive system of the poultry. The respiratory epithelial line is distorted which leads to inflammation and metaplasia that results in severe respiratory syndrome *i.e.,* chronic respiratory diseases (CRD) (Ley, 2003). MS causes subclinical infection in upper respiratory tract and also affect the synovial joints (Lockaby *et al*., 1999).

Microbial diagnosis employs three approaches: culturing, serological and molecular assays. Mycoplasmas culturing is considered as gold standard in avian mycoplasmosis diagnosis. However, it is not recommended in routine due to its fastidious nature and slow growth; sometime contaminants over grow and mask the growth of avian mycoplasma (Gracia *et al*., 2005). Serological analysis is more rapid and sensitive; but is easily misinterpreted due to cross-reaction between closely related species of mycoplasmas. Furthermore, serology can only be done after 1-3 week of post-infection which makes it a less effective tool for diagnosis and control (Feberwee *et al*., 2005).

Polymerase Chain Reaction (PCR) is well recognized and an important test for diagnosis as well as epidemiological studies. This has high sensitivity, specificity and rapidity (Ferguson *et al*., 2005). The 16s rRNA gene is the most significantly targeted gene for identification of mycoplasma, up to species level (Leuerman *et al*., 1993; Vitula *et al*., 2011). However, other targeted gene for PCR include 23s rRNA (Ramirez *et al*., 2006), *mgc2* (Gracia

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et al., 2005), and *vlh* gene (Hammonda *et al*., 2009). Moreover, modified PCR methods such as multiplex and nested PCR methods have also been employed for identification of mycoplasma (Reck *et al*., 2013; Mardassi *et al*., 2005). Multiplex PCR is cost-effective, specific and sensitive with higher throughput. Real time duplex PCR is also developed for diagnosis of mycoplasmas (Dijkman *et al*., 2013; Sprygin *et al*., 2010).

Direct PCR from clinical specimen produces false negative results due to undefined inhibitory substance (Molaic *et al*., 1998); false positive due to contamination during processing clinical samples (Ma, 1995). Alternatively, culture-enhanced PCR (CE-PCR) was introduced by Abele-Horn *et al*. (1998). The method allows enrichment of desired bacteria by diluting the inhibitors present in clinical samples and avoid the risk of false negative results associated with low number of mycoplasmas. This approach has successfully been developed for *Mycoplasmas pneumoniae* (Abele-Horn *et al*., 1998), genital mycoplasmas (Diaz-Gracia *et al*., 2004), *Mycobacteria* (Noussair *et al*., 2009) and *Actinobacillus* (Flemmig *et al*., 1995).

The tetra-PCR approach has been widely used for genotyping (Etlik *et al*., 2008) in human etc. However, in the present study, tetra-PCR with culture enhanced (CE) method was first time developed for avian mycoplasmas and the test efficacy for the identification and differentiation of avian mycoplasmas was evaluated.

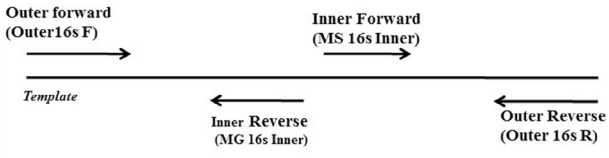

Fig. 1. Pictorial illustration of Tetra PCR mechanism.

MATERIALS AND METHODS

Mycoplasma culturing and isolation

A total of 70 field samples (trachea) from different commercial poultry farms (broilers, layers), submitted to clinical diagnostic laboratories suspected for avain mycoplasmosis, were cultured in mycoplasma broth based. All trachea samples were processed by four different culture techniques are: A) wetted swab was applied for swabbing on longitudinally open trachea and dipped into the broth and incubated for 2 h and then the swab was removed from broth. Broth was incubated till growth appears, B) tracheal pieces (from cranial, middle, and caudal thirds parts) incubated for 2 h in 3 ml broth and then tissue pieces discarded. Broth was incubated till growth appears, C) 200 µl Filtrate from minced trachea was added into broth. Broth was incubated till growth appears and D) 200 µl Filtrate from minced trachea were spread onto the surface of *Mycoplasma* modified Frey solid agar medium. After colonies development, piece of agar with positives clones (2 x 0.5 cm^2) was removed with sterile blade and transferred into 3 ml broth and incubated. Positive cultures were identified by tPCR.

After inoculation, 200 µl broth, from A and B each, samples were removed with different time intervals (4, 8, 12, 16, 24, 36, 48, 72, 96 h, or until color change or turbidity observed for DNA preparation and culturing on agar plates. Tracheal samples (n=10) collected from healthy birds (Culture and Seronegative) were also processed by the above four methods as negative control. All incubations were carried at 37ºC in humid environment. Prior to the experiments, the above culturing methods were optimized on clinical samples collected from experimentally inoculated chickens (Data not shown).

Primer designing

General and specie specific primers were designed from 16s were designed from 16s ribosomal DNA sequences (MG and MS). For specificity confirmation, the sequences were BLAST against NCBI database(http://blast.ncbi.nlm.nih.gov/Blast). For quality of sequence to be selected as primers were analyzed by Fast PCR tool (GC %, Linguastic complexity, Primer PCR efficiency, self-Dimerization, Hairpin structure etc.). The selected primers were synthesized from Integrated DNA technology (IDT), USA.

Optimization of PCR

For temperature Optima, gradient thermo cycler (Applied biosystem Veritti 96 well, USA) was used with temperature range of 56-66ºC. Tested annealing timings were 15, 30, 45, 60, 75, 90 sec. For optimization of conc. of $MgCl_2$, different concentrations *i.e.* 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.5, 3.75, 4.0 mM were evaluated. The evaluated Tetra PCR primer concentrations used were 20, 40, 80, 150, 300, 400 nM. Then the ratio of all primers was determined starting with equal ratios and up to 10 times difference in concentration between the outer and inner primers.

In a 25 volume, 5 µl PCR buffer (5x), 2.0 mM $MgCl_2$, 200 µM dNTPs, 1 U Taq DNA polymerase, 0.4 µM Inner F (MS), while other primers 0.04 µM (Outer F, Outer R and Inner R (MG) of each primer was used. 5ul template DNA and the final volume make up with nuclease free water.

Table I.- Tetra PCR primer sequences of MG and MS.

Primer name	Sequences	Position of sequence on 16s ribosomal DNA	Product size
Outer forward (UM16sF)	AAT-ACA-TAG-GTT-GCA-AGC-GTT-ATC	521-546	Outer F + Outer R=973 bps
Inner reverse (MG specific)	AAC-TGC-AGC-ACC-GAA-GTA-TTC-G-3'	830-855	Outer F + Inner R=334bps
Inner forward (MS specific)	ATG-ACT-AGT-TGA-TGG-AAA-CCA-TCG	812-839	Inner F + Outer R=682 bps
Outer reverse (UM16sR)	CCC-CAC-GTT-CTC-GTA-GGG	1477-1494	

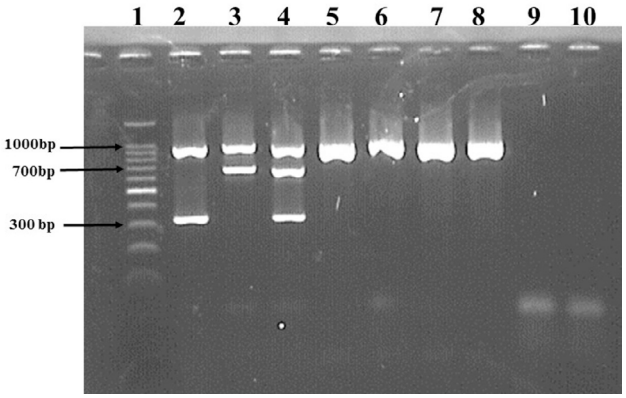

Fig. 2. Lane 1, *M. gallisepticum*; Lane 2, *M. synoviae*; Lane 3, both MG and MS; Lane 4, *M. gallinarium*; Lane 5, *Acholeplasma laidlawii*; Lane 6, *M. bovis* (ATCC 25230); Lane 7, *M. colloumbinsale* (field isolate); Lane 8, *Lactobacillus* spp.; Lane 9, *E. coli*; Lane 10, negative control.

Sensitivity determination

Tenfold dilution of the stock MG S6 strain culture, with an initial concentration of 3 x 10^7 colony-forming units (CFU)/ ml, were prepared up to 10^{-10} in Frey broth. One milliliter was taken from each dilution and centrifuged at 12000 x g for 5 min, and the pellets were suspended in 20 µl of sterile deionized water. Suspensions were transferred to thin-walled tube and heated at 100ºC for 10 min in heating block; 2 µl from this cell lysate was used as template in PCR.

Crude DNA preparation

We adopted three different methods for DNA extraction for the evaluation of their efficiency and rapidity from 200µl broth, removed from the broth culture samples. 1) Broth Dilution Method: broth culture were diluted to 1:5 or 1:10 in nuclease free water and heated in heating block at 100ºC for 10 min then centrifuged for 3 min at 12000 x g, supernatant was used as template, 2) PK and Triton x 100 method: 90µl Broth culture was pelleted at 12000 x g for 3 min and PK lysis and 0.5% Triton solution was added. Solution was incubated for 1 h and then heated up to 95ºC for 10 min (Raychilk *et al*., 1999) and 3) Alkaline lyses: 100 µl broth culture was centrifuged and resuspended in 15 µl alkaline lyses solution (0.25% SDS in 0.05N NaOH), solution was heated up at 95 ºC for 5 min and 90 µl water was added for neutralization (Sakenborg *et al*., 2006).

RESULTS

The optimized concentration of tPCR was 1: 1: 10: 1 (Outer F, Inner R, and Inner F and Outer R, respectively). $MgCl_2$ concentration did not show any affect and Optimum concentration was found to be 2.0 mM.

The overall positivity of A and B methods were the same for both MG (19) and MS (30) recovery. However, compared to MS most of the MG cultures were mixed with other non-glucose fermenter mycoplasmas (*M. gallinarium*).

Table II.- Strain of *Mycoplasma* used for the specificity evaluation of Tetra PCR.

Organisms	Strains
M. synoviae	WVU1853 (ATCC 25204)
M. gallisepticum	S6 (ATCC 19610)
M. gallinarium	KY077684)
M. gallinaceum	Field strain (KY077685)
M. iners	Field strain (KY077686)
Acholeplasma laidlewaii	Field strain (KY077687)
M. colloumbinsale	Field strain (KY077688)
M. bovis	ATCC 25523
A. oculi	Field strain
M. capriculum subsp. *capri*	Field strain
Salmonella enteriditis serovar enteriditis	Field strain
Lactobacillus spp.	Field strain

Table III.- Comparison of different culture methods for isolation of avian mycoplasmas from trachea.

Culture methods	Parameter	Time (h)								Total positivity
		12	24	36	48	72	96	168	336	
A	*Mycoplasma* (%)	29	78	91	100	100	100	100	100	70
	M. synoviae (n)	2	18	25	30	30	30	30	30	30
	M. gallisepticum (n)		12	17	19	19	19	19	19	19
B	*Mycoplasma* (%)	41	94	100	100	100	100	100	100	70
	M. synoviae (n)	6	25	30	30	30	30	30	30	30
	M. gallisepticum (n)	1	17	19	19	19	19	19	19	19
C	Mycoplasma (%)	0	6	26	40	50	67	75	81	42
	M. synoviae (n)	0	0	0	4	10	15	20	20	20
	M. gallisepticum (n)	0	0	0	1	4	5	7	9	9
D	*Mycoplasma* (%)	0	0	11	47	47	63	63	63	31
	M. synoviae (n)	0	0	0	0	0	11	11	11	11
	M. gallisepticum (n)	0	0	0	0	0	4	4	4	4

Table IV.- Comparison of conventional PCR and tetra PCR results with time interval.

Time interval (h)	PCR					PCR	
	Conventional		Tetra			Conventional	Tetra
	MS[a]	MG[b]	MS	MG	Mycoplasma	Total positive	Total positive
4	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
12	5	3	0	0	15	8	15
24	30	19	23	15	21	49	63
36	30	19	30	19	21	49	69
48	30	19	30	19	21	49	70

A, Lauerman (1993)16s rDNA based primer used; B, Garcia (2005) mgc2 based primer were used; Percentage indicated upon Fried-egg colony development that was streaked on agar medium upon relative color change or turbidity in the broth which either may be MG, MS or nonpathogenic ones. [2]*After typical fried-egg colony observation and confirmed by tPCR as MG and MS then Positivity percentage was calculated.

The B method comparatively produced growth more rapidly (at 36 h) than swabbing method (A) (at 48 h complete) but was somewhat laborious. Probably the swab may not absorbed enough material from the trachea. Samples with earlier color changed were mostly due to *M. gallinaceum* or turbidity due to *M. gallinarium* in both A and B as mostly single band (specific to genus) were observed in tetra PCR. The culture is not as much specific because sometime there is turbidity and color changed due to contamination of microorganism if the clinical samples not properly collected and shipped to laboratory. To avoid any contamination maximum concentration of antimicrobial agents (0.05 % thallium acetate and 0.1% Ampicillin).

Some samples were processed by C method produced growth even 9 days after inoculation. Though the nonpathogenic mycoplasma produced growth on agar medium after 18-24 h upon streaking but when processed by D method, growth was observed after 48 h without filtration. Moreover, there were higher chances of fungal and bacterial growth therefore we complemented the C and D method with filtration steps. The positivity percentage were very low 42% (20: MS and 9: MG) and 31% (11: MS, 4: MG) for C and D method, respectively. Nonpathogenic recovery were higher in C method (29) then in D method (28). The A and B method were more productive than C method (> 28%) and D method (>39%).

The growth percentage were calculated by colony counts as OD were found not reliable because of suspended materials carried on with tracheal swabs which hindered in calculating true OD of the samples while comparing with negative and positive control.

The four methods of culturing were optimized on confirmed clinical samples from experimentally inoculated birds (data not given here), then carried out the study on clinical tracheal samples submitted to veterinary diagnostic laboratory with complains of respiratory disturbance.

The Tetra PCR sensitivity compromised due to gradient concentration of all primers. Nevertheless when all primer were added in equal ratios, the sensitivity were

more than MG-mgc2 primer. The tPCR primer has the ability to produce the genus specific bands (980bp) that include both pathogenic and nonpathogenic mycoplasmas, it is the reasons of higher total positivity of tPCR.

Table V.- Comparison of different DNA preparation methods with culture.

Time interval (h)	Culture[a]	Different DNA preparation methods		
		Simple broth dilution[b]	PK+Triton X100[b]	Alkaline lyses[b]
0-8	0	0	0	0
10	20	0	0	0
12	50	50	20	30
24	80	70	50	50
36	100	100	100	100
48	100	100	100	100

A, the culture percentage calculated on the basis of turbidity and changed in broth color and produced fried colonies when inoculated onto agar medium; B, DNA preparation methods results were calculated on the basis of PCR result either genus specific or species band and presented as percentage.

The simple broth dilution method was higher in DNA yield and amplified by tPCR compared with other two methods. Though cultures were positive after 10 h of inoculation, none of the DNA preparation method produced template to be amplified by PCR. After 36 h, all DNA extraction methods yield 100%.

DISCUSSION

The direct PCR detection of avian mycoplasmas from field samples is vital for rapid diagnosis in breeding stocks, layers and broiler birds. However the direct detection has demerits of false negativity, first due to inhibitors carried within and secondly lower number of mycoplasmas number in clinical samples. There are many studies which have been carried out with direct clinical samples for mycoplasmas detection. During Cloacal swabs sampling used for *M. melagridis* detection, it has been found 40% inhibition of conventional PCR reaction in presence of Internal control (IC) in cocktail (Molaic *et al.*, 1998). Even Taqman real time PCR fidelity were compromised due to inhibitors carried with clinical samples in spite of DNA were extracted with commercial kits (Sprygin *et al*., 2010).

To overcome the false negativity due to lower number of microorganism, Pre-enrichment of the clinical samples result in increasing the quantity of targeted DNA template (Mardassi *et al*., 2005). Moreover, in a study (Lockabay *et al*., 1998) birds were experimentally inoculated with MS via eye drop and foot pad inoculation. The culture was reported sensitive over PCR because it had been found negative for viscera of food pad and lower respiratory tract from pool tissue samples because of lower number of mycoplasmas (100 cfu) to be detected by PCR.

To improve the efficiency of culture method we applied four different methods to be combined with PCR as CE-PCR. The A method which is routinely applied for the culturing of avian mycoplasmas in labs involved the swirling of swab in the media. Whereas, Zain and Bradbury (1995) reported upon quantitative analysis that up to 70% of mycoplasmas were still adhered to swab after swirling and were discarded. However we modified the protocol by retaining the swab for 90-120 min which resulted in a better yield, these results were in agreement to the findings of Zain and Bradbury (1995). Since several species of mycoplasmas reside in the same anatomical site and the pathogenic one may be present in very low number comparatively with commensal mycoplasmas species. Swabs could be retained in broth collected from respiratory tract and joints without incurring much contaminants (Zain and Bradbury, 1995). When we serially diluted the initial sample containing MG, the MG number considerably dilute out and the nonpathogenic mycoplasmas were got purified. So it were observed that lengthier the swab or biological sample in broth also get increases the chances of pathogenic recovery from field samples (Data not shown).

The B methods is not much common rather it has been used with filtration (0.45µ) steps for quantification of mycoplasmas on trachea during experiments in inoculated birds (Gates, *et al*., 2008). However, it was observed that A method require longer incubation period than culturing from direct tracheal pieces (method B). Similarly PCR from direct swabs from live birds produced none of positive from seropositive birds whereas tracheal samples collected by scraping the trachea from dead birds to remove complete epithelial to get enough mycoplasma cells to be detected by real-time PCR, produced 64% positivity (Carli and Eyigor, 2003). The method C (filtrate from minced trachea) is superior than both A and B in terms of culture purity of mycoplasmas (Cai *et al*., 2008). Moreover, direct culturing of agar medium provide increases chances of recovery of slow growing pathogenic strains and avoid the overgrowth of the saprophytic mycoplasmas as well (Dijkman *et al*., 2013). Nevertheless, it has very low productivity which may be due to some inhibitory substances released from the clinical specimen that did halt the growth of the mycoplasmas. The enzymes release from the tissue also one of the reason of the pH drop of the media. Dijkman *et al*. (2013) found just 16% sensitivity of direct plating compared with rPCR from synovial fluid. The filtration step (450 nm) considerably reduced the number of mycoplasma up to 2 $\log_{10}$ (if the initial number is 7 $\log_{10}$) (Bradbury, *et al*., 1993). One of the reason of lower positivity percentage of C and D methods.

The rapidity and accuracy of PCR method is highly

dependent on pre-PCR steps such as DNA preparation from the clinical samples. There are chances for false negative PCR results due to inhibitory substances when working with crude DNA preparation from clinical specimen rather purified DNA (Lantz *et al*., 2000). Three different crude DNA methods were evaluated for their efficiency. Simple broth dilution method being robust and high throughput in yield and was found superior to the other two methods being employed *i.e.* PK method and alkaline lysis method. Comparatively, it is also easiest for large number of sample DNA preparation. In this study we diluted the broth sample (1:5 or 1:10) before boiling because crude samples treated with proteinase K then heated to 95ºC may contained or produced some inhibitory substances. Moreover, upon dilution the template prepared by above three methods, Simple broth dilution method were superior. The PK method was not much productive reported in earlier studies because it was unable to overcome the inhibitors in spite of diluting (1:100) the DNA preparation (Molaic *et al*., 1998). Alkaline lyses method was next easier method with higher PCR productivity. Furthermore with serial dilutions to overwhelm the effect of inhibitors if there were, the PCR amplification product intensity was reduced which may due to dilution of the template but none of negative samples became positive. This result contradicts with the previous reports, where dilution has reduced the amount of inhibitors in the sample, thus produce a successful PCR (Al-Soud and Radstrom, 2001; Sprygin *et al*., 2010).

A variety of duplex and multiplex PCR protocol developed for the avian mycoplasmas which targeted two to four species of clinical significance. Moreover these technique could not as much of significance for other avian species diagnosis because they are many other mycoplasma species which cause mycoplasmosis. However they failed to detect in any 19 species of mycoplasmas. To detect all other species besides MG and MS in chicken we developed a tetra PCR approach. This approach made it easy to differentiate between pathogenic (MG, MS) and non-pathogenic mycoplasmas. The technique could be utilized in cell culture contamination utilized for avian origin virus proliferation and growth for research and vaccine preparation.

Primer concentration is the most critical parameter for Tetra PCR. Slight variation from optimized range leads towards unsuccessful PCR even if other parameters were efficiently optimized. As suggested ratio of outer-inner ratio (1:10) (Etlik *et al*., 2008) for tetra-primer found to not useful for because two bands were observed (Universal *Mycoplasma*; (975 bp) and *M. synoviae* (680 bp) while when the all used in equal ratio (1:1) contrast to two bands (Universal *Mycoplasma* 975 and *M. gallisetpicum*; 334 bp) have amplified. Then besides the outer primers the inner primer ratio individually evaluated (by 20, 40, 80, 150, 300 and 400 nM) and set up to 1:1:10:1 ratio (40, 40, 400 and 40 nM) (Outer F, Inner R, and Inner F and Outer R, respectively). By direct sequencing of the each individual amplified product the methodology were validated.

There is preferential amplification of outer primer compared to internal primer which may be due to secondary structures formation within target amplicon that make the later set of primers less efficacious. The problem was overcame by increasing the concentration of internal primers (Etlik *et al*., 2008). Moreover, the outer primers are genus specific while inner primers were specific for species. Since, commensals mycoplasmas also resides the upper respiratory tract with pathogenic one (Aviakin and Kleven, 1993), which is a major reason for preferential amplification of outer primers.

The detection level were 250 cfu and 100 cfu of MG and MS, respectively. The lower sensitivity due to lowest concentration of primer used in tetra PCR cocktail. While when used individual primer pair they have higher sensitivity level compared to reported primers.

PCR analysis of contaminated samples produces results because of its high specificity compared to culture. It has been reported that genus specific primer (van Kuppeveld *et al*., 1992) also amplified *E.coli* DNA produced positive results (Ongor *et al*., 2009). The genus specific primer developed in the study as Outer primer was specifically amplified the *Mycoplasma* from both avian (5 species) and bovine origin (3 species) during our study. The major demerit of PCR is it detects nucleic acid from both viable and nonviable mycoplasmas cells, whereas only alive cells should be considered as potential source of infection (Marois *et al*., 2000) by this CE-PCR approach also overcome this.

CONCLUSION

The PCR tool developed in the study has the potential to save veterinarian and researchers from loss of time to confirm the results within 24 h of non-conclusive culture test. Also direct samples provide result to reduced detection time, allow farmers to act quickly and prevent the spread of disease in flock or nearby flock. Our results indicated that primers based on 16s rDNA selected for the PCR assays with culture technique for screening of flocks is economically feasible approach in detection of MG and MS infections in Broiler and layers flocks which are important for control of the disease.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abele-Horn, M., Bushch, U., Nitschko, H., Jacobs, E., Bax, R., Pfaff, F., Scharffer, B. and Heesemann, J., 1998. Molecular approaches to diagnosis of pulmonary disease due to *Mycoplasma pneumoniae*. *J. clin. Microbiol*., **36**: 548-551.

Al-Soud, W.A. and Radstrom, P., 2001. Purification and characterization of PCR-inhibitory components in blood cells. *J. clin. Microbiol*., **39**: 485-493. https://doi.org/10.1128/JCM.39.2.485-493.2001

Avakian, A.P., Kleven, S.H. and Glisson, J.R., 1988. Evaluation of the specificity and sensitivity of two commercial enzyme-linked immunosorbent assay kits, the serum plate agglutination and the hemagglutination-inhibition test for the antibodies formed in response to *Mycoplasma gallisepticum*. *Avian Dis*., **32**: 262-272. https://doi.org/10.2307/1590813

Bradbury, J.M., Wahab, O.M., Yavari, S.A., Dupiellet, C.A. and Bow, J.M., 1993. *Mycoplasma imitans* sp. nov is related to *Mycoplasma gallisepticum* and found in birds. *Int. J. Sys. Bact*., **43**: 721-728. https://doi.org/10.1099/00207713-43-4-721

Cai, H.Y., Bell-Rogers, P., Parker, L., Ferencz, A. and Pozder, P., 2008. Development and field validation of a *Mycoplasma iowae* real-time polymerase chain reaction assay. J. *Vet. Diagn. Invest.,* **20**: 230-235. https://doi.org/10.1177/104063870802000216

Carli, K.T. and Eyigor, A.B., 2003. Real-time polymerase chain reaction for detection of *Mycoplasma gallisepticum* in chicken trachea. *Avian Dis*., **47**: 712-717. https://doi.org/10.1637/6041

Diaz-Garcia, F.J., Giono-Cerezo, S., Tapia, J.L., Flores-Medina, S., Lopez-Hurtado, M. and Guerra-Infante, F.M., 2004. Overnight enrichment culture improves PCR-based detection of genital mycoplasmas in urine samples. 11th ICID Abstract. *Int. J. Infect. Dis*., **8**: 130S.

Dijkman, R., Feberwee, A. and Landman, W.J.M., 2013. Validation of a previously developed quantitative polymerase chain reaction for the detection and quantification of *Mycoplasma synoviae* in chicken joint specimens. *Avian Pathol*., **42**: 100-107. https://doi.org/10.1080/03079457.2013.766669

Etilik, O., Koksal, V., Arican-Baris, S.T. and Baris, I., 2008. An improved tetra-primer PCR approach for the detection of the FGFR3G380R mutation responsible for achonroplasia. *Mol. Cell. Probes*., **22**: 71-75. https://doi.org/10.1016/j.mcp.2007.06.002

Feberwee, A., Mekkes, D.R., de Wit, J.J., Hartman, E.G. and Pijpers, A., 2005. Comparison of culture, PCR and different serological tests for detection of *Mycoplasma gallispticum* and *Mycoplasma synoviae* infection. *Avian Dis*., **49**: 260-268. https://doi.org/10.1637/7274-090804R

Ferguson, N.M., Hepp, D., Sun, S., Ikuta, N., Levisohn, S., Kleven, S.H. and Garcia, M., 2005. Use of molecular diversity by gene-targeted sequencing (GTS) and random amplified polymorphic (RAPD) DNA analysis for *Mycoplasma gallisepticum* epidemiology studies. *Microbiology*, **15**: 1883-1893. https://doi.org/10.1099/mic.0.27642-0

Flemmig, T.F., Rudiger, S., Hofmann, U., Schmidt, H., Plaschke, B., Stratz, A., Klaiber, B. and Karch, H., 1995. Identification of *Actinobacillus actinomycetemcomitans* in subgingival plaque by PCR. *J. clin. Microbiol*., **33**: 3102-3105.

Garcia, M., Ikuta, N., Levisohn, S. and Kleven, S.H., 2005. Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. *Avian Dis*., **49**: 125-132. https://doi.org/10.1637/7261-0812204R1

Gates, A.E., Frasca, S., Nyaoke, A., Gorton, T.S., Silbart, L.K. and Geary, S.J., 2008. Comparative assessment of a metabolically attenuated *Mycoplasma gallisepticum* mutant as a live vaccine for the prevention of avian respiratory mycoplasmosis. *Vaccine*, **26**: 2010-2019. https://doi.org/10.1016/j.vaccine.2008.02.010

Hammonda, P.P., Ramirez, A.S., Morrow, C.J. and Bradbury, J.M., 2009. Development and evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding vlhA and its value for strain typing. *Vet. Microbiol*., **136**: 61-68. https://doi.org/10.1016/j.vetmic.2008.10.011

Lantz, P.G., Al-soud, A.W., Knutsson, R., Hahn-Hangerdal, B. and Radstrom, P., 2000. Biotechnical use of polymerase chain reaction for microbiological analysis of biological samples. *Biotech. Annu. Rev*., **5**: 87-130. https://doi.org/10.1016/S1387-2656(00)05033-X

Lauerman, L.H., Hoerr, F.J., Sharpton, A.R., Shah, S.M. and van Santen, V.L., 1993. Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. *Avian Dis*., **37**: 829-834. https://doi.org/10.2307/1592037

Ley, D.H., 2003. *Mycoplasma gallisepticum* infection. In: *Disease of poultry* (eds. Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald and

D.E. Swayne). Iow State University Press, Ames, Iowa, USA, pp. 722-744.

Lockabay, S.B., Hoerr, F.J., Lauerman, L.H. and Kleven, S.H., 1998. Pathogenicity of *Mycoplasma synoviae* in broiler chickens. *Vet. Pathol*., **35**: 178-190. https://doi.org/10.1177/030098589803500303

Lockabay, S.B., Hoerr, F.J., Lauerman, L.H., Smith, B.F., Samoylov, A.M., Toivio-Kinnucan, M.A. and Kleven, S.H., 1999. Factors associated with virulence of *Mycoplasma synoviae*. *Avian Dis.,* **43**: 251-261. https://doi.org/10.2307/1592615

Kleven, S.H., 2008. Mycoplasmosis. In: *A laboratory manual for the isolation, identification, and characterization of avian pathogens* (eds. L.F. Zavala, D.E. Swayne, J.R. Glisson, J.E. Pearson, W.M. Reed, M.W. Jackwood and P.R. Woolcock). American Association of Avian Pathologists, Athens, GA, pp. 59-64.

Ma, T.S., 1995. Applications and limitation of polymerase chain reaction amplification. *Chest*, **108**: 1393-1404. https://doi.org/10.1378/chest.108.5.1393

Mardassi, B.B.A., Mohammed, R.B., Gueriri, I., Boughattas, S. and Malik, B., 2005. Duplex PCR to differentiate between *Mycoplasma synoviae* and *Mycoplasma gallisepticum* on the basis of conserved species-specific sequences of their hemagglutination genes. *J. clin. Microbiol*., **43**: 948-958. https://doi.org/10.1128/JCM.43.2.948-950.2005

Marois, C., Oufour-Gesbert, F. and Kempf, I., 2000. Detection of *Mycoplasma synoviae* in poultry environment samples by culture and polymerase chain reaction. *Vet. Microbiol*., **73**: 311-318. https://doi.org/10.1016/S0378-1135(00)00178-4

Molaic, P.Y., Gesbert, F. and Kempf, I., 1998. Utility of an internal control for evaluation of a *Mycoplasma melagridis* PCR test. *Vet. Microbiol*., **61**: 41-49. https://doi.org/10.1016/S0378-1135(98)00173-4

Noussair, L., Bert, F., Leflon-Guibout, V., Gayet, N. and Nicolas-Chanoine, M.H., 2009. Early Diagnosis of extrapulmonay tuberculosis by a new procedure combining broth culture and PCR. *J. clin. Microbiol*., **47**: 1452-1457. https://doi.org/10.1128/JCM.00066-09

Ongor, H., Kalin, R., Karahan, M., Cetinakaya, B. and Akan, M., 2009. Detection of *Mycoplasma* species in turkeys by culture and polymerase chain reaction. *Rev. Sci. Tech. Off. Int. Epiz*., **28**: 1103-1109. https://doi.org/10.20506/rst.28.3.1955

Ramirez, A.S., Naylor, C.J., Hammond, P.P. and Bradbury, J.M., 2006. Development and evaluation of a diagnosis PCR for *Mycoplasma synoviae* using primers located in the intergenic spacer region and the 23S rRNA gene. *Vet. Microbiol*., **118**: 76-82. https://doi.org/10.1016/j.vetmic.2006.06.021

Reck, C., Menin, A., Canever, M.F. and Miletti, L.C., 2013. Rapid detection of *Mycoplasma synoviae* and avian reovirus in clinical samples of poultry using multiplex PCR. *Avian Dis*., **57**: 220-224. https://doi.org/10.1637/10425-101712-Reg.1

Rychlik, I., van Kesteren, L., Cardove, L., Svestkova, A., Martinkova, R. and Sisak, F., 1999. Rapid detection of *Salmonella* in field samples by nested polymerase chain reaction. *Lett. appl. Microbiol*., **29**: 269-272. https://doi.org/10.1046/j.1365-2672.1999.00622.x

Sakenborg, T., Vicca, J., Butaye, P., Imberechts, H., Peeters, J., de Kruif, A., Haesebrouck, F. and Maes, D., 2006. A multiplex PCR to identify porcine mycoplasmas present in broth cultures. *Vet. Res. Commun*., **30**: 239-247. https://doi.org/10.1007/s11259-006-3226-3

Sprygin, A.V., Andreychuk, D.B., Kolotilov, A.N., Volkov, M.S., Runina, I.A., Mudrak, N.S., Borisov, A.V., Irza, V.N., Drygin, V.V. and Perevozchikova, N.A., 2010. Development of a duplex real-time Taqman PCR assay with an internal control for the detection of *Mycoplasma gallisepticum* and *Mycplasma synoviae* in clinical samples from commercial and backyard poultry. *Avian Pathol*., **39**: 99-109. https://doi.org/10.1080/03079451003604621

van Kuppeveld, F.J., van der Logt, J.T., Angulo, A.F., van Zoest, M.J., Quint, W.G., Niesters, H.G., Galama, J.M. and Melchers, W.J., 1992. Genus and species-specific identification of mycoplasmas by 16S rRNA amplification. *Appl. environ. Microbiol*., **58**: 2606-2615.

Vitula, F., Peckova, L., Bandouchova, H., Pohanka, M., Novotny, L., Jira, D., Kral, J., Ondracek, K., Oscikoval, J., Zendulkova, D., Rosenbergova, K., Treml, F. and Pikula, J., 2001. *Mycoplasma gallisepticum* infection in the grey patridge Perdix perdix: outbreak description, histopathology, biochemistry and antioxidant parameters. *BMC Vet. Res*., **7**: 34. https://doi.org/10.1186/1746-6148-7-34

Zain, Z.M. and Bradbury, J.M., 1995. The influence of type of swab and laboratory method on the recovery of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in broth medium. *Avian Pathol*., **24**: 707-716. https://doi.org/10.1080/03079459508419109