ORIGINAL RESEARCH PAPER

Veterinary Science

ISOLATION AND MOLECULAR IDENTIFICATION OF AVIBACTERIUM PARAGALLINARUM IN LAYERS BY USING SYBR GREEN REAL TIME PCR

KEY WORDS: *Avibacterium paragallinarum* ; chocolate agar; satellitism;SYBR Green real time PCR; Ct value

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ABSTRACT

Infectious coryza is an infectious disease, caused by *Avibacterium paragallinarum*, causing economic losses to commercial poultry Industry. The samples were collected from 6 commercial Infectious Coryza suspected layer farms includes, nasal swabs(119), ocular swabs(119) and tracheal swabs(119) were pooled farm wise and organ wise. On selective media the organism was produced satellite colonies after incubation of 48 hours at 37°c under anaerobic conditions. The organisms were showed positive test for sugar fermentation tests, negative for catalase test,Indole test and H2S production test. Further molecular confirmation was done by SYBR Green real time PCR by targeting the *HPG2* gene. In this study the Ct value below 35 was consider as positive cutoff for *Avibacterium paragallinarum*. All samples were screened by targeting HPG2 gene and found 100 percent positive for *Avibacterium paragallinarum*. To control the economic losses associated with the Infectious coryza, an early ,accurate and rapid diagnosis is essential. So the SYBR Green real time PCR is a rapid and highly sensitive technique which can substitute conventional methods. The antibiogram pattern revealed that the isolated pathogens were showed the sensitivity to enrofloxacin,ceftriaxone and amikacin. The diagnosis can be done by isolation of the pathogen on selective media and identification by biochemical tests, but less sensitive and time consuming. So the molecular detection based PCR tests are the most advanced, among the PCR tests, SYBR Green real time PCR test is the rapid, accurate and most sensitive test in detection of pathogens. Hence the present study was aimed that isolation and molecular detection of *Avibacterium paragallinarum* in commercial layers by using SYBR Green real time PCR by targeting *HPG2* gene.

INTRODUCTION

Infectious coryza is an acute, respiratory and contagious disease of several avian species, caused by *Avibacterium paragallinarum* (Blackall and Soriano-Vargas, 2013). The Infectious coryza is characterized by nasal discharge, facial swelling, sneezing, labored breathing and fetid smell of the exudates(Paudel, 2017). The disease occurs world wide and recovered birds becomes carriers which helps in spread of disease to healty flocks.The 10-40 per cent egg production loss and 10 per cent mortality in positive infectious coryza layers flocks was recorded by Paudel(2017). The economic losses can be controlled by rapid confirmarory diagnosis of the disease and inturn implementation of control strategies. In developing countries the diagnosis of infectious coryza is based on conventional methods such as clinical signs, demonstration of satellite colonies by culturing on selective media and confirmed by biochemical tests and conventional PCR (Corney,2008). However, the advanced molecular diagnostic tests like SYBR Green real time PCR test is an alternative in rapid and accurate confirmatory diagnosis of infectious coryza directly from clinical samples (Clotier *et al*.,2019). Hence the present study was taken for rapid detection of *Avibacterium paragallinarum* by SYBR Green real time PCR by targeting *HPG2* gene from the disease outbreak samples.

MATERIALS AND METHODS

Study area: The present research work was carried out on layer chickens from recent outbreaks in chittoor district of AndhraPradesh.

Collection of samples: A total of 6 poultrty farms, samples includes, 119 nasal swabs, 119 tracheal swabs,and 119 ocular swabs were collected pooled farmwise and organ wise from each suspected poultry farm near by Tirupati .The isolation and identification of pathogen was done by morphology, staining,cultural and biochemical properties. Age of the flocks was 45 to 73 weeks, isolated the organisms and sensitivity was studied with locally available antibiotics.

Isolation and identification of pathogen:

Cultural characteristics: Isolation of pathogen from suspected samples were carried out by culturing the samples on blood agar and chocolate agar plate, cross streaked with staphylococcus spp.(Wahyuni *et al* ., 2018).The inoculated plates were inubated at 37 °c under anaerobic conditions for 72 hours (Akhter *et al*., 2014). The identification of the bacterial agent from the pure culture were carried out based on the their colony characteristics,satellitism,and hemolytic patterns (Thenmozi *et al*.,2013).

Morphological characteristics: The colonies from pure culture were used for its morphological studies by grams method and capsular staining.

Biochemical characterization: Bichemical characterization was done by suger fermentation test, indol test,VP test, MR test, H2S production and motility tests for confirmation of pathogen.

Antibiotic sensitivity test:
Test was performed by using standard disc diffusion method.

Maintenance of stock culture:
Pure cultures were stored in 20% sterilised glycerin sealed with paraffin wax and stored at -80°c for future use.

Molecular detection tests:
Primers:*HPG*-2 Forward Primer 5′- GCAAAAGACT ACC AGCAAGGATAAT –3′) and reverse *HPG*-2 Primer 5′- CCTTACCCAAATATAATGTTCCACATT –3′) (Kuchipudi *et al.,*2021)

Nucleic acid extraction: DNA extraction was done by Trizol Method.

SYBR Green Real Time PCR:
The facilities in Department of Virology, SVIMS were utilized,

Aria Mx RealTime PCR machine, Maxima SYBR Green/ROX qPCR Master Mix(2x)(Thermo scientific) were used for this study.The Optimised SYBR Green Real Time PCR conditions for the amplification HPG2 gene was initial denaturation at 94°C for 3.59 mins, followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 min and final extension step was at 72°C for 10 mins.

RESULTS

Clinical signs: The clinical signs in infectious coryza affected flocks includes difficulty in breathing, dyspnoea, sneezing, severe oedema in the facial subcutis and eyelids, swollen infra orbital sinuses, nasal discharge, facial swelling, fetid smell of the exudates, mucoid nasal discharges with crest formation and dullness (Fig 1) was noticed in ailing birds.

Fig 1. showing swollen infra orbital sinuses, mucoid nasal discharges with crest formation and dullness was noticed in ailing birds.

Isolation and identification of *Avibacterium paragallinarum* from field samples:

Avibacterium paragallinarum organisms were isolated from the suspected field samples and identification was carried out by cultural, biochemical and molecular methods.

Isolation of *Avibacterium paragallinarum*

Out of 6 farm pooled samples, all 6 farms samples developed growth in nutrient broth media after 24 hours of incubation in anaerobic candle jar at 37°C with 90 per cent relative humidity . These cultures were plated on Blood agar medium and Chocolate agar medium streaked along with *Staphylococcus spp*. and were incubated in BOD incubator at 37°C with 90 per cent relative humidity for 72 hours in anaerobic candle jar to avoid drying of plates. After 2 days incubation, all 6 farm samples were grown on blood and chocolate agar media and showed characteristic satellite and non hemolytic colonies (**Fig 2**) .

Identification of *Avibacterium paragallinarum* by conventional methods:

Identification of *Avibacterium paragallinarum* was done based on colonial morphology on selective chocolate agar media, biochemical tests and molecular methods.

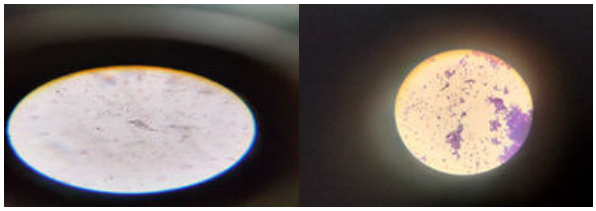
Colonial morphology:

Colonies of *Avibacterium paragallinarum* on chocolate agar medium revealed luxurious growth when streaked along with *Staphylococcus spp*. and which indicates characteristic satellitism of colonies and non hemolytic colonies on blood agar (**Fig 2, Table 1**). Grams staining of pure colonies revealed that gram negative coccobacillary organisms indicative of *Avibacterium paragallinarum*(**Fig 3)** and demonstrated the capsule with capsular staining(**Fig 3**)

Fig 2 Plates showing nonhemolytic pattern of growth on blood agar(A) and luxurious growth on chocolate agar(B)

A **B**

Fig 3 Showing gram negative pink color cocobacillary organisms(A,Grams staining) and capsulated organisms(B, Capsular staining)



A **B**

Table 1: Determination of V factor for the growth of *Avibacterium paragallinarum* by *Staphylococcus spp.*

Agar medium	Incubation period	Conditions	Colony morphology
Blood agar	after 48 hours of incubation	Anaerobic candle jar	Small dew drop like nonhemolytic colonies
chocolate agar	After 48 hours of incubation	Anaerobic candle jar	Luxuriantgrowth
chocolate agar cross streaked with *Staphylococcus spp*.	After 72 hours of incubation	Anaerobic candle jar	Satellite colonies

Biochemical tests:

Table 2: The following biochemical tests were used for confirmation of *Avibacterium paragallinarum*

Test	Result
Glucose	+
Sucrose	+
Lactose	+
Indol	-
Vp test	-
MR test	-
H2S production	-
Motility	-

Molecular detection of *Avibacterium paragallinarum* by SYBR Green Real Time PCR:

SYBR Green chemistry was used for detection of *Avibacterium paragallinarum* by Real Time PCR.

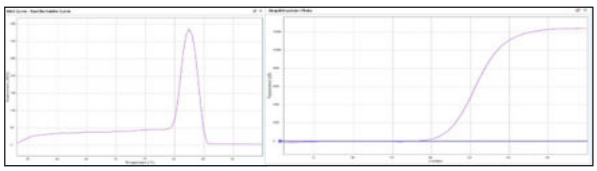
Standardization of SYBR Green Real time PCR for detection of *Avibacterium paragallinarum:* Real time PCR was standardized using primer specific for *HPG2* (HPG2 F and HPG2 R). The PCR was carried out in 25.0 µL volume in 0.2 mL PCR tubes. The PCR was standardised for different gradients of temperatures using Maxima SYBR Green-ROXq PCR Mastermix. Complementary DNA extracted from Nobilis Coryza Intervet vaccine was used as positive control and negative control was made up with nuclease free water. The reaction mixture was prepared by combining the following reagents in 25.0 µL volume comprising of 9.5 µL nuclease free water, 12.5 µL Maxima SYBR Green-ROXq PCR Mastermix, and 0.5µL (10 p*M*) of each of the forward and reverse primers (*HPG2* gene). Annealing temperature of 55°C for 45 sec and extension temperature 72°C for 1 min for HPG2gene gave optimal result (Fig 4).

SYBR Green Real time PCR was standardized for detection of using Infectious coryza specific primers targeting *HPG2* gene with varying degrees of temperature and time and optimized conditions were initial denaturation at 94°C for 3.59 mins, followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 min and final extension step was at 72°C for 10 mins.And the recorded ct values for *HPG2* gene was 18.42 and product Tm was 82.50(Fig 4).

Screening of field samples using SYBR Green Real Time PCR for detection of *Infectious coryza*

All the samples were screened for Infectious coryza by targeting *HPG2* gene and found that all 6 farms were positive for Infectious coryza infection. The amplification patterns were observed and the ct values ranged between 11.81to 30.28. The ct values directly correlates the concentration of DNA in that samples, high ct value indicates the low concentration of DNA and in NTCs there was no recorded ct value.The samples with less than 35 ct value were considered as positive and the recorded Tm values in positive samples ranged between 74.0 to 87.0 and the specificity was studied by using pox DNA.

Fig 4. Showing amplification plot and melt curve of HPG2 gene in SYBR Green real time PCR



DISCUSSION

The infectious coryza is one of the major economical disease to poultry industry.To control the disease, the proper isolation and identification is required for confirmation of disease. Hence the present study was aimed on isolation and identification of field isolate *Avibacterium paragallinarum* by using, morphology,cultural, biochemical and molecular based tests. In this study the ailing birds were showed the facial swelling, lacrimation, crust formation, nasal discharges, sneezing, fetid smell of the exudates and inflammation of infraorbital sinuses, similar symptoms was also recorded by Zhao *et al*.,(2010) and Atker *et al*.,(2016). We noticed the 30 per cent egg production was decreased in affected farms, similarly Zhang *et al*.,(2003) and Atker *et al*.,(2016) also recorded10-40 percent production loss. The recovered birds becomes permanent carriers and aids in the spread of the disease (Gallardo *et al*.,2020). The samples from all six affected flocks were processed and Out of 6 farm pooled samples, all 6 farms samples developed growth in nutrient broth media after 24 hours of incubation in anaerobic candle jar at 37°C with 90 per cent relative humidity . These cultures were plated on Blood agar medium and Chocolate agar medium streaked along with *Staphylococcus spp*. and were incubated in BOD incubator at 37°C with 90 per cent relative humidity for 48 hours in anaerobic candle jar to avoid drying of plates. After 3 days incubation, all 6 farm samples were grown on blood and chocolate agar media and showed characteristic satellite and non hemolytic colonies and gram negative pink color cocobacillary organisms(Grams staining) and capsulated organisms(Capsular staining)were observed.Our findings similar to observations recorded by Kridda *et al*., 2012 ; Falconi-Agapito *et al*., 2015; Jeong *et al*., 2017. The isolated organisms were showed positive for sugar fermentation test and negative for motility test similar to finding of Priya *et al*., 2012 and Akter *et al*., 2014. The antibiogram pattern showed the sensitivity to enrofloxacin, ceftriaxone and amikacin similar findings recorded by Thenmozhi *et al*.,2013.

The traditional methods like cultural and biochemical tests are time consuming and requires special media containing NAD for growth. A sensitive and specific polymerase chain reaction (PCR) was developed for identification of *Avibacterium paragallinarum* . In the recent years, real time PCR technique is being used for the diagnosis of infectious agents than the conventional PCR (Jarguin *et al*., 2009). More recently, a sensitive and rapid real-time PCR assay was developed for identification of pathogen (Ito *et al*.,2010). The fastidious nature of *Avibacterium paragallinarum* and the subsequent requirement for special media has made the isolation and identification of this organism a costly and laborious task. Furthermore, *Avibacterium paragallinarum* is a relatively slow-growing organism that can be easily overgrown by other contaminating bacteria that commonly inhabit the nasal and upper respiratory passages. Another approach for diagnosis of infectious coryza is direct PCR on clinical samples. *HPG*-2 gene targeting in PCR was developed for this purpose with very promising results, and in Australia, the DNA from presumptive *A. paragallinarum* cultures was tested in a previously conventional PCR (Chen *et al*.,1998 and a real-time PCR (Corney *et al*., 2008) found equivalent to culture . In developing countries problems like improper sampling, delayed transport, and poor quality of media could result in higher failure rates in isolation of pathogen. In contrast to culture, direct PCR can also be performed on swab samples that were stored at 4 °C or –20 °C for up to 180 days for Infectious coryza (chen *et al*.,1998), therefore, *HPG*-2 SYBR Green real time PCR has also proven to be very useful in these situations and Usage of antibiotics, especially sulfonamides, contamination can increase the failure rate of bacterial isolation (Erfan *et al*.,2019). In the present study direct SYBR Green real time PCR was applied to all clinical samples to overcome the stated issue and also isolated the organisms from samples and tested.

In this study the higher the ct value, lower is the concentration of DNA in the sample and vice versa. A ct value of ≤ 35 is considered as the cut off in real time PCR similarly Phillips *et al.* (2009) also taken same cut off value for detection of Rota viral infections by using Real time PCR.

CONCLUSION:

This SYBR Green realtime PCR assay provides a high-throughput detection method for chickens with respiratory disease and may provide more accurate and rapid detection needed for the characterization of the epidemiology of Infectious coryza in chicken populations. It can also serve as a screening method for birds prior to entry into a new flock as well as allow producers to initiate interventions, such as quarantine and vaccination, to limit the spread of disease.

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