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Molecular Characterization of Indigenous Isolates of *Avibacterium paragallinarum* and Media Optimization of its Growth for Vaccinal Seed Production

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Key words
***Avibacterium paragallinarum*, Avian infectious coryza, Brain heart infusion, Diagnostic tests**

ABSTRACT

The current study was conducted for isolation, identification, and rapid detection of *Avibacterium paragallinarum*, the causal agent of Infectious Coryza (IC), from layer chickens in various farms in Pakistan. A total of 46 isolates were obtained from a total of 244 clinical samples from 122 sick and moribund or dead layer chickens showing signs of IC like slight swelling of infra orbital sinuses and nostril mucus membranes. Two sampling sites were chosen, squeezing the nostrils for live birds and infra orbital sinus for moribund killed with later providing more live bacterial isolates. Isolation, biochemical identification, species-specific PCR tests and classical serotyping were performed. Two types of swab samples were used, one for direct PCR detection and other for the conventional diagnosis procedure. All isolates were identified as *Avibacterium paragallinarum* and required nicotinamide adenine dinucleotide phosphate (NAD) for their growth. The isolates were typical for *Av. paragallinarum* as they were not able to ferment neither galactose nor trehalose. Performing PCR of direct swab samples provided higher detection level as compared to the PCR from isolated colonies following conventional diagnostic procedure. In the current study, page serotyping demonstrated that all isolated *Av. paragallinarum* belonged to page serovars B. Among the media evaluated for growth of the isolated strain BHI/SN appeared to be the optimum media supporting the maximum growth rate and providing the maximum cfu count and the highest value of specific growth rate during the exponential phase of bacterial growth curve were obtained at 8 h. Thus the current study demonstrates that diagnostic approach using direct swab samples for the detection of *Av. paragallinarum* was found to be more accurate, rapid and sensitive than routine, culture-based PCR analysis and isolated *Av. paragallinarum* can be grown in BHI/SN to achieve maximum biomass, a trait required for vaccine production, using brain heart infusion broth media.

INTRODUCTION

Avibacterium paragallinarum previously known as *Haemophilus paragallinarum*, is the causative agent of a disease condition in chickens internationally called Infectious Coryza (IC) (Patil *et al*., 2017) but other avian specie are also known to be affected (Thenmozhi and Malmarugan, 2013). The site of action for the pathogen is primarily upper respiratory tracts of birds causing various symptoms such as nasal discharge, facial swelling and lacrimation. IC induces severe economic losses in the poultry sector causing increased culling, decreased production of eggs, death of young birds, and production of severe secondary bacterial infections (Blackall and Soriano-Vargas, 2019).

Avibacterium paragallinarum is a fastidious microorganism which needs complex nutrient media supplemented with blood serum and nicotinamide adenine dinucleotide phosphate for its growth, so a nurse culture, containing a bacterium which can produce NAD, such as *Staphylococcus aureus* or *Staphylococcus epidermidis* is required for cultivation of this bacterium on agar plates (Feberwee *et al*., 2019). However, NAD independent isolates having typical growth characteristics have also been described (Mangwani *et al*., 2014). The distinguishing features of *Av. paragallinarum* include lack of catalase and

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inability to ferment galactose and trehalose making the differentiation easy from rest of the species of the genus *Avibacterium* (Araya-Hidalgo *et al*., 2017).

For the detection and identification of *Av. paragallinarum* various techniques including conventional methods such as, selective plate culturing, biochemical identification, microscopy and modern molecular investigations using conventional and real-time PCR are usually performed (Chen *et al*., 1998; Corney *et al*., 2008). Furthermore, serotyping is also done for characterization of *Av. paragallinarum* isolates (Cabrera *et al*., 2011). The vaccination and strict biosecurity are the most appreciated control and preventive procedures against infectious *coryza* (Bragg, 2004). While trying to develop vaccine the most important and foremost step is the cultivation of required microorganism and for that purpose the selection of optimal growth media is imperative.

Preparation and selection of nutrient medium is of vital importance for the cultivation of microbial suspension. *Av. paragallinarum* being a very fastidious microorganism that requires complex nutrient media and sensitive cultivation conditions for optimal growth required for vaccine production. Rapid and sensitive detection is very important for the timely and effective control of the infectious agent in case of any disease. This current study reports the evaluation of diagnostic methods for *Av. paragallinarum* isolates obtained from outbreaks of infectious coryza in various farms in Pakistan. Isolation, biochemical identification, species-specific PCR tests and classical serotyping were performed. For rapid detection procedures authors have evaluated the PCR based detection using two different approaches, DNA extraction using direct swabs or using the cultures. In this study various media and media components have also been evaluated for the optimal growth of *Av. paragallinarum* strain isolated in outbreaks in Pakistan to be further used for autogenous vaccine production.

MATERIALS AND METHODS

Sample collection

For bacterial isolation, a total of 244 clinical samples were collected from respiratory tract of 122 sick and moribund or dead layer chickens showing signs of IC, like slight swelling of infra orbital sinuses and nostril mucus membranes. The sampling from chickens was done from various poultry farms in Pakistan, where IC outbreak occurred. Samples were collected from November 2016 to January 2017 and were brought to University diagnostic laboratory, UVAS, Lahore. Aseptically collected swab samples from swollen infraorbital sinus and tracheal swabs were used as laboratory specimens.

Isolation and purification of bacteria

Bacteriological samples obtained from nasal, tracheal and infraorbital sinus swabs of live and dead birds from various layer poultry farms with IC outbreaks were transported to the lab maintaining the cold chain. They were inoculated separately in glycerol-enriched phosphate buffered broth supplemented with NAD (final concentration 0.15 g/l) and on blood agar media which is cross streaked with *Staph. epidermidis*. *Staph. epidermidis* as nurse culture supplies NADH (V Factor). They were incubated anaerobically (candle jar method) at 37°C for 24 h to allow maintenance and growth of only NAD dependent organism as followed by (Byarugaba *et al*., 2007). Next day the enrichment culture from phosphate buffered broth (supplemented with NAD) was inoculated in blood agar media and cross streaked with *Staph. epidermidis* followed by incubation at reduced oxygen (candle jar method) at 37°C for 24 h as mentioned by (Byarugaba *et al*., 2007). Later on, plates were observed for typical tiny dewdrop colonies (Cabrera *et al*., 2011) to evaluate NAD-dependent or NAD-dependent colony growth. The dew drops like colonies showing satellite growth around feeder culture were further streaked onto BA/SN agar plates and blood agar plates cross streaked with *Staph. epidermidis*. BA/SN agar plates having composition; (Blood agar supplemented with 0.25 percent of 1 percent NADH (25 µg/mL medium), 1 percent of 0.05 percent thiamine HCL, 5 percent of oliec albumin complex and 1 percent filtered horse serum (serum was heat inactivated at 56 °C for 1 h) (Cabrera *et al*., 2011). Petri plates were kept in incubator at 5 percent CO_2 tension at 37 °C for 24 h. After purification, the isolated strain was processed for further analysis of microscopic and morphological characteristics.

Biochemical identification of bacteria

The isolated strains were tested for their ability to ferment various sugars such as sorbitol, sucrose, mannitol, maltose, D-glucose, L-arabinose, D-arabinose, lactose a nd Urease. Nitrate reduction tests was also performed. Other than this fermentation of galactose, trehalose and the presence of catalase was also tested (Feberwee *et al*., 2019).

Molecular diagnosis and characterization

For bacterial DNA extraction two types of approaches i.e., DNA extraction from swab preparation and DNA extraction from colonies grown on agar plates were adopted to test better quantification. It was ensured that the swabs should be free of blood. The swab samples taken from expressed mucus out of the nasal cavities were collected by gentle squeezing of the sinus of live birds. From killed chicken swabs were taken from mucus present

in sinuses of cut heads. Swabs were soaked in 400 µL phosphate buffer solutions (PBS) pH 7 for 1 h at room temperature in 1.5 mL microfuge tube followed by being vortexed and then centrifuged at 13000 rpm for 15 min. Supernatant was discarded and pellet was resuspended in 20 µL PBS or stored at -20 °C. Later on about 2 µL of solution was taken into 18 µL lysis buffer in a PCR tube (The lysis buffer would contain 880 µL H_2O, 100 µL Tris 1M at pH 8.3, 10 µL Proteinase K (20mg/mL), 5 µL Tween 20 and 5 µL NP40) and incubated at 56 ˚C for one hour followed by heating in a thermal cycler at 95°C for 10 min to inactivate proteinase K. The solution was held on ice for 10 min before being used in the PCR reaction (Chen *et al*., 1998).

In the second approach one loopful of isolated strain was suspended in 200 µL of PBS in a 0.6 mL PCR tube followed by being vortexed and then heated at 98 ˚C on heat block for 5 min. The mixture was later on centrifuged in a bench top microfuge at 13,000 rpm for 5 min. Supernatant was discarded and pellet was resuspended in 20 µL PBS or stored at -20 °C. The PCR test was performed with the whole sample. About 1µL was used as DNA template in 50 µL reaction (Chen *et al*., 1998). The quantification of genomic DNA was done using Nanodrop-2000 (Thermo Scientific®).

The genomic DNA extracted from the above-mentioned strategies were used as template in PCR amplifications of HPG-2 region of 16s rRNA gene of *Av. paragallinarum*, using bacterial primers, N1 (5' TGA GGG TAG TCT TGC ACG CGA AT 3') and R1 5' CAA GGT ATC GAT CGT CTC TCT ACT 3'). Each PCR reaction mixture was about 50 µL, which further contained about 31.75 µL milliQ water, 10x PCR Buffer (2 mM $MgCl_2$) 5 µL, dNTPs 8 µL, N1 primer 2 µL, R1 primer 2 µL, template 1 µL, Taq DNA polymerase 0.25 µL (1.25 units). The final concentration for each dNTPs was set at 200 µM in the reaction. The concentration of template DNA would be 100 ng/mL (Chen *et al*., 1998). The PCR reaction conditions were as follows: denaturation of DNA at 98°C for 2.5 min followed by amplification cycle with denaturation at 94°C for 1 min, annealing at 65°C for 45 s and extension at 72°C for 2 min. The reaction was further incubated at 72 °C for 7 min. All the collected samples we processed for DNA extraction and after confirmation through PCR we further propagated and stored isolates.

The 500 bp specific PCR products were separated on agarose gels and visualized using ethidium bromide (EtBr) staining to detect specific amplification. Gel was 0.7 percent agarose NA which contained ethidium bromide (6 µL of 10 x ethidium bromide in 120 mL of melted gel) in TAE buffer (75 µL of 10 x ethidium bromide in 1.5 L of TAE buffer in tank). The PCR products 10 µL were run with 2 µL loading buffer. The voltage of electrophoresis apparatus was set at 80 V and duration was about 30 min. Afterwards the products were visualized under UV light (Chen *et al*., 1998). A fully characterized isolate of *Av. paragallinarum* in this study was used as the positive control and a negative control without template in all of the PCR assays. A ladder of 100-bp was used to gauge the size of the product.

Serotyping of isolates

Serotyping of isolates was performed according to Page scheme using hemagglutination inhibition (HI) test. For this reference serum of *Av. paragallinarum* serovar A, serovar B and serovar C were used (Blackall and Soriano-Vargas, 2019).

Preparation of hemagglutinin antigen from TMB grown bacteria

Freshly grown *Av. paragallinarum* culture were inoculated into 100 mL of TMB supplemented broth medium and incubated overnight at 37°C. Broth was centrifuged twice at 4,000 g for 10 min and supernatant was discarded, and pellet was resuspended in 1 mL of PBS followed by microfugation for 2 min and pellet was suspended in PBS containing 0.01 percent thimerosal. Antigen was held at 4 °C for 3 days. In case the antigen didn't not show any HA activity, it was treated with hyaluronidase to show HA activity.

Hyaluronidase treatment

About 0.5 mL of antigen was taken and centrifuged at 4,000 g for 10 min with pellet resuspended in phosphate buffer pH 6.0 containing 100 units of hyaluronidase/ mL and incubated at 37°C for 2 h followed by washing twice in PBS. The pellet was resuspended in PBS containing 0.01 percent thimerosal to the original volume of antigen. The HA activity of the antigen was then tested (Blackall and Soriano-Vargas, 2019).

Preparation of chicken erythrocytes

Glutaraldehyde-fixed erythrocyte (GA-fixed RBC) suspension (30%) in distilled water added with 100 mg/ mL of thimerosal was prepared and kept in the dark at 4°C. When needed, this solution was diluted 1:30 in a working buffer consist of PBS (pH 7.2) with 0.1 percent of bovine serum albumin and 0.001 percent of gelatin.

Hemagglutinin antigen titration

Phosphate buffered saline solution supplemented with about 50 µL of PBSS, BSA 0.1 percent, and gelatin diluent 0.0001 percent was added in all wells of U bottomed microtiter plate. About 50 µL of HA antigen was added to

the wells. About 50 µL of 1 percent GA-RBCs was added to each well containing HA antigen. It was also added to the control well that contained only 50 µL of diluent. The plate was incubated at room temperature for 30-60 min. HA titer was read as the highest antigen dilution giving at least 50 percent HA activity. This titer would be divided by four. This would give HA antigen dilution that contains 4 HAs (Blackall and Soriano-Vargas, 2019).

Performance of hemagglutination inhibition test

HI test was performed using the three Page antisera-A, B and C as described below. About 1/100th dilution of each of three reference antisera were prepared in PBSS, BSA, and gelatin. For each antigen, doubling dilution of each antiserum across three wells (1/200th, 1/400th, 1/800th) was done in working buffer. About 50 µL of antigen (containing 4HAs) were added to all wells of the antiserum dilution. A control well was also set up which contained 50 µL of diluent and 50 µL of adjusted antigen. The plate was left at room temperature for 20 min. About 50 µL of 1 percent GA-fixed RBCs were added to all wells. Another control well was set up which contained 50 µL of red blood cells and 50 µL of diluent. HI titer was read. The HI titer was the most diluted antiserum that inhibits HA. The hemagglutinin serovar of each isolate was assigned according to the highest HI titer.

Nutrient medium selection and optimization of Av. paragallinarum *growth conditions*

One of the isolated strains *Avibacterium paragallinarum* Avpk 17 was used for optimization of media for obtaining maximum biomass. For cultivation of *Av. paragallinarum* the following media were used: (BHI/SN) brain heart infusion broth (Sigma Aldrich, Germany) supplemented with 1 percent horse inactivated serum (Sigma Aldrich, Germany), Oliac albumin complex and thiamin (Fisher Scientific, USA) and 0.0025 percent NADH (Fisher Scientific, USA); BHI/SN 0.01: (BHI/SN 0.01) brain heart infusion broth supplemented with 1 percent horse inactivated serum, Oliac albumin complex and Thiamin and 0.005 percent NADH; BA/SN 0.005 percent: (BHI/SN 0.005) brain heart infusion broth supplemented with 1% horse inactivated serum, Oliac albumin complex and thiamin and 0.005% NADH. For the three compositions of the media under consideration, separate flasks of brain heart infusion broth supplemented with the above-mentioned ingredients for each composition were prepared in triplicates. To prepare chocolate brain heart infusion (CBHI) media about 3.9 g of media was taken into 100 mL of distilled water in a 250 mL Erlenmeyer flask and autoclaved at 121°C for 20 min under 15 lbs pressure. After the autoclave it is allowed to cool at 40°C. About 7 mL of lyzed horse blood was added into it. Lysed equine blood is prepared by holding fresh equine blood at 56°C for 40 min with occasional stirring. The lysed horse blood is held at –20°C. This all is done in laminar flow hood. To prepare CASMAN/SN media, Casman Broth Base M766 (Thomas Scientific, USA) was supplemented with 1 percent horse inactivated serum, Oliac albumin complex and thiamin and 0.0025 percent NADH. To prepare CBHI/IsS (CBHI, Serum, and Isovitalex (Sigma Aldrich, Germany) chocolate brain heart infusion broth was supplemented with hemoglobin powder 1 percent, IsoVitaleX enrichment 1 percent, chicken serum 1 percent. Bacteria were cultivated in a liquid nutrient medium in conical flasks in an orbital shaker-incubator at 150 rpm for 24 h at 37 °C under elevated carbon dioxide conditions (5 percent). After the incubation, bacterial growth was measured after every 4 h interval that is at 4, 8, 12, 16, 20, and 24 h by plate count method by using serial dilutions of the broth culture. All the experiments were done in triplicates. BA/SN agar supplemented with 0.0025 percent NADH and 1 percent horse serum was used as solid agar in plate count method and cfu/mL was estimated.

Statistical analysis

To evaluate statistical significance of various parameters studied in the current study, analysis of variance (one-way ANOVA), and Tukey's HSD (honestly significant difference) test were performed using SPSS Inc. version 26 (IBM Corp., NY, USA) and p-value set at <0.05 was considered as statistically significant. Data are reported as mean and standard deviations.

RESULTS AND DISCUSSION

A total of 244 clinical samples were collected from respiratory tract of 122 sick and moribund or dead layer chickens showing signs of IC like slight swelling of infra orbital sinuses and nostril mucus membranes. Aseptically collected Swab samples from swollen infraorbital sinus and tracheal swabs were used as laboratory specimens. From a total of 103 live layers chickens, about 35 *Avibacterium* isolates were obtained, while out of 19 killed moribund chicken layers, about 11 *Avibacterium* isolates were obtained. From this study it could be mentioned that respiratory tract is the main source of sample to be collected and both sick and moribund or dead layer chickens can be used for sampling, in this study the percentage of obtaining isolates was more in dead layer chickens. All isolates required NAD for their growth. Bacteria were stained as gram negative and showed rod shaped appearance and were suspected as *Av. paragallinarum* (Kaur *et al*., 2004). About 46 isolates were tested biochemically and all

isolates were catalase and indole negative and fermented glucose, sucrose, maltose and mannitol and produced acid. The isolates were typical for *Av. paragallinarum* as they were not able to ferment neither galactose nor trehalose. The lack of ability to ferment both galactose and trehalose and lack of catalase differentiates *Av. paragallinarum* from other species of the *Avibacterium* genus (Blackall and Soriano-Vargas, 2019).

The blood agar media with and without NADH (V Factor) and in the presence or absence of *Staphylococcus epidermidis* as nurse culture was used in isolation and it was revealed that the organism grew more frequently with typical dewdrop colonies when cultured with BA containing both NADH and feeder organism. These findings are in complete agreement with previous studies from literature (Akter *et al*., 2014; Feberwee *et al*., 2019).

As reported in literature, a species-specific PCR has been developed for rapid and accurate detection of the *Av. paragallinarum* by (Chen *et al*., 1996). Based on this a PCR assay targeting a smaller region of the gene called HPG-2 of *Av. paragallinarum* was developed in 2008 (Corney *et al*., 2008).

For the molecular detection of *Av. paragallinarum* two approaches were used to test better quantification i.e. DNA extraction from direct swab preparations and DNA extraction from bacterial colonies. It was ensured that the swabs should be free of blood. The swab samples taken from expressed mucus out of the nasal cavities were collected by gentle squeezing of the sinus of live birds. From killed chicken swabs were taken from mucus present in sinuses of cut heads. From live layers 35 isolates were confirmed by PCR using DNA extracted from bacterial culture and 57 isolates were confirmed by using Direct PCR on swab samples. While in case of killed moribund layers 11 isolates were confirmed by PCR using DNA extracted from bacterial culture and 17 isolates were confirmed by using Direct PCR on swab samples. Statistically, there was no significant difference in prevalence of *Av. paragallinarum* isolates from live layers ($p > 0.05$) and killed moribund layers among different farms ($p > 0.05$) Tables I and II.

There was a significant difference in detection level of *Av. paragallinarum* from live and killed moribund layers among various farms by using different PCR techniques. As detection level was higher while doing PCR from direct swab samples as compared to PCR from isolated colonies ($p < 0.05$).

This approach using direct swab samples for the detection of *Av. paragallinarum* was found to be more accurate, rapid and sensitive than routine, culture-based PCR analysis (Towner et al., 1998). There are previous

Table I. Detection of *Av. paragallinarum* from live layers among different farms in Pakistan.

Farm No.	No. of samples	No. of *Av. paragallinarum* detected by PCR on extracted DNA from culture	Detection of *Av. paragallinarum* by direct PCR on swab samples	Serotyping by HI test
1	26	7	11	B
2	18	9	14	B
3	22	5	9	B
4	20	8	13	B
5	17	6	10	B
Total	103	35 ^{ns}	57 ^{ns}	B ^{ns}

*Represent a significant difference in detection of *Av. paragallinarum* isolates among different farms (P< 0.05), ns: no significance difference (P <0.05).

Table II. Detection of *Av. paragallinarum* from killed moribund layers among different farms in Pakistan.

Farm No.	No. of samples	No. of *Av. paragallinarum* detected by PCR on extracted DNA from culture	Direct PCR confirmed isolates from swab samples	Serotyping by HI test
1	3	1	3	B
2	5	3	5	B
3	4	2	2	B
4	4	4	4	B
5	3	1	3	B
Total	19	11 ^{ns}	17 ^{ns}	B ^{ns}

*Represent a significant difference in detection of *Av. paragallinarum* isolates among different farms (P< 0.05), ns: no significance difference (P <0.05).

reports for the rapid detection of bacteria with similar recommendations (Louie *et al*., 2002; Shrestha *et al*., 2003). Statistically speaking, there was no significant difference in detection of *Av. paragallinarum* by direct PCR technique from swabs of live layers ($p > 0.05$) and killed moribund layer ($p > 0.05$) among different farms in Pakistan.

There was significant difference in detection of *Av. paragallinarum* by culture-PCR technique (on suspected colonies) and direct PCR technique on swabs from live layers ($p < 0.05$) and killed moribund layers ($p < 0.05$). The eventual goal was to rapidly detect *Av. paragallinarum* from swab samples; we found that using PCR from direct swab samples was a sensitive, accurate, and rapid strategy for detection of *Av. paragallinarum* from direct surveillance swab specimens.

To determine the efficacy of vaccine, especially in case of vaccine failures, serotyping is very crucial. For *Av. paragallinarum* serotyping is usually based on the Page and Kume serotyping schemes. The Page scheme uses agglutination test and identifies three serovars A, B and serovar C (Page, 1962) while the Kume scheme uses haemagglutination inhibition and identifies nine serovars and three serogroups (I, II and III). The three Page serogroups correspond with the three Kume serogroups (Blackall and Soriano-Vargas, 2019). According to the cross-protection studies there is a good correlation between serovar and immunotype and usually vaccine failures is reported due to immunologically different isolates. In one of the reports by (Terzolo *et al*., 1997). Vaccine failures are reported to happen due to the presence of serovar B isolates, immunologically diverse from other known serovar B isolates. In the current study Page serotyping demonstrated that all isolated *Av. paragallinarum* belonged to Kume serovars B.

The main reason for determining the optimal conditions for the cultivation of *Av. paragallinarum* was to achieve the maximum biomass concentration. Selection of optimum nutrient medium was based on the evaluation of *Av. paragallinarum* growth kinetics on media of varying concentrations. As Figure 1 shows, among the media BHI/SN appeared to be the optimum media supporting the maximum growth rate and providing the maximum cfu count and the highest value of specific growth rate during the exponential phase of bacterial growth curve were obtained at 8 h after which a gradual decline in the growth rate was observed, there was significant difference in the cfu count at different time points ($p > 0.05$). Followed by this BHI/SN 0.005 percent, BHI/SN 0.01 percent and CBHI supplemented with Isovitalax, serum with dry Hb powder appeared to be the optimum media supporting the maximum growth rate and providing the maximum cfu count and the highest value of specific growth rate during the exponential phase of bacterial growth curve were obtained at 12 h after which a gradual decline in the growth rate was observed. There was significant difference in growth rate of *Av. paragallinarum* in different media used ($p < 0.05$).

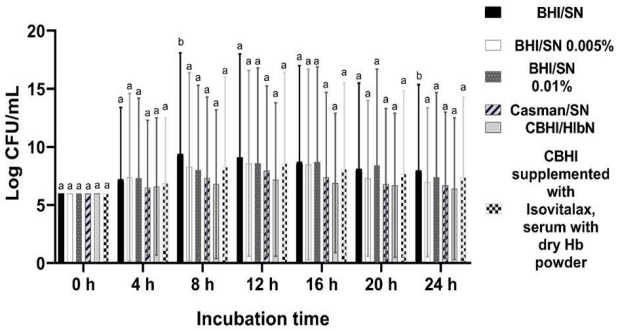

Fig. 1. Growth rate of *A. paragallinarum* at different incubation hours in various media. Results were the average of triplicate trials (mean ± SEM). Superscripts with different letters at the same incubation hour represent a significant difference in CFU between various media ($p < 0.05$).

Brain heart infusion broth is considered as one of the most widely used media for the growth of fastidious microorganisms. There are numerous reports in the literature for the use of BHI broth for the growth of *Av. Paragallinarum* (Terzolo *et al*., 1997; Feberwee *et al*., 2019). So the current study demonstrates that isolated *Av. paragallinarum* can be grown in BHI/SN to achieve maximum biomass, a trait required for vaccine production, using brain heart infusion broth media.

CONCLUSION

The current study found that using PCR from direct swab samples was a sensitive, accurate, and rapid strategy for detection of *Av. paragallinarum* from direct surveillance swab specimens.

In the current study the optimal nutrient medium for *Av. paragallinarum* cultivation was evaluated and it is demonstrated that isolated *Av. paragallinarum* can be grown in BHI/SN to achieve maximum biomass, a trait required for vaccine production, using brain heart infusion broth media.

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IRB approval

The study was approved by Advanced Studies and Research Board (ASRB), UVAS (DAS/4917-230216).

Ethics statement

During the samples collection, animals were handled according to the approved guidelines provided by University of veterinary and animal sciences, Lahore ethical institutional review board.

Statement of conflict of interest

The authors have declared no conflict of interest.

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