Indian Journal of Poultry Science (2024) 59(2): 171–175, Research Article
https://doi.org./10.56093/ijps.v59i2.165169

A multiplex PCR assay for simultaneous detection of common respiratory pathogens of poultry in Andhra Pradesh

NAGENDRA REDDY THOPIREDDY*, SREEDEVI BOLLINI AND VINOD KUMAR NAGARAM

Department of Veterinary Microbiology, College of Veterinary Science,
Sri Venkateswara Veterinary University, Tirupati-517502

(Received on February 23, 2024; accepted for publication on November 17, 2024)

ABSTRACT

Thopireddy, N.R., Bollini, S. and Nagaram, V.K. 2024. A Multiplex PCR assay for simultaneous detection of common respiratory pathogens of poultry in Andhra Pradesh. Indian Journal of Poultry Science, 59(2): 171-175.

The present study was undertaken to screen the common respiratory pathogens like avian *mycoplasmas* (*Mycoplasma gallisepticum* and *Mycoplasma synoviae*), *Infectious laryngotracheitis* virus (ILTV) and *Infectious bronchitis virus*(IBV) in poultry farms of Andhra Pradesh by developing suitable diagnostic tool i.e. Multiplex PCR assay for rapid detection and differentiation of these pathogens. Mixed respiratory infections are common in commercial poultry due to intensive rearing in large numbers. Among the respiratory pathogens avian *mycoplasmas*, IBV and ILTV are the major etiology causing significant economic losses to poultry industry. To reduce the losses, early detection and differentiation of common respiratory pathogens, the at a time detection and differentiation of common respiratory pathogens by application of multiplex PCR assays have been regularly used in poultry. In our study we standardized a multiplex PCR assay by targeting individual gene for each pathogen and used this test for rapid screening of above agents. 228 pooled cloacal swabs, 228 pooled nasal swabs, 228 pooled tracheal swabs samples from the ailing birds and 152 pooled tracheal tissues, 152 pooled lung tissues and 76 pooled oviduct samples from the dead birds were collected from the suspected 19 poultry farms in A.P. as labelled accordingly. For extraction of Nucleic acid the Trizol method was used. All nineteen pooled clinical samples and were subjected for PCR individually. A Multiplex PCR test was developed for in a single tube detection of all common four pathogens by choosing individual genes i.e., N gene for IBV, ICP4 gene for ILTV, 16S r RNA gene for MG and vlhA gene for MS. The results revealed that 10 farms (MG), 7 farms (MS), 5 farms (MG AND MS) and 2 farms were found to be positive for ILTV and none of them found to be positive for IBV. Among the 17 positive farms, 60 cloacal swabs were positive for MG, 120 tracheal swabs, 96 nasal swabs, 72 tracheal swabs, 64 lung tissues were found were found positive for MG, MS and 8 oviduct samples were found positive for MS. Among the 2 positive farms, twenty-four tracheal swabs and sixteen tracheal tissues were found positive for ILTV.

Keywords: Nucleic acid extraction, MG, MS, IBV, ILTV, MULTIPLEX PCR

INTRODUCTION

Indian poultry industry is one of the leading and regularly growing fields in egg and meat production and stands 3rd and 7th position in the world. As per census the Indian poultry population is 851.81 million (20th livestock census, 2019) with an overall growth rate of 16.81 per cent in total poultry population. The latest census also recorded an increase of 45.78 per cent and 4.5 per cent respectively in backyard and commercial poultry over the previous census. Andhra Pradesh is in number one position in egg production. As a top producer still demand and need target is their but to achieving need targets poultry is facing problems like diseases. Poultry industry and farmers are facing major problems like disease risk, production related problems, decreased feed conversion ratio and mortality problems. Because of evolution of pathogens and use of Live vaccines the re-emerging is common. The poultry industry facing more economic losses due to respiratory diseases which is responsible for *Mycoplasma*, *Infectious bronchitis virus* and *Infectious laryngotracheitis* virus. To control these diseases early detection and differentiation by using multiplex PCR assay and further carrying out standard strategies is helpful to reduce losses.

The continuous change in the pathogenicity of the respiratory pathogens may cause increased virulence. *Mycoplasma gallisepticum* is one of the respiratory pathogens affecting chicken and causes Chronic Respiratory Disease (CRD). The *Mycoplasma gallisepticum (*MG*)* pathogens, could modify their surface antigens, may help in persistency in their hosts. Persistent infections lead to entry of other pathogens like *E. coli*, as *E. coli* alone cannot invade the air sacs. *Mycoplasma synoviae(MS)* infections most commonly appears as subclincal and confined to upper respiratory and airsacs. MG and MS pathogens spread rapidly both vertically and horizontally via the eggs and contaminated fomites. MS and MG may cause Egg Apex Abnormalities (EAA) and joint infections along with other complications. Poultry farms infected with MS caused severe financial losses by reduced weight gain, poor conversion ratio, culling of lame birds due to synovitis, tremendous drop in egg production (20-30%), eggshell abnormalities and mortality. The percentage upto 25 depending on the stage of production and age of the birds. Once affected the flock were produced abnormal eggs

*Corresponding author Email: tnreddy32@gmail.com

throughout life. The economic impact resulted from eggshell breakage which parallel the occurrence of defective eggshell apices. The economic damage due to EAA was occurred at the farm level and at egg packing station due to breakage of eggs.

Avian infectious bronchitis is an acute, highly contagious upper respiratory tract viral disease in chickens. The IBV strains are causing substantial pathological damage to female reproductive tracts, delayed onset set of production, poor peak in egg production, high percentage of false layers and poor-quality eggs. The IBV infected chickens also showed upper respiratory tract signs, along with reproductive and urinary system involvement leading to economic losses to industry. The mortality can increase due to immunosuppression, Mycoplasmosis and other secondary bacterial infections caused by various accompanying infectious agents like *E. coli*, *Ornithobacterium rhinotracheale* and *Bordetella avium*. The mortality rates up to 20-30 percent or higher was recorded in 5 to 6 weeks of aged IBV infected chicken. During first month, 10 to 20 percent mortality was recorded due to IBV outbreak in broilers. Worldwide IBV is one of the main causes of respiratory disease leading to reduction in poultry production. In IBV endemic areas of poultry pockets, the mortality rate may be reaching up to 100 percent, while the mortality rates differ from 20 to 30 percent. More commonly mixed infections due to *Mycoplasma* and *E. coli*, up to 25 percent mortality was reported in young chicks. Rapid spread of IBV *via* direct and indirect contact occur with infected materials and the severity of infection is influenced by the agent, environment, and biosecurity. Different serotypes and genotypes of IBV identified worldwide and the cross protection among them is very minimum. Once IB affected, the birds becomes not return to normal production levels.

The ILT is a upper respiratory tract infection which is a contagious disease of poultry and annually causes impactful production losses worldwide. Virulent strains can cause highest mortality, recovered birds can result in latent carriers of the virus. The mortality levels may reach 70 percent and morbidity up to 100 percent and decreased growth rate in broiler chickens and egg production in hens are seen. In an ILT virus infected layer flock, recorded 30 % production loss was recorded. The maximum mortality was observed in 10-20 weeks age group (41%) followed by 20 to 30 weeks (35%) and the average mortality percent recorded in layer was 28.60 (Xie *et al.,* 2014). The disease occurs in densely populated poultry areas and increases susceptibility to other pathogens. The severe form is characterized by bloody mucus in the trachea with high mortality, while mild form shows nasal discharges, conjunctivitis, reduced weigh gain and egg production.

The respiratory tract of poultry is contains suitable environment for many pathogenic and non-pathogenic bacteria and viruses. In this environment the organisms interact each other e either synergistic or may be antagonistic and indicating the disease severity. These respiratory disease problems in poultry common due to rearing in intensive and closed environment. Multifactorial respiratory diseases were commonly caused by coinfection with bacteria (MG, MS, *E. coli* and *Avibacterium spp*.) and viruses, such as IBV and ILTV. Various studies reported that the mixed respiratory infections are common in poultry mainly due to IBV, ILTV, MG and MS (Thopireddy *et al.,* 2021; Reddy *et al.,* 2022; Thopireddy, 2023). All the pathogens may exihibit similar clinical signs and lesions leads to misdiagnosis, to avoid the complications in diagnosis accurate early diagnostic at a time detection and differentiation test is highly required for implementing control of mixed diseases.

To overcome in misdiagnosis of mixed respiratory infections this study was aimed to detect and differentiate the common respiratory pathogens of poultry in Andhra Pradesh (AP) with reference to Infectious bronchitis, Infectious larygotracheitis and *Mycoplasma* and to detect and differentiate the common respiratory pathogens by application of advanced molecular technique multiplex PCR.

MATERIALS AND METHODS

Positive controls

The Vaccines The infectious bronchitis and NDV combined vaccine (Nobilis H120), the modified live infectious laryngotracheitis vaccine (Merial), the *Mycoplasma gallisepticum*, live, freeze dried vaccine (Nobilis MG 6/85) (Intervet) and the *Mycoplasma synoviae* live vaccine (Bioproperties Pvt Ltd, (MS-H strain)) were used as positive controls.

Samples

Collected samples from suspected farms with history of vaccination against IB and RD in different poultry pockets of Andhra Pradesh. From a total of 19 suspected farms, a total pooled samples of all the poultry farms includes tracheal swabs (228), cloacal swabs (228), nasal swabs (228), trachea from dead birds (152), lungs from dead birds (152) and oviduct from dead birds (76) were labelled separately as per farm.

Nucleic acid extraction

MG-DNA and MS -DNA was extracted from the samples using TRIsol (TRIsoln of Genei, Banguluru) reagent (Baert *et al.,* 2007) and ILT- DNA was extracted from the samples using Wizard Genomic DNA purification kit (Promega, USA) reagent (Ahmed *et al.,* 2007).Viral RNA was extracted from the samples using TRIzol LS reagent (Ahmed *et al.,* 2007).The cDNA synthesis was done using QIAGEN kit.4.PCR:PCR

mixture optimised for the amplification of *Mycoplasma gallisepticum*(MG), Variable lipoprotein hemagglutinin A (*VlhA)*, IB N, and ICP4 genes contains template DNA of each 2 µL,forward primer of each gene 0.5 µL, reverse primer of each gene 0.5 µL, Emerald Amp GT PCR Master Mix 25 µL and Nuclease free water(NFW)8.5 µL. Optimised PCR conditions for all genes Initial Denaturation at 94°C for 3 min, Denaturation at 94°C for 1 min, Annealing at 55°C for1.5 min, extention at 72°C for 2 min and final extension 10 min total reaction kept for 35 cycles. Primers, IBV- *N* gene (Andreasen *et al.,* 1991)-F5'-TCATGGCAAGCGGTAAGG-3',R5'-TTCAGGTTAGCGGCTGGTC-3':*ICP4* gene –ILTV (Chacón and Ferreira, 2009)-F 5^1-CTTCAGACTCCAGCTCATCTG-3^1, R 5^1-GTCATGCGTCTATGGCGTTGAC-3^1: *vlhA* gene-MS (Thopireddy *et al.*,2023)-F5'-TACTATTAGCAGCTAGTGC-3', R5'-AGTAACCGATCCGCTTAAT-3'and*16S rRNA* gene-MG (Reddy *et al.*, 2022)- F5'-ACACCAGAGGCGAAGGCGAGG-3': R 5'-CGGATTTGCAACTGTTTGTATTGG-3'.

RESULTS AND DISCUSSION

Respiratory infections are common due to intensive rearing methods of poultry and causing significant economical losses to poultry industry worldwide. These respiratory pathogens most of the times result in mixed infections causing severe complications and mortality. Respiratory pathogens like Avian *mycoplasmas* including MS and MG, viruses like IBV and ILTV are common etiological agents in commercial poultry. These etiological agents have the capacity to cause disease individually and co-infection with one or more etiological agents. The mixed respiratory infections cause severe losses when compared to one etiological agent. To control the economic losses from mixed respiratory diseases, the early confirmatory diagnosis is required. The diagnosis based on the conventional methods are time consuming and costly, so the alternative methods as the molecular based diagnostic methods are more sensitive and rapid. For at a timely detection and differentiation of mixed respiratory infections, a multiplex PCR assay can be used. So, A multiplex PCR assay was developed, standardised and used for at a time detection and differentiation of common respiratory infections which includes MG, MS, IBV and ILTV by targeting individual genes for each pathogen (Fig1). The clinical signs including respiratory rales dyspnoea, sneezing, mucoid nasal discharges, swelling of lower eyelids, oedema of facial sub-cutis, growth retardation and lameness were observed during sample collection, similarly reported by Ley *et al.* (2003) and Ramadass *et al*. (2006).The clinical findings suggestive of respiratory complications like *Mycoplasma*, ILT and IB (Nagendra Reddy *et al*., 2022; Thopireddy *et al.,* 2023). Some of the birds in the affected farms also exhibited lameness due to joint infections and facial oedema suggestive of mycoplasma infections (Thopireddy *et al*., 2023). In this study the Multiplex PCR test was standardized for detection of MG, MS, IB and ILTV using specific primers targeting 16S rRNA, *vlhA*, N and ICP4 genes respectively and the optimised conditions were initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min 30 seconds and extension at 72°C for 2 Min and final extension step was at 72°C for 10 Min. A thirty-five cycle PCR with annealing temperature of 55°C for 1 min 30 seconds was found to be optimum for the amplification of 530 bp of MG, 350-400 bp of MS, 316 bp of IBV and 635bp of ILTV PCR product in positive DNA samples (Vaccines). The negative control kept in the reaction did not produce any amplification. Similarly, Thopireddy *et al*. (2021); Thopireddy *et al*. (2022) developed a multiplex SYBR Green Real time PCR assay for simultaneous detection of MG, MS,IBV and ILTV for rapid screening by targeting individual gene. Nguyen *et al*. (2023) used a similar type of multiplex assay for simultaneous detection of ILTV and ORT infections in respiratory infections of poultry. After screening by the multiplex PCR assay developed for at a time detection and differentiation of IBV, MS, MG and ILTV in a single test, it was seen that 10 farms were positive for MG, 7 for MS, 5 for MG and MS and 2 farms were positive for ILT infections based on the predicted size of product produced (Fig2, 3, 4). Among the 17 positive farms, 60 cloacal swabs were positive for MG,120 tracheal swabs, 96 nasal swabs, 72 tracheal swabs, 64 lung tissues were found were found positive for MG,MS and 8 oviduct samples were found positive for MS. Among the 2 positive farms, twenty-four tracheal swabs and sixteen tracheal tissues were found positive for ILTV. So multiplex PCR technique is useful for simultaneous detection of common respiratory infections in poultry farms. By using the results strict control

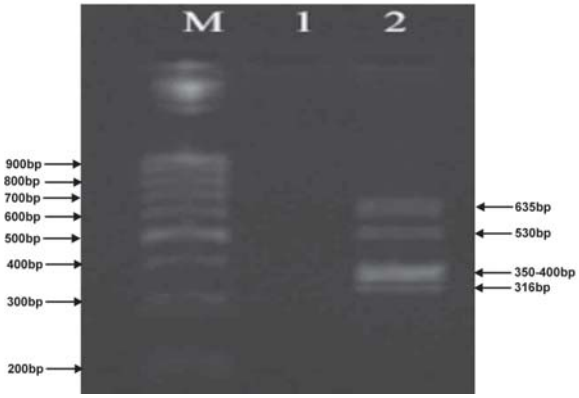

Fig. 1: Standardization of multiplex PCR for simultaneous detection of MG, MS, IB and ILTV
Lane M: 100bp ladder
Lane 1: Negative control (NFW)
Lane 2: All positive controls (ILT, MG, MS & IB Vaccines)

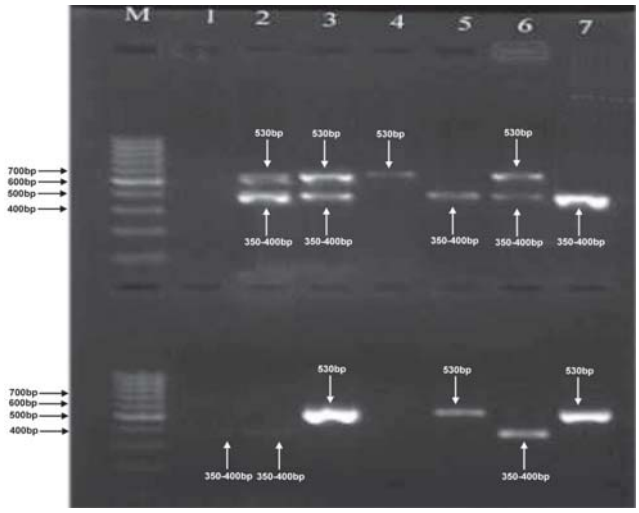

Fig. 2: Screening of field samples for MG, MS, IB and ILTV by Multiplex PCR

Bottom
M: 100bp ladder
Lane 1, 2, 6: Field sample (MS)
Lane 3, 5, 7: Field sample (MG)
Lane 4: Negative Field sample

Top:
M: 100bp ladder
Lane1: Negative sample
Lane 2, 6: Field sample (MG, MS)
Lane 3: Field sample (MG, MS)
Lane 4: Field sample (MG)
Lane 5, 7: Field sample (MS)

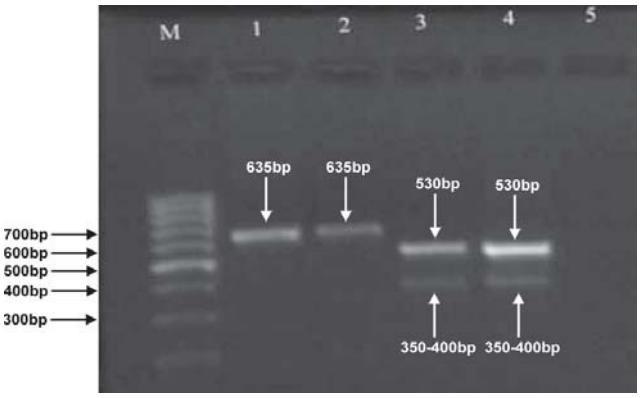

Fig. 3: Screening of field samples for MG, MS, ILT and IB by multiplex PCR
M: 100bp ladder
Lane1, 2: Positive sample (ILT)
Lane3, 4: Positive sample (MS, MG)
Lane5, 6, 7: Negative sample

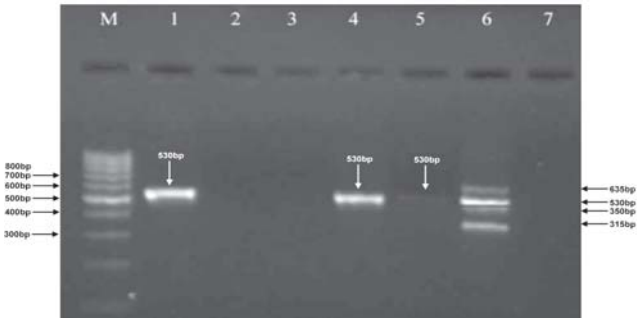

Fig. 4: Screening of MG, MS, ILT and IB by multiplex PCR
M: 100bp ladder
Lane1, 4, 5: Positive sample (MG)
Lane2, 3: Negative sample
Lane6: Positive control
Lane7: Negative control (NFW)

strategies can be implemented for the control of common respiratory infections. The multiplex PCR the test can be used as rapid diagnostic tool in poultry infections for confirmatory and quick diagnosis, similarly by Pang *et al.* (2002) ;Buim *et al.* (2009); Rashid *et al.* (2009); Xie *et al*. (2014); Mettifogo *et al*. (2015); Zhang *et al*.(2021);Nagendra Reddy Thopireddy *et al*.(2021);Thopireddy *et al*.(2022) were used to study the prevalence of respiratory bacterial and viral infections by Multiplex PCR. Our multiplex PCR approach that could able to identify and discriminate 4 main common respiratory pathogens that cause poultry illnesses, including ILTV, IBV, MG and MS with excellent specificity, sensitivity, and repeatability. As a result, preventative measures may be put in place as soon as feasible to minimize financial damage.

CONCLUSION

In poultry the involvement of more than one agent causing diseases are common and leads to production losses to reduce losses this multiplex PCR approach helpsin rapid detection and differentiation is possible to take necessary control measures. This multiplex PCR test is useful for field application for simultaneous detection and differentiation of MG, MS, IBV and ILTV.

REFERENCES

Ahmed, Z., Naeem, K. and Hameed, A. 2007. Detection and sero-prevalence of *Infectious bronchitis virus* strains in commercial poultry in Pakistan. *Poultry science*, **86**(7): 1329-1335.

Andreasen Jr, J.R., Jackwood, M.W. and Hilt, D.A. 1991. Polymerase chain reaction amplification of the genome of infectious bronchitis virus. *Avian diseases*, 216-220.

Baert, L., Uyttendaele, M. and Debevere, J. 2007. Evaluation of two viral extraction methods for the detection of human noroviruses in shellfish with conventional and real time reverse transcriptase PCR. *Letters in applied microbiology*, **44**(1): 106-111

Buim, M.R., Mettifogo, E., Timenetsky, J., Kleven, S., and Ferreira, A.J.P. 2009. Epidemiological survey on *Mycoplasma gallisepticum* and *M. synoviae* by multiplex PCR in commercial poultry. *Pesq. Vet. Bras*, **29**(7): 552-556.

Chacón, J.L. and Ferreira, A.J.P. 2009. Differentiation of field isolates and vaccine strains of *Infectious laryngotracheitis virus* by DNA sequencing. *Vaccine*, **27**(48): 6731-6738.

Ley, D.H. 2003. *Mycoplasma gallisepticum* infection. In: *Diseases of Poultry*, Saif Y.M. *(ed.), 11th edition, Iowa State Pres, USA*, 722-744.

Mettifogo, E., Buzinhani, M., Buim, M.R., Timenetsk, J., Ferreira, A. and Piantino, J. 2015. Evaluation of a PCR multiplex for detection and differentiation of *Mycoplasma synoviae*, *M. gallisepticum*, and *M. gallisepticum* strain F-vaccine. *Pesquisa Veterinária Brasileira*, **35:** 13-18.

Thopireddy, N.R., Sreedevi, B. and Nagaram, V.K. 2021. Simultaneous detection of mycoplasma gallisepticum, mycoplasma synoviae, infectious bronchitis virus and infectious laryngotracheitis virus in poultry by multiplex SYBR green Real Time PCR. *EAS Journal of Veterinary Medecine Science*, **3**(6): 72-76.

Nagendra Reddy, T., Sreedevi, B., Vinod Kumar, N., Srilatha, CH., Madhava Rao, T. 2022. Molecular detection of mycoplasma species from common respiratory infections of poultry in Andhra Pradesh. Research & Reviews: *Journal of Veterinary Science and Technology,* **11**: 4-8.

Nguyen, V.G., Cao, T.B.P., Le, V.T., Truong, H.T., Chu, T.T.H., Dang, H.A. and Huynh, T.M.L. 2023. A multiplex PCR method for simultaneous detection of *Infectious Laryngotracheitis Virus* and *Ornithobacterium rhinotracheale*. *Veterinary Sciences,* **10**(4): 272.

Pang, Y., Wang, H., Girshick, T., Xie, Z. and Khan, M.I. 2002. Development and application of a multiplex polymerase chain reaction for avian respiratory agents. *Avian Disease,* **46:** 691-699.

Ramadass, P., Ananthi, R., Senthil Kumar, T.M.A., Venkatesh, G., and Ramaswamy, V. 2006. Isolation and characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from poultry. *Indian Journal Animal Science,* **76:** 796-798.

Rashid, S., Naeem, K., Ahmed, Z., Saddique, N., Abbass, M.A. and Malik, S.A. 2009. Multiplex polymerase chain reaction for the detection and differentiation of avian influenza viruses and other poultry respiratory pathogens. *Poultry Science,* **88:** 2526-2531.

Reddy, T.N., Sreedevi, B. and Kumar, N.V. 2022. Molecular detection and differentiation of *Mycoplasma gallisepticum* field isolates of poultry in Andhra Pradesh. *Indian Journal of Poultry Science*, **57**(2): 181-186

Thopireddy, N.R. 2023. Isolation and molecular detection of *E.coli* from common respiratory infections of poultry in A.P. *EAS Journal of Veterinary Medecine Science,* **5**(1): 1-6.

Thopireddy, N.R., Bollini, S. and Nagaram, V.K. 2022. Multiplex SYBR green Real-Time PCR assay for detection of common respiratory pathogens of poultry. *Newest Updates in Agriculture and Veterinary Science,* **1**: 64-73.

Thopireddy, N.R., Bollini, S. and Nagaram, V.K. 2023. Comparative study of molecular variations in Mycoplasma synoviae field isolates from common respiratory infections of poultry in Andhra Pradesh. *Indian Journal of Poultry Science*, **58**(2): 159-163.

Thopireddy, N.R., Bollini, S., Nagaram, V.K., Srilatha, C.H. and Raot, M. 2022. Isolation and identification of Mycoplasma spp. from common respiratory infections of poultry in Andhra Pradesh. *Haryana Vet.,* **61**(2): 110-112.

Wang, H., Fadl, A.A. and Khan, M.I. 1997. Multiplex PCR for avian pathogenic mycoplasmas. *Molecular and Cell Probes,* **11:** 211-216.

Xie, Z., Luo, S., Xie, L., Liu, J., Pang, Y., Deng, X. and Khan, M.I. 2014. Simultaneous typing of nine avian respiratory pathogens using a novel GeXP analyzer-based multiplex PCR assay. *Journal of Virological Methods*, **207:** 188-195.

Zhang, Z., Wang, X., Wei, X., Zheng, S.W., Lenhart, B.J., Xu, P. and Liu, C. 2021. Multiplex quantitative detection of SARS-CoV-2 specific IgG and IgM antibodies based on DNA-assisted nanopore sensing. *Biosensors and Bioelectronics*, **181**: 113-134.