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Isolation, characterization and preparation of inactivated vaccine against avian Infectious Bronchitis virus

Minahil Sajid[1*], Atta ur Rehman[1] and Misbah Ijaz[2]

[1]Institute of Microbiology, University of Agriculture, Faisalabad-38040, Pakistan.
[2]Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad-38040, Pakistan.

*Correspondence: minahilsajid3@gmail.com

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ABSTRACT

The goal of this research is to isolate and identify the infectious bronchitis virus in order to create an inactivated vaccine. Infectious bronchitis is a very contagious upper respiratory disease that affects young poultry. Infectious bronchitis virus (IBV), the chicken coronavirus, is one of the leading causes of economic loss in the poultry business, affecting both meat-type and egg-laying birds. It is a member of the Coronavirus family, the genus Gamma coronavirus, which has a single-stranded RNA genome surrounded by a nucleocapsid and envelope. Clinical samples (trachea, bronchi, and caeca) were collected from the Clinical Diagnostic Lab of UAF. The samples were screened by HA, HI test for the presence of virus. IHA was performed for IBV because IBV has no glycoprotein on their surface. That's why add some mask reagent to form agglutination. The positive IBV samples were cultured in specific pathogen-free (SPF) embryonated hen's eggs. Virus isolation was performed by inoculation of 9–11-day-old eggs with 200µl of 10% tissue homogenates and incubation at 37°C for 72 hours. For vaccine, the confirmed virus was cultivated in embryonated chicken eggs. Sixty broiler chicks will be divided into 3 groups, having 20 each. First group was injected by the prepared vaccine. Second group was injected by a commercially available vaccine while the third was negative control. Standard statistical analysis was applied, and treatment means were estimated.

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Introduction

Young hens are susceptible to the acute, extremely contagious upper respiratory infection known as infectious bronchitis. One of the major causes of monetary loss in the cattle business is IBV, often known as the chicken coronavirus. All birds that lay eggs and generate meat are impacted by IBV in terms of productivity. The virus also replicates in numerous additional tissues, such as those in the kidney, oviduct, and testes, in addition to the epithelium of the lower and upper respiratory tract tissues. It can be found in both respiratory fluid and excretion. More and more research suggested that IBV can infect birds besides chicken (Henry 2020)

IBV is an RNA virion with a positive-sense, non-segmented genome that is around 27.6 kb in size. It has at least 10 open reading frames (ORFs) and has 5' and 3' of untranslated parts. These ORFs are listed in the following order: 3a-3b-3c-M-5a-5b-N-Poly (S1, S2)-3a-3b-3c-M, -3a-1a-1b-S. Two glycoprotein subunits, S1 and S2, make up the spike protein of the IBV virus. Neutralizing antibodies mostly target the S1 subunit because it has distinctive antigen markers for serotype identification (Pourbakhsh et al. 2000). The S2 protein's C-terminal portion interacts with the viral envelope and helps the S1 protein adhere to the membrane. IBV is a member of the Coronavirus

family's Gamma coronavirus genus. This genus includes nucleocapsid-encased single-stranded RNA genomes (Zhang et al. 2021).

One of these newly discovered genotypes is variation 2 (GI-23/IS-1494), a neuropathogenic genotype that first surfaced in Israel in 1994. Reports of this genotype have since spread to several Middle Eastern nations, such as Iran, Afghanistan, Iraq, Oman, Turkey, Jordan, and Europe. According to cross-protection tests, the protection level after using a vaccination of the Massachusetts type is around 25%. Nonetheless, the immunization program's combined use of the Mass type and 793/B vaccines raises the protection rate by up to 80%. It is thought that IBV cannot spontaneously infect different animals. The infectious bronchitis virus can spread in nursing mice if it is given intracerebrally (30). On the other hand, it seems that this type of virus passage results in the selection of a virus population that is safe for chicks. Intracerebral IBV injection can potentially harm suckling rabbits and guinea pigs. The infectious bronchitis virus is temperature-sensitive; most strains are typically inactivated at 56°C for 15 to 45 minutes or at 45°C for 90 minutes, however individual strains may vary in this regard. The virus can only survive at room temperature for a few days. Storage at 4°C has an impact on viral infectivity, and -70°C is advised for long-term storage. For at least 21 The virus's infectivity is affected by 4°C storage; for long-term storage, -70°C is suggested. Lyophilized virus kept at 4°C will stay infectious for at least 21 months. A wide range of widely used disinfectants, such as iodine, 70% ethanol, 1% phenol, 1% formalin, and chloroform, can readily inactivate the infectious bronchitis virus. A virus is more stable at pH 3.0 than it is at a high pH. The stability of the virus lasts for 14 days. Water quality, IBV lifetime, and immunization administration are extras or contents. The virus appears to be able to endure for a considerable length of time in feces-filled litter.

Another potential method of indirect long-distance transmission is through contaminated tools and materials (Bhuiyan et al. 2021). Depending on the infectious dose and mode of infection, the IBV incubation time might change. For instance, the incubation time is around 36 hours when the virus is delivered by ocular inoculation, but infection through the trachea may result in a shorter duration of 18 hours (Boroomand et al. 2012). The Harderian gland, trachea, bronchi, and air sac epithelia of the host become first infected. From there, the infection travels to the kidneys and urogenital tract, causing systemic disease. The nature and clinical features of IB are determined by the particular organ or system involved. Infection in the respiratory system can cause symptoms like nasal discharge, lassitude, tracheal rales, gasping, and sneezing. Weight loss and the concentration of birds close to a heat source are potential additional symptoms (Bande et al. 2016).

The combination of HVR1-3-specificprovideassays and IBV N-specific RT-qPCR assays provides an accurate, sensitive, and precise tool for undertaking additional genetic characterization of viruses as well as for screening suspect samples. HVR-specific PCRs enable genotyping directly from clinical specimens, negating the requirement for virus isolation, where the RNA sample quality is appropriate and viral loads are sufficient (Najimudeen et al. 2002). Several risk factors, such as different vaccination schedules, gaps between vaccines, and the difficulties in managing possible immunosuppression in birds, can be blamed for the poor efficacy of IB immunization (Yan et al. 2013).

Materials and Methods

Sample Collection

Clinical samples of oral, tracheal, and ceacal swabs were collected from a large number of sick birds in Faisalabad and placed in sterile zipper bags. Once it has been collected and transported, the sample was maintained in a transport medium.

The transportation medium was sent to the Institute of Microbiology at the University of Agriculture in Faisalabad for further processing. For prolonged storage, samples could be stored at -80°C (OIE 2013).

Processing of Sample

After cutting the tissues from the samples that were taken, normal saline was applied. It will then be homogenized in a homogenizer and centrifuged for 5 minutes at 4000 rpm. The pallets were thrown away, and the supernatant kept for future use. Falcon tubes containing processed samples and tissue suspensions of IBV were labeled and stored at -20°C accordingly (Hammad et al. 2022).

Isolation of Infectious Bronchitis Virus (IBV)

With 200 µl of 10% tissues homogenates, eggs that are 9 to 11 days old were candled for viability before being incubated for 72 hours at 37°C. Every day, eggs were candled to check for any mortality. Allantoic fluid from the eggs were collected and kept after three days (Tran et al. 2020).

Allantoic Fluid Sterility Test

The IB virus's allantoic fluid unwasderwent sterility testing. Allantoic fluids were centrifuged for 5 minutes at 1500 rpm for this purpose.

Sterility Testing

Sterility of the vaccine was tested on Nutrient agar, MacConkey agar and Sabouraud Dextrose agar.

Safety Testing

Three-week-old birds were used in the safety tests. Three groups of five birds each were formed from the fifteen total birds. Three groups each received one m of subcutaneous inoculation with each of the three vaccinations.

Inactivation of Virus

The vaccination will be rendered inactive using formaldehyde and heat. An inactivated virus won't

be totally gone for at least 60 minutes. The virus will be thermally killed during the preparation of the vaccine using heat, UV radiation, or gamma radiation (Al-Ebshahy et al. 2019).

Preparation of Monovalent Vaccine

To improve the effectiveness of vaccination under continual centrifugation, inactivated oil adjuvant monovalent vaccines were made by combining 3 parts of Inactivated antigen and 7 parts of Montanide adjuvant ISA-206 VG.

A single dosage of 0.1 ml of IBV (field isolates) was administered intranasally to fifty percent of the birds in each group at the age of 35 days. The clinical findings (mortality and morbidity rates) were documented for 10 days following the challenge. The proventriculus, lungs, liver, spleen, kidneys, and intestines all had lesions that required close inspection.

Experimental Design

All experimental birds were divided into three groups (I, II, III) with each group having 20 birds that were one-day old and healthy. Birds were immunized on the 0th day (14 days of age) and the second dose was injected at 7th day (21 days of age). Group 1 was injected subcutaneously with commercially available monovalent vaccine of IBV whereas, experimentally prepared vaccine was administered through sub-cut route of inoculation in the birds of group II. Group III was inoculated with Phosphate Buffer Saline and oil adjuvant (mock vaccine) subcutaneously and served as a control group (Mahmood and Sabir 2021) (Table 1).

Statistical Analysis

Graph Pad Prism 5 software was used to compute the geometric mean titer (GMT) against IBV antibodies for each experimental group on the seventh and twenty-first days, respectively. Through analysis of variance of means using a totally randomized design and paired t-test, the data on antibody titer were examined.

Results

Haemagglutination (HA) assay of Infectious Bronchitis Virus (IBV) showing agglutination of chicken red blood cells (CRBCs) up to 7th well (1/128th dilution of antigen). Last well showing haemagglutination was referred to as one HAU which was used to calculate 4HAU (4/128=1/32). 4HAU of IBV was 1/32 it means that IBV antigen was diluted 32 times to be used further in HI assay (Table 2).

HI titers and Log$_2$ HI titers against IBV at 7th day of vaccination (21 days of age)

On the 7th day PV, the antibody titers against IBV of randomly selected 5 birds from each group ranging from 2 to 7. GMTs of group I, II and III were recorded as 42.2, 84.4 and 4.6 respectively. Statistical difference in the GMTs of group I and II was non-significant whereas vaccinated groups had a significant difference as compared to non-vaccinated (Fig. 1).

Table 1: Experimental design for immunization of chickens

Groups	**No. of Birds**	**Treatment**	**Dose**	**Route**
I	20	Commercial Vaccine	0.5 ml	Sub-cut
II	20	Experimental Vaccine	0.5ml	Sub-cut
III	20	PBS+Oil Adjuvant	0.5ml	Sub-cut

Hemagglutination Inhibition (HI) assay of Infectious Bronchitis Virus (IBV) showing button formation (settling down of CRBCs) up to 5th well (1/32nd dilution of anti-IBV antibodies). Last well showing button formation was referred to as end point and is the final HI titer of antibodies.

The sterility test to check Allantoic fluid was uncontaminated, as evidenced by the lack of bacterial and fungus growth on Nutrient, MacConkey and Sabouraud agar. Allantoic fluid of Infectious Bronchitis Virus (IBV) when inoculated on Nutrient and MacConkey agar following overnight incubation at 37^{o}C showed no bacterial growth on both media plates (Stenzel et al. 2017). Sabouraud agar plates after inoculation and 3-4 days incubation were observed. There was lack of any fungal growth on Sabouraud agar. It means that the allantoic fluid of IBV was free from any bacterial and fungal contamination.

Table 2: GMT of IBV at 7th day of Vaccination (21 days of age)

Samples	**Group I**		**Group II**		**Group III**	
	HI Titer	**Log$_2$ HI Titer**	**HI Titer**	**Log$_2$ HI Titer**	**HI Titer**	**Log$_2$ HI Titer**
1	32	5	128	7	4	2
2	64	6	64	6	4	2
3	32	5	64	6	8	3
4	32	5	128	7	4	2
5	64	6	64	6	4	2
GMT	42.2		84.4		4.6	

HI titers and Log$_2$ HI titers against IBV at 14th day of vaccination (28 days of age)

At 14th day PV, the antibody titers against AIV of randomly selected 5 birds from each group ranging from 2 to 8. GMTs of group I, II and III were recorded

as 97.0, 168.9 and 5.3 respectively. Statistical difference in the GMTs of group I and II was non-significant whereas, vaccinated groups had significant difference as compared to non-vaccinated.

The vaccination will be rendered inactive using formaldehyde and heat. An inactivated virus won't be totally gone for at least 60 minutes. The virus will be thermally killed during the preparation of the vaccine using heat, UV radiation, or gamma radiation (Al-Ebshahy et al. 2019).

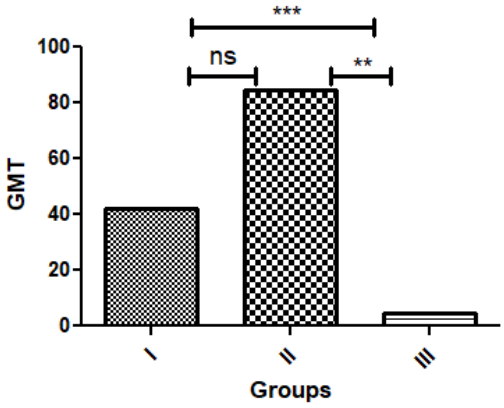

Fig 1: GMT of IBV at 7th day of Vaccination (21 days of age)

All experimental birds were divided into three groups (I, II, III) with each group having 20 birds that were one-day old and healthy. Birds were immunized on the 0th day (14 days of age) and the second dose was injected on the 7th day (21 days of age). Group 1 was injected subcutaneously with commercially available monovalent vaccine of IBV whereas, experimentally prepared vaccine was administered through sub-cut route of inoculation in the birds of group II. Group III was inoculated with Phosphate Buffer Saline and oil adjuvant (mock vaccine) subcutaneously and served as a control group (Mahmood and Sabir 2021).

Discussion

The Acute, highly contagious viral respiratory illness of chickens known as infectious bronchitis (IB) has been described as having tracheal rales as one of its main symptoms of having a cough and a cold. Considering that it might lead to bad economic outcomes, it has also been described as a significant sickness. Broilers get air saculitis because of weight increase, inadequate diet, and maybe a combination of illnesses. It could affect egg production and quality in hens and other layers. Secondary bacterial infections, which happen after IBV has damaged the respiratory tract and spread to the rest of the body, are typically the cause of death. IBV strains with strong nephropathogenicity have been shown to have the ability to kill up to 30% of young birds.

IBV, the infectious bronchitis virus, is a member of the Coronaviridae family and the genus Coronavirus in the class Nidovirales. A non-segmented, positive-sense virus Genome of single-stranded RNA. It is an encapsulated virus with club-shaped surface projections (Spikes) about 20 nm or less in length. It also includes three key structural proteins. The illness may be controlled affordably and effectively with vaccination. The development of the immune system affects the chickens' response to vaccination at every stage. Birds that are three to six weeks old respond well to the health challenge in terms of their immune systems (Ginkel et al. 2015). The quality of the chicken bursa and the thymus's development are both necessary for the T-cell immune response and the synthesis of antibodies, respectively. After vaccination, chickens have two different types of immunological reactions: humoral and cell mediated. In this work, the immunokinetics of the chicken humoral response to the live and dead IBV vaccine was examined (Spackman 2021). Following the immunization, blood samples and cloacal swabs were taken at regular intervals of three days. Birds exposed to vaccination challenges repeatedly see a fast rise in immunoglobulin levels as a result of immunologic memory. (Chhabra et al. 2015). IgA is connected to mucosal immunity and has two types: monomer form present in serum and dimeric form present in secretions like tears, saliva, mucous, and gastrointestinal tract. IgM and IgG are present in serum that neutralize the antigen and aid in its phagocytosis by macrophages. To measure the IgA response to immunization, cloacal swabs were washed as was done before (Merino-Guzmán et al. 2017).

After vaccination, titration of antibodies was used to measure the humoral immune response. After one day has passed since the immunization, IgA, IgM, and IgG titers first begin to progressively rise. Similar findings published by (Amer et al. 2018: Charabeh et al. 2013) show that IgM levels in serum grow until 12 days after vaccination and then gradually start to decline. Following a 6-day immunization period, IgG levels start to rise. The amount of IgG begins to rise quickly after the booster dosage is administered and continues to do so until the experiment is complete. Immunoglobulin G (IgG) titers were greater following secondary immunization than following first vaccination, according to Liu et al. (2012). By neutralizing viral receptors, mucosal antibodies IgA produced in mucosal secretions prevent IBV from attaching to them. The IgA extracted from cloacal swab processing starts increasing in the first week after immunization and increases progressively, according to Bakema and Van Egmond (2011).

The vaccines are created from locally prevalent, altered viral strains in a way that prevents sickness

and instead triggers a defense mechanism. Compared to live vaccination, the dead vaccine results in somewhat delayed antibody titers. In comparison to the control group, the challenge protection test reveals complete protection in both the live and killed vaccination groups. In comparison to the unvaccinated groups, IBV-vaccinated birds showed 80 to 100% protection, according to (Sultan et al. 2019).

It has been found that live vaccines induce a quicker immune response than inactivated vaccines. Economically, a live vaccination offers a better defense against IBV as the virus begins to replicate within the host (Gurjar et al. 2013; Okino et al. 2014). However, there is a potential that a virus can revert to its original form or that a pathogenic strain would contaminate a vaccination strain, which could result in sickness. Since the virus is so feeble and the vaccination induces a protective immune response, the killed version is safer but more expensive and requires numerous booster doses. According to the experiment, live IBV vaccinations induce a greater immunological response in chickens than inactivated vaccines.

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Ethical Statement
No Ethical permissions were required for this article.

Availability of Data and Material
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Consent to Participate
All the authors gave their consent for equal participation.

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All the authors gave their consent for publication.

Competing Interest
The authors declare that they have no relevant financial or non-financial interests to disclose.

Author Contribution
MS, AUR, and MI conducted the experiment MI analyzed the data and performed statistical Analysis. MS prepared the manuscript for publication.

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