

## *In vitro* physicochemical characterization of nephropathogenic strain of infectious bronchitis virus isolated from poultry

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### Abstract

Infectious bronchitis virus (IBV) is an important pathogen in the poultry industry causing avian infectious bronchitis that is, an acute but highly contagious disease affecting the upper respiratory tract, kidneys and reproductive tract. The 3<sup>rd</sup> passage of a polymerase chain reaction confirmed nephropathogenic IBV isolate was used for this study. Heat stability for 5, 10, 15, 20, and 30 min at 56.00 °C, pH sensitivity at pH 3.00, 7.00, 9.00, and 11.00 ultraviolet (UV) irradiation for 10, 15, 20, and 30 min, and chloroform sensitivity were studied. The IBV isolate was found to be susceptible to a temperature of 56.00 °C for 5 min and above, UV irradiation within 10 min, chloroform treatment and to pH 11.00 while being resistant to pH 3.00 and 9.00. The second part of the study investigated *in vitro* effectiveness of the disinfection potential of several commercially used disinfectants in Pakistan against the IBV isolate. For this purpose, Virkon S, Bromosept, and Beloran were employed for the virus inactivation test. Following the IBV challenge for contact time of 1, 5, 10, and 30 min, we counted the number of embryos that died after incubation. Results showed that suitable dilution of disinfectant for the recommended contact period could kill the virus. The maximum susceptibility was seen in the case of Virkon S which killed the virus in just 1 min. Thus, IBV could be killed using commercially available Virkon S, Beloran, and Bromosept after being used in recommended concentrations for recommended contact time.

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### Introduction

The history of poultry is 150 million years old. In Pakistan, poultry is considered to be the second-largest industry contributing to 1.30% national gross domestic product. A huge economic loss is being faced by the poultry producers during the last decade due to outbreaks of many poultry diseases.<sup>1</sup> Infectious bronchitis disease is one of the major avian diseases that are prevalent in Pakistan. The disease is of great economic importance because it causes heavy production losses throughout the life of the bird although infectious bronchitis disease is more prevalent in birds of young age.<sup>2</sup>

Infectious bronchitis is an acute highly contagious respiratory disease of poultry caused by infectious bronchitis virus (IBV), a member of the family *Coronaviridae*, sub-family *Coronavirinae*, genus *Gammacoronavirus*.<sup>3</sup> The IBV is an enveloped virus containing round to pleomorphic shape and lipid envelope around the capsid.

The virus has a diameter of approximately 120 nm having a crown shape due to the presence of surface spike proteins.<sup>4</sup> The genome size of the virus is 27.60 kbps and encodes 15 non-structural proteins from nsp2-16 as well as four structural proteins including spike protein, small membrane protein, membrane and nucleoprotein in the following order: 5' - ORF1 a / b S - E - M - N - 3'.<sup>5</sup>

The birds show clinical symptoms within 24-48 h, however, it can be 18 hr in intratracheal inoculation.<sup>6</sup> Aerosol, as well as mechanical transmission of virus, is seen between birds, houses in farms. The virus can spread through large distances by indirect transmission by the contaminated litter, farm visits, clothing, footwear, utensils, fertilizer and equipment.<sup>7</sup> The IBV is shed in droppings and tracheobronchial exudates of infected chickens.<sup>8</sup>

Tracheal lesions are usually seen in respiratory infection, whereas, nephropathogenic strains also cause kidney lesions having 25.00% mortality in broilers. Mortality rate can be increased by complications in

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co-infection with certain other bacteria. Nephropathogenic IBV cause apoptosis in kidney cells which is a major contributor to the pathogenicity of the virus. Virus also induce renal endoplasmic reticulum stress in birds.<sup>9</sup> Recovery starts after one week in uncomplicated cases, although flocks may test positive and shed virus for another 15 - 20 weeks.<sup>10</sup>

Avian infectious bronchitis is mainly a respiratory disease of chicken but damage to the reproductive system as well as nephritis is also observed. Respiratory signs include sneezing, coughing, difficulty in breathing, tracheal rales, puffy swollen eyes, congested lungs, depression and weight loss in young chickens.<sup>11</sup> Reproductive system damage results in a decline in egg production, weak and broken shelled eggs, low quality of eggs and oviduct damage in adult hens.<sup>12</sup> Nephropathogenic form of IBV is characterized by mild respiratory infection followed by diarrhea, depression, excessive water intake, reluctance to move, wet litter and death after 5 to 7 days of infections.<sup>13</sup>

First nephropathogenic IBV infection case was reported in United States in 1960. Nephropathogenic strains of IBV have been the most prevalent strains of IBV in recent years.<sup>14</sup> The IBV has a large number of serotypes, owing to the numerous point mutations and recombination events present in RNA viruses. This study aimed to provide the first comprehensive physicochemical characterization of locally isolated nephropathogenic IBV strain and evaluated the efficacy of commercially used disinfectants (Virkon S, Beloran, Bromosept) against the IBV isolate, offering novel insights into IBV management and control strategies.

## Materials and Methods

**Revival and identification of virus.** The 3<sup>rd</sup> passage of a polymerase chain reaction (PCR) confirmed nephropathogenic IBV isolate was used for this study. The sample was processed and passaged on 9-days old embryonated eggs. The study was conducted according the institutional ethical committee of University of Veterinary and Animal sciences Lahore Pakistan's guidelines after approval from the concerned department (No. DR/403, 29/11/2021).

**Confirmation of virus.** Genome of the virus was extracted by using QIAMP Viral RNA Mini Kit (Qiagen, Hilden, Germany). Confirmation of virus was made by reverse transcription- PCR using Verso-One Step RT-PCR Kit (Thermo Scientific, Waltham, USA) using the primers set forward 5'-CCCAATTTGAAA ACTGAA CA-3' and reverse 5'CCTACTAATTTACCACCAGA-3' targeting *S1* gene.<sup>15</sup> The reaction mixture was prepared by mixing 5.50  $\mu$ L nuclease free water, 12.50  $\mu$ L master mix, 1.00  $\mu$ L of each primer and 5.00  $\mu$ L of extracted RNA, having a total volume of 25.00  $\mu$ L. The mixture was incubated at 55.00 °C for 20 min for cDNA preparation followed by initial

denaturation at 94.00 °C for 5 min and 35 cycles of denaturation at 94.00 °C for 1 min, annealing at 49.00 °C for 1 min and extension at 72.00 °C for 3 min and one final extension step at 72.00 °C for 7 min.

**Physicochemical characterization.** Physicochemical characterization of nephropathogenic IBV isolate was carried out by inoculating virus in 9-days old embryonated egg after applying following physicochemical treatment on the virus.

**Heat stability.** Extracted allantoic fluid containing IBV was subjected to heat treatment at 56.00 °C for different time intervals of 5, 10, 15, 20 and 30 min using a water bath. After each treatment tubes were removed and placed in an ice bath. Virus viability was determined by titrating of heat treated allantoic fluid in 9-days old embryonated eggs.<sup>16</sup>

**pH stability.** Virus stability to different pHs was determined by mixing IBV infective allantoic fluid in phosphate buffered saline whose pH was adjusted to 3.00, 7.00, 9.00 and 11.00 by adding 1.00 N HCL or by adding a small amount of 1.00 N NaOH. After incubation for 180 min at 4.00 °C treated allantoic fluids were inoculated in 9-days old embryonated eggs. Viral viability was determined.<sup>17</sup>

**Ultraviolet (UV) stability.** The nephropathogenic IBV infective allantoic fluid was irradiated by placing it 30.00 cm under a 30.00-Watt UV lamp to irradiate the virus. After irradiation for 10, 15, 20 and 30 min the viral viability was determined by titrating of irradiated allantoic fluid in 9-days old embryonated eggs.<sup>18</sup>

**Chloroform susceptibility.** Chloroform susceptibility was checked using 4.80% reagent-grade chloroform (Sigma-Aldrich, St. Louis, USA). The IBV infective allantoic fluid was treated with chloroform at a final concentration of 4.80%. Mixture was shaken at 4.00 °C for 10 min and then centrifuged at 500 *g* for 5 min. Topmost transparent layer was recovered and viral viability was assayed by titrating this layer in 9-days old embryonated eggs.

**Evaluation of disinfectants.** *In vitro* efficacy of different routinely used disinfectants in poultry sheds like VirkonS (Lanxess, Cologne, Germany), Beloran (Vetoquinol SA, Lure, France) and Bromosept (Daesung Microbiological Labs. Co. Ltd., Gyeonggi-do, Korea) for virus inactivation was assayed. Evaluation was made by recording characteristic lesions, stunting of growth, curling or death of embryo/five inoculated 9-days old embryonated eggs.<sup>19</sup>

**Efficacy of Virkon S.** Virkon S was diluted at 1:100 dilution in sterile distilled water as per the manufacturer's recommendations. Half mL of  $1.00 \times 10^5$ , 50.00% egg infectious dose virus was treated with 0.50 mL of 1:100 diluted Virkon S at room temperature for a time of 30 sec, 1-, 5- and 30-min. Efficacy of Virkon S was checked by inoculating this treated mixture in five 9-days old embryonated eggs by chorioallantoic route in Quality

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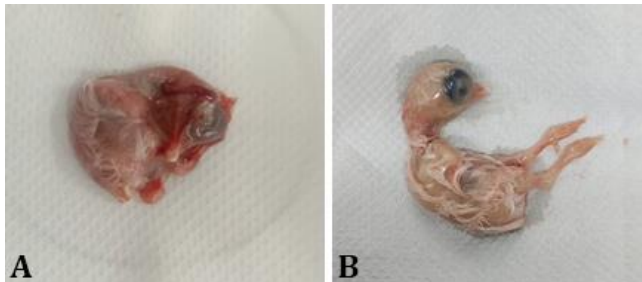
**Efficacy of Beloran.** As per the manufacturer's recommendations a dilution of 0.50% Beloran was made. Half mL of this dilution was mixed with 0.50 mL of allantoic fluid containing  $1.00 \times 10^5$ , 50.00% egg infectious dose virus and was incubated at room temperature for 30 sec, 1, 5 and 30 min. The solutions were treated in 9-days old embryonated eggs.

**Efficacy of Bromosept.** The 0.25% dilution of 10.00% bromosept was made in sterile distilled water as per manufacturer's recommendations. A 0.50 mL of this dilution was mixed with 0.50 mL of allantoic fluid containing  $1.00 \times 10^5$ , 50.00% egg infectious dose virus at room temperature for 30 sec, 1-, 5- and 30-min. Efficacy of bromosept was checked by treating this mixture in 9-days old embryonated eggs.

## Results

**Sample revival and confirmation.** A sample was found positive for IBV and showed typical characteristic lesions of IBV which were stunting of growth, curling and dwarfism of embryo on inoculation of virus in 9-days old embryonated egg followed by 5 days incubation period as shown in Figure 1A and a negative control in Figure 1B. Samples were processed for RNA reverse transcription-PCR using Verso-one Step PCR kit. The primers were considered to have a product size of 1700 bp and by visualizing the gel we found the band with the same amplicon size which confirmed that the sample was positive for IBV (Fig. 2).

**Heat stability.** Like all corona viruses IBV is also sensitive to heat. Virus viability was reduced by exposing virus at 56.00 °C for 5 min. The more exposure time increased, the more virus' viability declined, and even 5 days after inoculation, no distinctive lesions or embryo deaths were seen.



**Fig. 1. A)** Embryo showing characteristic IBV symptoms after inoculation of virus via chorioallantoic route in 9- day old embryonated egg, and **B)** Chicken embryo as negative control at 14-day of incubation (embryonated egg).

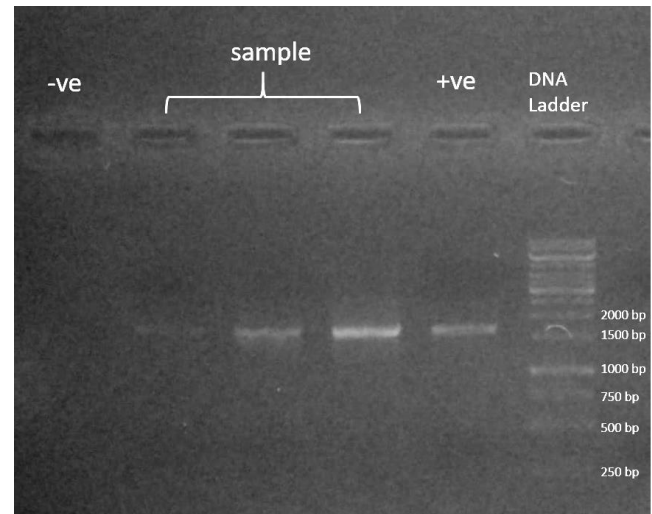
**pH stability.** Isolated nephropathogenic IBV isolate was relatively resistant to pH = 3.00, 7.00 and 9.00. The IBV withstood low pH and survived in acidic, neutral and slightly basic pH showing characteristics IBV lesions on

embryo. However, IBV got inactivated when exposed to pH = 11.00.

**Ultraviolet stability.** The nephropathogenic IBV isolate was found highly sensitive to UV radiations. Virus was killed after being exposed to UV radiation as less as 10 min from a 30.00 cm distance. More exposure time showed the same results and the isolate was found sensitive to UV radiations.

**Chloroform susceptibility.** The nephropathogenic IBV isolate was found to be highly susceptible to chloroform. Virus did not survive after being treated with 4.80% reagent-grade chloroform for 10 min. No distinctive lesions or mortality was seen in inoculated embryos.

**Disinfectants results.** Different disinfectants have been used in poultry sheds for disinfection such as Virkon-S, Beloran, and Bromosept that were tested for their *in vitro* effectiveness in inactivating IBV. Embryos showing characteristic lesions of IBV such as stunting of growth, curling or death of embryo were considered as survival of virus to disinfection. No characteristic lesions and survival of embryo was recorded as susceptibility of virus to disinfectant. The results of IBV inactivation using several types of disinfectants with exposure durations are shown in Table 1.



**Fig. 2.** Reverse transcription-polymerase chain reaction amplicon of infectious bronchitis virus on 1.50% agarose gel while moving from right to left 1<sup>st</sup> well; DNA ladder, 2<sup>nd</sup> well (+ve); positive control, 3<sup>rd</sup>-5<sup>th</sup> well; 1,700 bp band of infectious bronchitis virus (3<sup>rd</sup> and 4<sup>th</sup>) and 6<sup>th</sup> well (-ve); negative control.

## Discussion

Physicochemical characters in addition to being a mean of virus detection also provide details on the appropriate means of virus eradication. To evaluate the effect of various physicochemical treatments on viability of the nephropathogenic IBV isolate the third passage of IBV isolate was used because it consistently produced characteristic IBV embryonic lesions, stunting of growth

**Table1.** Number of dead and live embryos/five inoculated eggs after incubation.

Disinfectants	Dilutions (%)	Exposure time (min)	No. of died embryo	No. of survived embryo	Age survival of embryos (%)
Virkon S	0.01	1	0	5	100
		5	0	5	100
		10	0	5	100
		30	0	5	100
		1	3	2	40.00
Beloran	0.25	5	2	3	60.00
		10	1	4	80.00
		30	0	5	100
		1	1	4	80.00
		5	0	5	100
Bromosept	0.50	10	0	5	100
		30	0	5	100
		-	5	0	0.00
		-	0	5	100
Positive	-	-	5	0	0.00
Negative	-	-	0	5	100

and curling of the embryo.<sup>20</sup> The locally isolated nephropathogenic strain of IBV is shown to be thermosensitive when heated for 5 min or above at 56.00 °C. Proteins of IBV, like those of other corona viruses, were unable to tolerate high temperatures, and as a result, the virus was killed and the embryo survived. In the present investigation, it was found that the IBV isolate was resistant to both slightly basic and acidic pH. Virus survived on treatment at pH 3.00, 5.00 and 9.00 for 180 min at 4.00 °C and, it was considered that IBV strain was resistant to this pH. However, the virus was sensitive to highly alkaline pH as 11.00 as the virus was not inactivated and the embryo showed the typical lesions of the IBV. Virus was found sensitive to UV irradiation within 10 min when placed 30.00 cm under a 60.00-Watt UV lamp. Electromagnetic energy is released by UV light and certain triggered photochemical reactions inactivate the virus when placed under UV for 10 min, and the results are supported by various studies.<sup>21,22</sup> As demonstrated by other investigations with different IBV strains, the viral strain has been found to be sensitive to chloroform, indicating that lipid is a key component of infectious bronchitis virus.<sup>23</sup> The isolate was found to be sensitive at 56.00 °C for 5 min, to chloroform, UV light within 10 min, sensitive to pH 11.00 but resistant to pH 3.00, 7.00 and 9.00. The results of present investigation about sensitivity of virus showed that the IBV strain was sensitive at 56.00 °C for 5 min and were consistent with reports of others.

In this study, we looked into the effectiveness of a number of commercially used disinfectants in Pakistan, such as Virkon S, Beloran, and Bromosept against nephropathogenic IBV isolate. Disinfectants were used according to the manufacturers' recommendations. The outcomes showed that the virus must have been inactivated with the right concentration and contact duration. However, a variety of factors, including concentration of disinfectant, contact time, activity in organic material or protein-containing materials, temperature and quantity, affected disinfection efficiency that must be considered prior to use of disinfectants as describe by others.<sup>24</sup> The inactivated

effectiveness to IBV for Virkon S appeared to be in the first rank. Virus was inactivated when Virkon S at the concentration of 1:100 was contacted with IBV for at least 1 min. The IBV inactivated efficacy was held by Bromosept whose 0.25% dilution for a contact time of 5 min eliminated the entire virus. The IBV was inactivated completely when 0.50% concentration of Beloran was used for 30 min. Pathogen decontamination on surfaces of objects is crucial for preventing infectious infections.

This study offered valuable insights into the physicochemical susceptibilities and optimal disinfection strategies for managing nephropathogenic IBV. The virus was effectively inactivated by Virkon S, Bromosept, and Beloran when applied at precise concentrations and contact durations. These findings underscored the necessity of implementing evidence-based disinfection protocols to mitigate IBV transmission and enhance biosecurity measures in poultry management.

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### Conflict of interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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