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Research Article
Deactivation of Avian Infectious Bronchitis Virus H120 Using Natural Oil Blend on *in vitro* Medium

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Abstract

Background and Objective: Avian Infectious Bronchitis Virus (AIBV) is a highly infectious pathogen of chicken. There is no medication and vaccine developed yet due to its highly mutable nature. The objective was to formulate a blend that can be effective against the viral infection in chicken. **Material and Methods:** Several natural essential oils, like, *Gardenia jasminoides*, *Commiphora myrrha*, *Boswellia serrata*, *Foeniculum vulgare* and *Daucus carota* with anti-viral properties were blended and tested against Avian Infectious Bronchitis Virus H120 (AIBV H120) strain using *in vitro* medium. The trial was conducted in an allantoic fluid medium. Different concentration of NOB was inoculated into the allantoic-fluid to challenge with the lethal dose of virus. **Results:** The Natural Oil Blend (NOB), at its minimum concentration up to 0.01 mL of 0.1%, was effective against the AIBV H120 strain. The NBO concentration lower than 0.1% was not sufficient to deactivate the AIBV H120 strain. The embryos up to 0.1% of NBO treated groups and negative control embryos were alive and tested negative to AIBV H120 strain, whereas the embryos in positive control died and tested positive. **Conclusion:** The *in vitro* trial proven that the essential oil Blend can deactivate the Avian Infectious Bronchitis Virus H120 (AIBV H120) in *in vitro* medium.

Key words: Avian infectious bronchitis virus (AIBV) H120, *in vitro* test, natural oil blend, allantoic fluid, human corona virus (COVID-19), respiratory disease, RNA virus

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Avian Infectious Bronchitis Virus (AIBV), commonly called Chicken SARS Corona Virus, is an acute avian gamma coronavirus. It is only infectious to chickens. The sign of infection in diseased birds can recognize by respiratory signs, decreased egg production and egg quality. The other species, like pheasants and peafowl, reported being subclinically infected. The AIBV is an enveloped virus with a single positive-stranded RNA genome, which replicates in the host cell cytoplasm[1]. Proteins encoded by AIBV include the viral RNA polymerase, structural spike proteins, membrane and nucleocapsid and various other regulatory proteins. The spike glycoprotein mediates cell attachment and plays a significant role in host cell specificity[2]. The AIBV has a worldwide distribution with many antigenic types that can cocirculate in a given region. The variation in AIBV historically arises from mutations accumulating over time as the virus replicates (genetic drift). The AIBV is transmittable through shedding off by diseased chicken in respiratory discharges and faeces. It spreads by aerosol, ingestion of contaminated feed and water and even in contact with contaminated farm equipment and accessories. The incubation period of Chicken AIBV is generally 24-48 hrs, with the peak in the excretion of the virus from the respiratory tract lasting 3-5 days after infection[3].

Medication is not available to alter the course of AIBV infection. Due to differences in antigenic types of the AIBV causing the disease, do not cross-protect, complicating control efforts. The vaccination for AIBV is only partially successful due to the continual emergence of antigenic variants.

It is crucial to understand how coronavirus infects the host and identifying genes involved in resistance. The importance is not only for the poultry industry, but it has significant implications for human health as diseases like SARS, COVID-19[4,5].

The role of natural oil blend against enveloped viruses are well known. The natural essential oils, like, *Gardenia jasminoides*, *Commiphora myrrha*, *Boswellia serrata*, *Foeniculum vulgare* and *Daucus carota*, have anti-viral and immune-modulating properties. These natural oils blended and tested using *in vitro* medium successfully against the Avian Infectious Bronchitis Virus (AIBV) H120 strain.

MATERIALS AND METHODS

Research station: The *in vitro* trial was conducted at the biosecure laboratories of Faculty of Pharmacy, Universitas Padjadjaran (Padjajaran University), Indonesia, Faculty of Medicine, Universitas Padjadjaran (Padjajaran University), Indonesia and PT. Biotis Prima Agrisindo, Bogor Indonesia, during February-March, 2020.

Natural Oil Blend Formulation (NOBF) Preparation and Composition: Each single oil *Gardenia jasminoides*, Commiphora myrrha, *Boswellia serrata*, *Foeniculum vulgare* and *Daucus carota* obtained from the vendors who comply with the strictest industry practices: Demeter Agro Research and Improvements Pty Ltd. New Directions Australia Pty Ltd. and Australian Botanical Products Pty Ltd. Each essential oil is obtained through a steam distillation process and should undergo thorough checking for the quality and chemical compositions. After the essential oils are declared to pass the quality checking, the mixture of the NOs conducted with the following sequence and percentage: *Gardenia jasminoides*, *Commiphora myrrha*, *Boswellia serrata, Foeniculum vulgare* and *Daucus carota* are added in equal quantities to form the oil mixture they mixed with extra light olive oil, so we get 1% concentration of the final product. The efficacy of natural oil blend in its different concentrations tested against the Avian Infectious Bronchitis Virus (AIBV) H120 strain.

Embryonated chicken egg preparation: Pre-trial was subjected to several passages to allow the Avian Infectious Bronchitis Virus (AIBV) H120 strain to adapt and replicate to high titer in the allantoid cavity of 9-11 day-old chicken embryo. The purified virus was inoculated into the allantoid cavity of specific pathogen-free eggs and incubated at 36-37°C. The allantoic fluid was harvested after 72 hrs from eggs that were chilled overnight and tested for the presence of AIBV using serological tests. After 6 days, inoculated eggs were opened and observed for characteristic AIB lesions such as curling and dwarfism of the infected embryo.

Culture avian infectious bronchitis virus strain H120: The AIBV H120 strain prepared for the trial following the steps of the OIE Terrestrial Manual[6,7]. The procedure summarized as below:

- Samples were placed in cold transport media containing penicillin (10,000 International Units [IU] mL^{-1}) and streptomycin (10 mg mL^{-1}) and kept on ice and are frozen as soon as possible
- Suspensions of tissues (10-20% w/v) prepared in sterile phosphate-buffered saline (PBS) for egg inoculation

Table 1: Natural oil blend doses against the AIBV treatment groups (T_1 to T_{12}), NOB control 1, NOB control 2, positive control and negative control

	Treatments												EOB		Control	
Materials	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}	Control 1	Control 2	Positive	Negative
Virus/Allantoic fluid (mL)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	0
NOB (mL)	2.5	1.25	0.625	0.3125	0.156	0.08	0.04	0.02	0.01	0.005	0.0025	0.00125	5	0.16	0	0
Saline Water (mL)	2.5	3.75	4.375	4.6875	4.998	4.92	4.96	4.98	4.99	4.995	4.9975	4.99875	0	4.84	5	10
NOB (%)	25	13	6.3	3.1	1.56	0.8	0.4	0.2	0.1	0.05	0.025	0.0125	100.0000	1.6000	0.0000	0.0000

- The suspension was clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 μ) before the injection of SPF embryonated chicken eggs
- The 0.1-0.2 mL of sample supernatant inoculated into the allantoid cavity of 9-11 day old embryos

Saline solution: Total 0.85% of saline solution was freshly prepared.

Trial design: The trial conducted in 12 different concentrations (T_1-T_{12}) of Natural Oil Blend (NOB) by combining NOB and saline solution in a different ratio. The controls were prepared, NOB Control 1 (inoculated with 0.2 mL 100% NOB), NOB Control 2 (inoculated with 0.2 mL 1.6% NOB), Positive Control and Negative control. The detailed design is in Table 1. The 0.2 mL of AIBV H120 was inoculated in positive control and treatment groups, whereas no inoculation made in the negative control.

Inactivation: The diluted Natural Oil Blend solution mixed using vortex for ±30 sec. Then, gently transferred the NOB solution to the Erlenmeyer flask antigen contained. We incubated the specimen in a 37°C shaker incubator for 16-18 hrs.

Embryonated chicken egg incubation: Prepared 10 day-old chicken embryo for 12 groups of treatment groups (T_1-T_{12}), NOB Control 1, NOB Control 2, Positive control and negative control group. The 0.2 mL of purified virus AIBV H120 was inoculated into the allantoid cavity of eggs and incubated at 36.5°C. Eggs with dead embryos within 24 hrs of post-inoculation discarded as non-specific. The eggs were candled daily to monitor embryo viability. The allantoic fluid harvested after 72 hrs from eggs that were chilled overnight and tested for the presence of AIBV using HA tests. After 6 days, inoculated eggs were opened and observed for characteristic AIB lesions such as curling and dwarfism of the infected embryo.

Embryo viability test: Visual observation was performed to test the embryo viability. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros and embryo mortality. Infective allantoid fluids were kept at -20°C after lyophilization.

RESULTS AND DISCUSSION

Avian infectious bronchitis virus strain H120: The extracted and purified Avian Infectious Bronchitis Virus strain H120 showed high similarities with other IBV strains in the gene bank (Fig. 1). The complete sequence is attached to the manuscript.

Natural oil blend as anti-AIBV H120 supplement: The natural oils used to formulate the essential oil blend are having anti-viral properties. *Gardenia jasminoides* reported working efficiently against influenza virus strain A/FM/1/47-MA *in vivo*[8] and H5N1 strain[9] and WSSV in crayfish[10,11].

The *Commiphora myrrha*, commonly known as resins, has anti-viral, anti-fungal and antioxidantproperties[12-14]. *Boswellia serrata* have shown anti-viral properties as reported by Rhein *et al.*[15]. *Foeniculum vulgare* has shown anti-viral properties against Potato Virus X, Tobacco Mosaic Virus and tobacco ringspot virus[16]. The oil of *Daucus carota* has anti-microbial properties, especially against gram-positive bacteria and the Candida group of bacteria[17]. The previous trials conducted by the co-authors on the animal viral and bacterial pathogen have proven that essential oils are having anti-viral and anti-bacterial properties[18-21]. The obtained results have also shown immunomodulating properties of essential oils.

Embryo viability test: The portion of the shell above the air cell was removed using the sterile forceps. The embryo was taken out and put on the petri dish for observation. The embryos of Negative control and NOB-treated groups were alive up to T_9, whereas mortality and deformity were observed in T_{10}, T_{11} and T_{12} groups (Table 2 and Fig. 2). The embryos of positive control showed several defects, such as dehydration, curly legs, stunting and other abnormal features (Table 2 and Fig. 2). All the embryos died in NOB Control 1 group, probably due to the oil layer at the top.

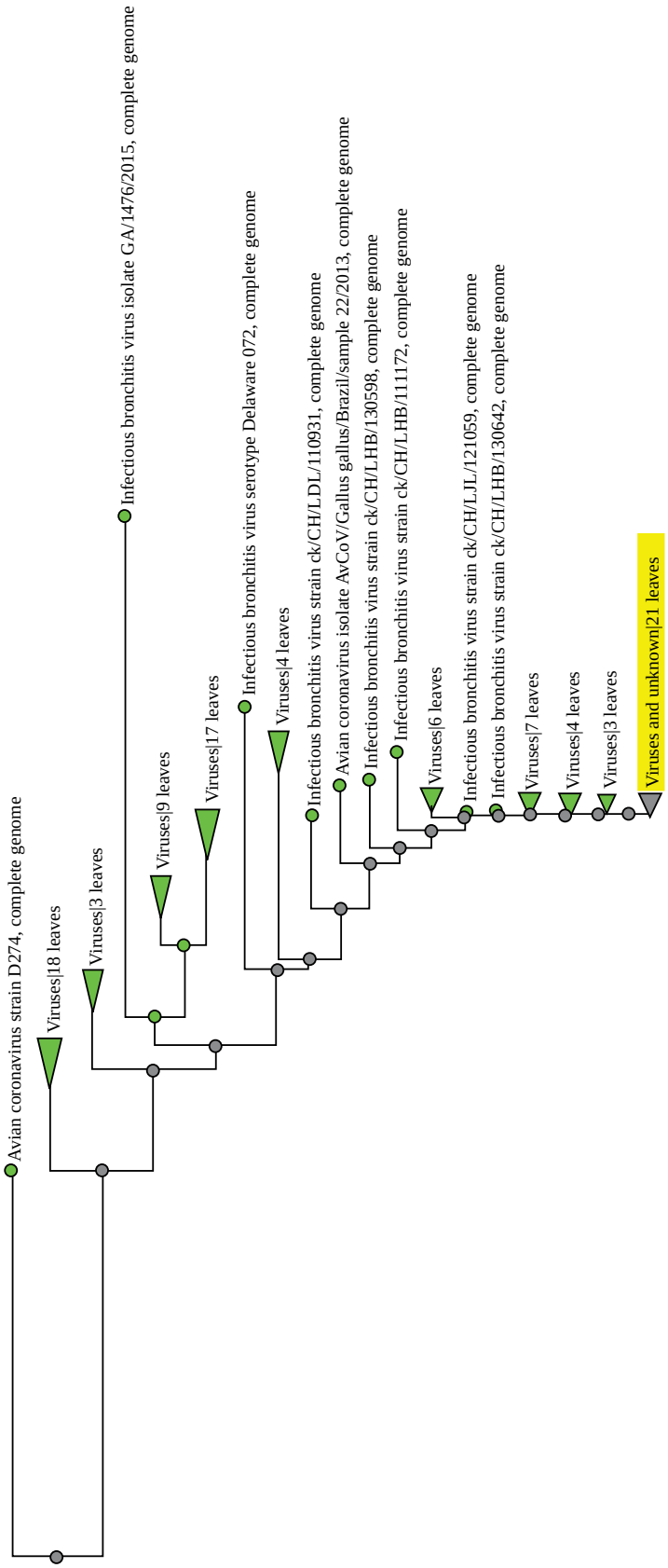

Fig. 1: Avian infectious bronchitis virus strain H120 showing high similarities with other IBV strains in blast analysis

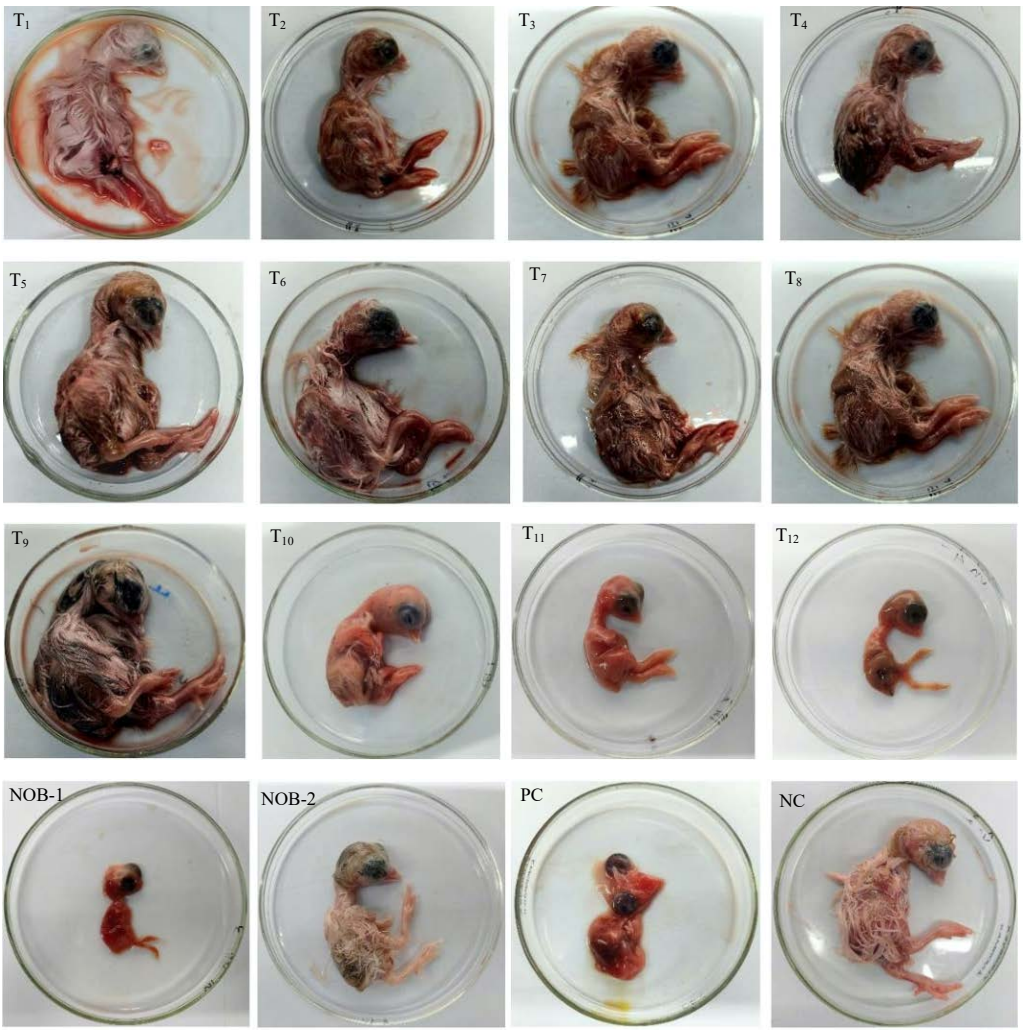

Fig. 2: Visual observation of embryos of different trial groups

Treatment groups (T_1-T_{12}), NOB control 1, NOB control 2, Positive control and Negative control groups. NOB: Natural oil blend

Table 2: Visual observation of embryos of different trial groups

		Embryos observations		
Groups	Total embryos	Viable embryos	Dead embryos	Abnormal/Undeveloped embryos
T_1	5	5	-	-
T_2	5	5	-	-
T_3	5	5	-	-
T_4	5	5	-	-
T_5	5	5	-	-
T_6	5	5	-	-
T_7	5	5	-	1
T_8	5	5	-	-
T_9	5	5	-	-
T_{10}	5	5	1	2
T_{11}	5	5	2	3
T_{12}	5	5	4	1
NOB control 1	5	-	5	-
NOB control 2	5	-	2	3
Positive control	5	2	2	1
Negative control	5	5	-	-

NOB: Natural Oil Blend, Treatment groups (T-1 to T-12), NOB control 1, NOB control 2, Positive control and Negative control groups

Two embryos died and 3 showed abnormalities in NOB Control 2. The data showed that the Natural Oil Blend concentration starting from T_{10}, which is 0.005 mL or 0.05% are less effective in inhibiting the Avian Infectious Bronchitis Virus (AIBV) to propagate and cause abnormalities and death to the embryos efficiently. In this study, we also found that natural oil blend treatment is inhibiting the growth of embryo when given at 100% concentration.

The findings prove that the Natural Oil Blend (NOB) has potent anti- Avian Infectious Bronchitis Virus (AIBV) strain H120 properties. The recent findings from Drosten *et al.*[4] and Ksiazek *et al.*[5] have identified and discovered that the Avian Infectious Bronchitis Virus (AIBV) has high genetic similarities with Human Corona Virus (COVID-19) and that it uses the same infection mechanism.

The obtained results give strong evidence that the current formulation using blend oil has anti-viral properties, especially against the RNA group of viruses. It paves the path to work further on a similar group of viruses like COVID-19. The formulation is more comfortable to develop and durable. The recommendations would be to apply and test the product on a large scale in commercial farms to have better feedback. The success of commercial-scale farms will enhance the limit and application of the formulations.

CONCLUSION

Avian Infectious Bronchitis Virus (AIBV) is one of the deadly diseases of chicken. Natural Oil Blend is a combination of the following oils Gardenia jasminoides, Commiphora myrrha, Boswellia serrata, Foeniculum vulgare, and Daucus carota with the purpose of viral inactivating properties. The in vitro trial results showed that various Natural Oil Blend doses were effective against the Avian Infectious Bronchitis Virus (AIBV) H120 strain. Natural Oil Blend is a potential anti- Avian Infectious Bronchitis Virus (AIBV) candidate. The large scale trial will pave the path to determine the formulation effectiveness against other viruses and overall contribution to the production of healthy animals and their products.

SIGNIFICANCE STATEMENT

This study discovered the anti-Avian Infectious Bronchitis Virus (AIBV) properties of the natural oil blend. The *in vitro* level test proved the efficacy of the NOB against AIBV up to the lowest dilution level. This study will help the researchers uncover the critical areas of infectious lung diseases of poultry and other animals that researchers have not been able to explore yet. Thus a new theory on uses of natural blend oil against AIBV will help to minimize the incidence of similar diseases.

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