



Assessment of antigenic specificity of polyclonal antisera raised against *Avibacterium paragallinarum* by ELISA

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ABSTRACT

Lack of availability of commercial antibodies against whole-cell antigen or an antigenic epitope of *Avibacterium paragallinarum* (*Av. paragallinarum*) has hindered the development of novel immunoassays for the diagnosis of infectious coryza (IC). In this study, we raised polyclonal antisera against *Av. paragallinarum* and evaluated its antigenic-specificity using enzyme linked immunosorbent assay (ELISA). We standardized antigen coating concentration(s), antibody detection limit, and optimal range of dilutions of primary antisera and secondary conjugated antibody. Our results show the development of antigen-specific antibody response in rabbits following repeated antigenic exposure with 0.5% formalinized antigen over a period of four weeks. Further, we showed its possible applicability in detection of pathogens in tissues by immunohistochemistry for confirmatory disease diagnosis and disease pathogenetic study.

Introduction

Avibacterium paragallinarum (*Av. paragallinarum*), the etiologic agent of Infectious Coryza (IC) in chicken is widely prevalent in Indian poultry operations (Rajurkar *et al* 2009; Patil *et al.*, 2017). The pathogen primarily affects upper respiratory tracts of birds causing discomfort accompanied with nasal discharge, facial swelling and lacrimation, especially in intensive poultry rearing operations. IC causes significant economic losses to poultry industry as it leads to reduced egg production, culling of young birds, and increased risk of serious respiratory disease conditions in chicks by secondary bacterial infections resulting in pneumonia and air sacculitis (Blackall, 1999; Blackall and Soriano 2008). The disease has also been reported to affect other avian species than chicken (Priya *et al.*, 2012, Thenmozhi *et al.*, 2013) with an initial settlement across the nasal passages during naturally acquired infection. The presence of infection in flock is largely determined by estimation of haemeagglutination inhibition (HI) antibody titer (Blackall, 1995). Intriguingly, the pathogen *Av. paragallinarum* has a distinction of possessing varied genetic combinations, which accounts for poor cross immune protection among flocks when infected with varied serotypes (Terzolo *et al.*, 1997). The use of polyclonal antisera in the plate agglutination test is one of the traditional methods used to identify the patho-serotype involved in a particular outbreak

(Page 1962; Kume *et al.*, 1983), thus only allowing its restricted usage to pathogen's subtypes detection (Bragg *et al.*, 1996; Terzolo *et al.*, 1997; Blackall, 1999). Contrary to its conventional use, we have given an attempt to use polyclonal antisera for immunolocalization studies of *Av. paragallinarum* across the upper respiratory tract of birds by immunohistochemistry and obtained some novel information about the migration pattern of the pathogen through nasal passages and turbinates (Balouria *et al.*, 2018). Therefore, with the objective to establish the specificity and the antigenic avidity of the used polyclonal antisera, enzyme linked immunosorbent assay (ELISA) was developed. ELISA is one of the most sensitive and specific laboratory tests among various immunological assays (Tizard 1996; Beck and Rice, 2003). Since ELISA is largely employed for screening of antibody specificity in commercial production and is immensely utilized to identify specific infections or immunological status of ongoing clinical infections (Barrette *et al.*, 2006), we therefore standardized this method to test the specificity of the polyclonal sera. We aim to use this ELISA protocol in our future studies to measure infection-induced mucosal and systemic immune responses and to verify the vaccination status/ frequency of bird flocks as an optimized method other than Haemeagglutination and Haemeagglutination inhibition test (HA-HI test).

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2. Materials and Methods

2.1. Bacterial culture

Avibacterium paragallinarum isolate was obtained from Poultry Diagnostic and Research Centre, Venkateshwara Hatcheries India (Pvt) Ltd, Pune, India. The organism was grown in *Haemophilus* test medium broth and agar plate (Hi Media Laboratories Pvt. Ltd, Mumbai, India) supplemented with 1% (v/v) filter-sterilized, heat-inactivated horse serum and 0.0025% (w/v) reduced nicotinamide adenine dinucleotide for the maintenance and propagation of bacterial culture. Additionally, 10% sheep blood agar with *Staphylococcus aureus* was used as a feeder culture (Quinn et al., 1994) to evaluate the purity of growth. All incubations were performed at 37°C with 5% carbon dioxide. Species level confirmation and serotyping by PCR (Chen et al., 1998; Sakamoto et al., 2012) confirmed the isolate as *Avibacterium paragallinarum* serovar B. The culture was then preserved as 20% glycerol stocks at -80°C. The stock culture was utilized for polyclonal antibody production as well as for coating of ELISA plates.

2.2. Antigen preparation

About 10 ml of *Haemophilus* broth was inoculated with *Av. paragallinarum* and incubated in a candle jar at 37°C for 48h. Following incubation, the broth was centrifuged at 5000 rpm for 10 min. The pellet was washed three times with Phosphate Buffered Saline (PBS) and then re-suspended in 10 ml PBS (pH 7.4) and adjusted to a final concentration of 4×10^8 colony forming units (CFU)/ml. Approximately 50 µl of formaldehyde and 25 mg sodium thiosulphate were added to the suspension to prepare 0.5% formalinized antigen, which was then stored at 4°C, until further use.

2.3. Animals and ethical approval

Two rabbits (Soviet Chinchilla; aged > 6 months of age; male) were used for developing polyclonal antisera. Animals were acclimatized for one week before the start of the experiment and were monitored for general well-being. The animals were housed in individual cages and allowed free access to *ad libitum* feed and water during the period of the experiment. The ethical approval related to the use of rabbit in the experimental trial was obtained from Institutional Animal Ethical Committee (IAEC), GADVASU, Ludhiana, India and performed according to the regulations and guidelines on animal ethics.

2.4. Polyclonal antibody production

0.5 ml of formalinized *Avibacterium paragallinarum* antigen with 0.5ml Freund's complete adjuvant (FCA) was subcutaneously administered bilaterally to one rabbit on day one. The other rabbit was injected with only 0.5 ml FCA. Subsequently, boosting inoculations (immunization) were carried out with same amount of antigen with incomplete Freund's adjuvant on 6, 14 days following priming while a gradual increment in antigen dose @ 0.5 ml was kept upto 1.5 ml on 21 and 28 days post priming respectively. Approximately 2 ml blood was collected from the marginal ear vein without anticoagulant at the indicated time point prior to the antigen inoculations to monitor generation of specific antibodies. Final bleeding was carried out upto two weeks after the last antigen inoculations. The collected blood was kept in slant position for 10 min at room temperature, brought to laboratory on ice and immediately centrifuged at 6500 rpm for 10 min. The serum was collected and transferred to sterile vials and stored at -20°C.

2.5. Avibacterium protein preparation and estimation

Avibacterium paragallinarum was grown in *Haemophilus* broth for 48 h. After washing thrice with PBS at 5000 rpm for 10 min, the cells were

resuspended in PBS. Then 5µl of 1% SDS was added to the suspension, which was then sonicated thrice using 30s pulses/burst with 15s pause for cooling (Soniprep 150, MSE, UK). The sonicated suspension was used for indirect ELISA detection. The protein content in the whole-cell sonicated bacterial suspension was estimated using a commercial kit (BCA Protein Assay Kit II, BioVision Incorporation, San Francisco Bay Area, USA) as per the manufacturer's instructions. The protein concentration was estimated spectrophotometrically (Synergy H1 Hybrid Reader, BioTek, Winooski, VT, USA) and the antigenic suspension was preserved at -20°C.

2.6. Measurement of rabbit anti-Avibacterium antibody

Polystyrene 96-well flat-bottom microtitre plates (Maxisorp™ Nunc, Roskilde, Denmark) were coated overnight with 100 µl of serially two-fold diluted known concentration of sonicated whole-bacterial cell lysate (ranging from 5 µg to 0.3125µg) in bicarbonate coating buffer. The plates were then washed three times with 0.1% (v/v) Tween 20 in PBS (pH 7.2). The non-specific sites were blocked with blocking buffer (2% BSA containing 0.1% Tween 20 in PBS (pH 7.2) for 1 h at room temperature. Thereafter, plates were washed with washing buffer (0.1% (v/v) Tween 20 in PBS, pH 7.2). The coated plates were wrapped in aluminium foil and kept at -20°C, until further use. On the day of usage, ten-fold serially diluted positive rabbit antiserum (1:10 to 1:10,000) in BSA buffer (0.1% BSA containing 0.1% Tween 20 in PBS, pH 7.2) was added to one half of the coated plates. On the other half of the plate, antisera from adjuvant treated rabbit was added in similar dilutions. The microtitre plates were sealed and incubated overnight at 4°C. Next day, after washing the plates, goat-anti-rabbit HRP conjugated antibody (Abcam, Cambridge MA USA) diluted in BSA buffer (1:1000 to 1:100000) was added and incubated for 1 h at room temperature. After washing, 100µl of O-phenylenediamine (OPD) substrate was added and plates were incubated at room temperature for 10-15 min. The reaction was stopped by 100µl 1N HCL. The release of chromogenic substrate was measured at 490 nm using ELISA reader (BioTek). The optimal concentration of commercial secondary antibody was established through checkerboard titration experiments (*data not shown*). The antibody titer was calculated as the highest dilution giving an optical density (OD) value more than 1 based on ratio between positive and negative antisera (adjuvant treated rabbit antisera) following background correction (OD absorbance values of BSA buffer). Further, the antigenic specificity of raised antisera was determined from paired sera samples collected at weekly interval from positive rabbit with two different dilution ranges i.e. 1:2000 and 1:4000. The OD value for 1:4000 dilution was determined at 490 nm wavelength.

2.7. Antibody detection limit

The antibody detection limit was obtained from the plates coated with serially diluted sonicated antigen ranging from 0.3215 to 5µg. Positive antisera collected from first bleeding point i.e. 35th day of sampling was tenfold diluted (1:10 to 1:10,000) and added on plates coated with antigen on three different days in triplicates. The limit of detection for the assay was determined as the concentration corresponding to the OD value three times the standard deviation above the BSA buffer (blank/background corrected wells) (Andersen et al., 2012). The antibody titer was considered positive when the ratio exceeded more than 1 and was found to be in convincing range between 1:1000 and 1:4000 dilutions at each and every occasion. Any positive to negative ratio (P/N), which is highest among the dilution range, was considered for further determination of antigenic specificity.

2.8. Intra-plate and Inter-plate assay precision

Intra-plate assay coefficient of variation (CV) was determined for 5 duplicates of 1: 2000 and 1:4000 dilutions on the same assay plate. For

inter-assay CV, 6 quadruplets of 1: 2000 and 1:4000 dilutions of anti-*Avibacterium* antibodies were tested on individual plates run on several days over a span of 5 days.

2.9. Antibody specificity test

In order to assess the specificity of raised polyclonal antisera against *Av. paragallinarum*, paired serum samples collected on day 0, 14, 21, 28, 35 and 42 from the immunized (antigen inoculated) rabbit was subjected to indirect ELISA test. The serum samples were diluted 1:2000 or 1:4000 and assayed in triplicates. All the plates bear control as serum blank i.e. diluent only and goat-anti-rabbit HRP conjugated antibody only. In one of the test plate, the serum samples obtained from rabbit injected with *Salmonella* Gallinarum antigen was used as negative control to ensure minimal or no cross reactivity of anti-*Salmonella* antisera with *Av. paragallinarum* antigen.

2.10. Immunohistochemistry

Few sections of nasal turbinate tissues were obtained on the poly-l-lysine coated slides and dried in an oven at 60°C for 1h. Deparaffinization of sections was performed and subsequently rehydrated through graded series of ethanol solutions (100%, 100%, 96% and 70%) and washed with Tris-buffered saline (TBS, pH 7.6). The endogenous peroxidase activity of the tissues was quenched with 3% hydrogen peroxide in TBS for 10 min and antigen were retrieved by incubating slides in Tris-EDTA buffer (10mM Tris Base, 1mM EDTA, pH 9.0) in microwave oven for 2.5 min at 800W. This was followed by microwaving at 400W for 2.5 min and then two more rounds at 400W for 5 min each. The slides were allowed to cool at room temperature (RT) for 30 min. To block non-specific binding of primary antibody, the slides were incubated in 2% Bovine serum albumin (BSA) (Hi-Media Laboratories Pvt.Ltd, Mumbai, India) prepared in TBS for 10 min at RT. Sections were then covered with primary antibody (non-filtered raised polyclonal antiserum) diluted to 1:1000 in 1% BSA at 4°C overnight. Following a brief TBS wash, the sections were incubated with HRP-conjugated goat anti-rabbit IgG (Abcam, UK) for 45 min. Finally, bound antibody was visualized with ImmPACT DAB chromogen (Vector laboratories, USA) for 15 sec. Sections were counterstained with Mayer's hematoxylin for 3 min and subsequently mounted with DPX. Infected tissues slides where 1% BSA was used instead of the raised polyclonal antiserum as primary antibody during staining served as the negative controls.

2.11. Statistical analysis

The Graph Pad Prism® software package v 4.0 (Graph Pad Software

Inc, La Jolla, CA, USA) was used to assess the variability between 1:2000 and 1:4000 dilutions of sera collected from the antigen injected and adjuvant treated rabbit. The correlation between samples collected at different time points and corresponding OD values was also evaluated. The significance level was set at 0.05. The mean values and standard deviation were calculated for positive serum samples collected at each time point and were assessed by one-way ANOVA and Tukey's post-hoc test.

3. Results

3.1. Protein concentration of *Avibacterium paragallinarum* sonicated antigen

The crude protein content of *Avibacterium paragallinarum* antigen in the sonicated whole-bacterial cell lysate was evaluated by BCA protein assay kit (BioVision Incorporation, San Francisco Bay Area, USA). BSA was used as a reference and the standard curve was prepared. We used undiluted and two-fold dilution of the crude antigenic suspension to establish the linearity of signal as a function of dilution at 562 nm wavelength. A decrease in optical density following the antigen dilution was observed (*results not shown*). The undiluted crude protein concentration was estimated as 1413 µg/ml. Based on our pilot titration experiment results (*results not shown*), we decided to use crude sonicated antigen at a concentration of 0.3125 µg for coating on ELISA plate because of its reasonable range of OD absorbance value estimates. The P/N ratio, when calculated at 2 fold dilution from 1:1000 onwards revealed highest value at 1:4000 at 450 nm wavelength. Nonetheless, the ratio (P/N) from 1:2000 dilution ranges remained very close to 1:4000 dilutions as second in fiddle. So we decided to include both the dilution ranges for subsequent paired sera sample titration, regression analysis as well as for determination of antigenic specificity.

3.2. Sera titration

On every occasion, the microtitre well was coated with 0.3125µg protein (antigen) and antisera was added starting with 1:1000 dilution onwards, which revealed reasonable range of OD absorbance value (mean ± SEM: 2.25 ± 0.695). Interestingly, a linearity of signal as a function of dilution was appreciated in the tested dilutions (*data not shown*). Therefore, we decided to narrow down the dilution range of primary antisera from 1:1000 onwards with further 2-fold serial dilutions till 1:8000 in the test set up. We noted a convincing range of OD value between 1:1000 and 1:4000 dilutions and therefore decided to select 1:2000 and 1:4000 dilutions range for determination of antigenic specificity. The titration curve and regression analysis between positive paired sera samples showed a goodness of fit at $r^2 = 0.8358$ and 0.7411

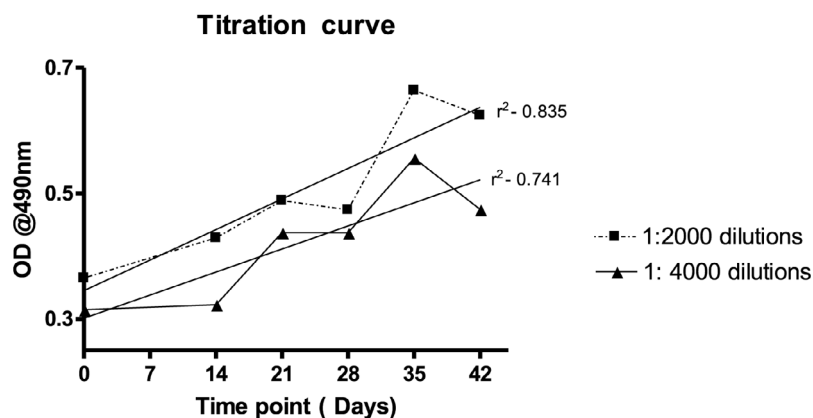


Fig. 1. Titration curve and regression analysis (r^2) of rabbit antisera samples collected at various time points against 0.5% formalinized whole-cell *Avibacterium paragallinarum* antigen. The samples were diluted 1:2000 and 1:4000.

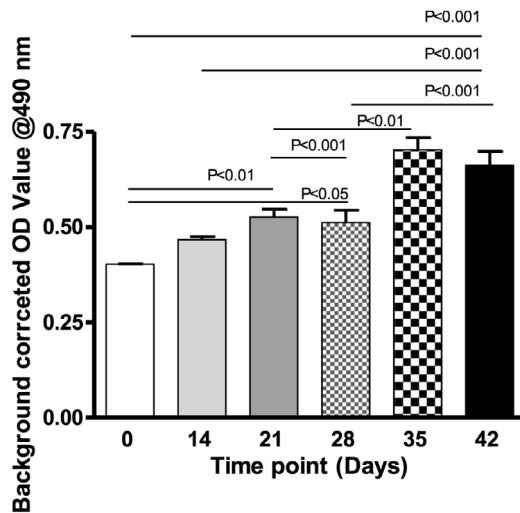


Fig. 2. Specific antibody response to repeated exposure of 0.5% formalinized whole-cell *Avibacterium paragallinarum* antigen. The samples were diluted 1:2000. The titres were calculated from triplicate samples as Mean \pm SD for each time point. ANOVA analysis of ELISA was performed on the measured absorbance value. Asterisks with indicated value defines significant difference between sera samples as well as from the day 0 samples i.e. samples collected before injection of antigen (*, $P=0.05$; **, $P=0.01$; ***, $P=0.001$).

at 1:2000 and 1:4000 dilution, respectively reflecting a clear cut antigen specificity (Fig. 1). The 1:2000 dilution showed higher correlation ($r=0.91$) as compared to 1:4000 dilution ($r=0.86$) and was selected to evaluate the end-point antibody titer.

3.3. Antigen-specific antibody responses

The serum samples obtained at different time points after immunization were analyzed independently (assayed in triplicates) for measuring the antigen-specific antibodies (Fig. 2). The highest OD absorbance values were observed on 35th day of serum sampling (one week after the last immunization). As shown in the figure, no significant difference between non-immunized and positive serum samples was obtained up to day 14 of sampling i.e. two weeks after the first immunization. However, a gradual and significant rise in antigen-specific antibody titers was observed following day 21 of sampling. The antibody responses decreased on the day 42 of sampling i.e. two weeks after the last immunization. No cross-reactivity was detected with sera samples obtained from rabbit injected with *Salmonella Gallinarum* antigen.

The limit of detection for this polyclonal antibody detection assay was found to be at an OD absorbance value of 0.046 to 0.051 (based on three independent assays performed on three different days). The analysis of 5 duplicates of day 28 serum samples at 1:2000 and 1:4000 dilutions yielded an intra-plate CV of $\leq 4.48\%$ and $\leq 6.11\%$ and an inter-assay CV of 1.3% and 4.9%, respectively.

3.4. Immunohistochemistry

The immunopositive reaction was evident as brown granular like deposits diffusing within the superficial layer of modified keratinized epithelium of anterior nasal turbinates as well as in respiratory epithelium of middle turbinates (Fig. 3). At some places, intact staining of single bacterium was spotted. Gradual decline in immunoreactivity against the bacterial antigen was noticed, only when there was a decreasing number of bacteria were present. However, section treated with 0.1% BSA instead of primary antibody, as negative control didn't show positive immuno-reaction.

4. Discussion

Lack of availability of commercial antibodies against different serotypes of *Av. paragallinarum* as compared to other poultry pathogens has restricted the applicability of various immunodiagnostic assays in case of infectious coryza. Only a few laboratories in the world have been able to produce such antibodies, possibly due to stringent legislatures, which cannot be accessed easily by others for experimental purposes (Blackall, 1999). Therefore, the purpose of this study was to produce in-house polyclonal antibody against *Av. Paragallinarum* and assess its antigenic specificity by ELISA. Among the various immunological assays that are used for validating the antigenic specificity of raised polyclonal antisera, ELISA offers certain advantages like low cost, is easy to perform, less labor intensive, and is highly sensitive (Barrette et al., 2006; Nollens et al., 2007) where results can be obtained within relatively shorter period of time. ELISA also offers determination of specific antibodies in serum and plasma in many species including lower vertebrates and marine mammals (Tizard 1996; Beck and Rice, 2003; Nollens et al., 2007).

In this study, we estimated minimum concentration of coated antigen to detect specific antibody and evaluated the optimal dilution range of the primary antisera and the secondary antibody. These parameters are important to validate the specificity of raised antibody for detection of proteins or antigens of interest in biological samples even during extreme denaturation or fixation steps normally employed during IHC in tissue sections. The minimum antigenic concentration that estimated antigen-specific antibodies in positive serum was found to be 0.3125 μg as against the least concentration of 0.25 μg , normally considered for any ELISA format (vanderHyde et al., 2007). The selected optimal dilution range of 1:2000 of primary antibody (polyclonal antisera) was based on regression analysis and was applied for the evaluation of titer development over the course of immunization in rabbit. The resultant analysis revealed a time-dependent significant increase in specific antibody levels following repeated antigenic exposure. The antibody responses wane down after withdrawal of antigenic exposure, possibly due to diminishing levels of circulating antigen (Quan et al., 2009). Although the magnitude of the antibody response noted was not very high and was limited only to post-antigen inoculation period, antigen-specific antibody production was observed in the study. ELISA confirmed specific antibody production owing to strong immuno-reactivity of lymphoid system against the circulating antigen and antigenic repertoire already available during the subcutaneous exposure.

Intriguingly, we used this polyclonal antisera to identify the precise location of antigen in tissue sections following IHC procedure after decalcification, which describes the diagnostic robustness of antisera even during extreme denaturation protocol. This observation could explain molecular configurational stability of the polyclonal antisera during harsh IHC protocol without any prior pretreatment or purification process. There was only little inter-plate and intra-plate variation during ELISA, which supports higher chances of repeatability and consistency. Similar research work was reported against pathogens, recombinant proteins or certain class of species-specific antibodies (Sangdee et al., 2012; Abdi et al., 2012; Nollens et al., 2007), where commercial antibodies are not available. Application of a monoclonal antibody-based ELISA in evaluating pathogen exposure in chicken against *Av. paragallinarum* has already been established (Zhang et al., 1999). However, the poor validity of the monoclonal antibody denigrated its use in detecting host's sero-conversion against vaccine antigen or natural exposure.

In summary, we show the possibility of generation of polyclonal antibody against *Av. paragallinarum* in rabbit with strong antigenic specificity through ELISA. IHC performed with the antisera further ensures its ability to resist harsh procedural conditions, thereby improving its chances of being utilized in other immunological assays. Our next goal is to diagnostically validate this ELISA assay for the estimation of

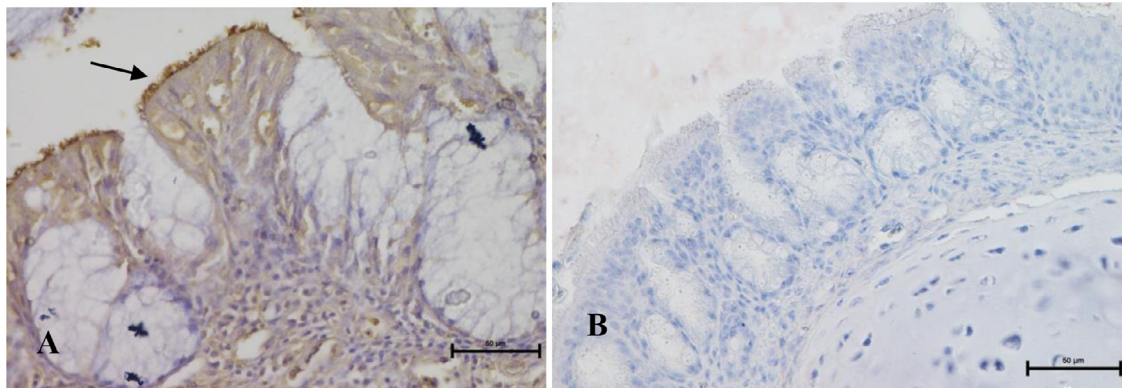


Fig. 3. Nasal turbinates showing bacterial antigen staining as brown granular like deposits. This brown colouration is due to the precipitation of 3' 5' Diaminobenzidine (a chromogenic substrate) along the zone of specific antigen-antibody complex. Note there is no non-specific background staining observed. X40, IHC. Negative control slides lack the brown immunopositive staining X40, IHC.

specific antibody titer from ailing bird or suspected flock against infectious coryza or following vaccination.

Ethical Approval

We declare that the ethical approval related to the use of rabbit in the experimental trial was obtained from Institutional Animal Ethical Committee (IAEC), GADVASU, Ludhiana, India and performed according to the regulations and guidelines on animal ethics (Approval reference no. GADVASU/IAEC/23/006)

Declaration of Competing Interest

We declare that we have no conflict of interest.

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