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Detection of Influenza A virus contamination in Newcastle Disease live virus vaccines and their pathological effects on visceral organs

Munir Hussain [1], Muhammad Saeed Imran [1], Asim Aslam [1], Nafeesa Yasmeen [1], Sajjad Hussain [2], Shahzad Munir [3], Mubashir Hussain [4], Ahmad Kamran Khan [5], Muhammad Usman [6], Abdullah Jalal [7], Muhammad Ameen Jamal [8], Mir Zulqarnain Talpur [9], Irfan Ahmed [9,*], Zulqarnain Baloch [10,*]

[1]Department of Pathology, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan
[2]Veterinary Research Institute Lahore-54000, Pakistan
[3]Faculty of Plant Protection, Yunnan Agricultural University, Kunming 650201, Yunnan, China
[4]Department of Microbiology, Kohat University of Science and Technology Kohat, KP, 26000, Pakistan
[5]Department of Entomology, University of Agriculture Faisalabad, Pakistan
[6]Livestock and dairy development department, Government of Punjab, Pakistan
[7]Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan
[8]Department of Animal Breeding, Genetics and Reproduction, Yunnan Agricultural University, Kunming 650201, Yunnan, PR China
[9]Yunnan Provincial Key Laboratory of Animal Nutrition and Feed, Yunnan Agricultural University, Kunming, Yunnan Province, People's Republic of China
[10]College of Veterinary Medicine, South China Agricultural University, 483 Wushan Road, Tianhe District, Guangzhou, 510642, China
*corresponding author e-mail address: ***znbalooch@yahoo.com, irfanahmad166@yahoo.com***

ABSTRACT

To investigate this perception that the NDV live virus vaccine could be the source of Avian Influenza A virus (H9) contamination. Sixteen samples of ND live virus vaccines were purchased from the local market. Prior to use in birds, the samples were tested for Avian Influenza A virus contamination through RT-PCR and used in live birds for any gross pathology and histopathology changes. All the samples were negative against Avian Influenza A virus. Furthermore, these vaccines were also used in broiler and desi chicks at day 5 and day 21 through eye drop and drinking water route respectively. Then, these birds were slaughtered at day 10, 20, 30 and 40 for any gross pathological and histo-pathological changes against Avian Influenza (H9). There were no macroscopic and microscopic lesions observed in visceral organs like trachea, lungs, liver and spleen for Avian Influenza. The results of the study using RT-PCR indicated that the ND live virus vaccine both (local and imported) was free of Avian Influenza A virus (H9). There was a perception among some technical persons that some Avian Influenza outbreaks in the field might be through the source of Newcastle Disease live virus vaccine. This theory regarding contamination of Avian Influenza A virus in Newcastle Disease live virus vaccines found to be wrong on the basis of this study and these commercial vaccines placed in the market are safe to use against Newcastle Disease and are not source of Avian Influenza outbreaks in the field.

Keywords: *Live vaccines; Broilers; Avian Influenza; PCR; Histopathological changes.*

1. INTRODUCTION

Avian Influenza viruses typically produce Syndromes ranging from asymptomatic infection to respiratory disease and drops in egg production to severe, systemic disease with near 100% mortality [1]. AI viruses in domestic poultry produce clinical sign reflect abnormalities in the respiratory, digestive, urinary, and reproductive organs [2]. To date, naturally occurring virulent influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5, H7 and H9 subtypes. Influenza A viruses of subtype H9 are now considered to be widespread in poultry and have demonstrated the ability to infect humans [3, 4]. All outbreaks of the highly pathogenic form have been caused by influenza A viruses of the subtypes H5 and H7. The disease is transmitted horizontally by direct contact through contamination. There is little or no evidence of vertical transmission (egg-borne infection). However, eggshell surfaces can be contaminated with the virus [5]. Wild and domesticated waterfowl is the major natural reservoir of influenza A viruses. Representatives of all the different subtypes of avian influenza A virus have been isolated from birds, particularly from aquatic species such as ducks, geese, and gulls [6]. Wild birds such as geese, ducks and game birds; they can be carriers of even highly pathogenic strain H5N1 shedding the virus in their feces without clinical sign of disease. Vaccination is one of the most effective ways to prevent the poultry birds from the specific diseases. Usually the biological substance is avirulent, the live disease organisms, which are capable to protect the bird against the particular disease by producing an immune response. Presence of these organisms (antigen) in the blood stimulates the body's defense mechanism to produce antibodies that neutralize the disease-causing organisms when the bird is exposed to them [7]. A danger of such type of live vaccines are that the live microbes can back mutate to a virulent form. While dead vaccines that contain whole killed (usually by formalin or phenol) microbes are safe. They may contain little or no extraneous material and therefore tend to produce fewer adverse effects. Due to capability of influenza virus mutation into a form that is more transmissible among humans, enhance the pandemic risk and mass mortality event [8]. The vaccines that contain dead organisms are safe with respect to residual virulence and are easy to store, since organisms are already dead. While live vaccines may possess residual virulence for the animal by

reversion of avirulent organisms to fully virulent type or spread to non-vaccinated animals.

Dead vaccines have very little risk of 'alive' contamination, while live vaccines always run the risk of contamination with unwanted organisms; for instance, outbreaks of reticuloendotheliosis in chickens in Japan and Australia have been traced to contaminated Marek's disease vaccine [9]. Thus, the present study was carried out to examine the viral contamination (Influenza A virus) in poultry vaccines manufactured locally and imported from different countries to Pakistan. The findings of the study have helped us to see the Avian Influenza A virus contamination in vaccines which are used in field conditions and also help to evaluate the purity of vaccines.

2. MATERIALS AND METHODS

Total sixteen samples of ND live virus vaccines were collected, out of which, the 4 samples were collected from company A (Local Manufacturer), 4 samples from company B (Local Manufacturer), 4 samples from company C (Imported) and 4 samples from company D (Imported) as shown in Table 1. Samples were brought in thermopole with ice bags to Quality Operation Lab, WTO University of Veterinary & Animal Sciences, Lahore-Pakistan and were kept in cold chain at the temperature 2-8 C° which is prescribed for storage. Each sample was diluted by adding 0.5 ml distilled water in each vial of 1000 doses for the proceeding of RT-PCR in safety cabinet. Viral RNA was extracted with TriZol LS reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. NanoDrop was used for the analysis of RNA both to quantify the RNA concentration and to determine its purity. Thermo Scientific NanoDrop™ 2000/2000c Spectrophotometer measure 0.5- 2 µL samples with high accuracy and reproducibility. Pure RNA of sample 1 from company A typically yielded a 260/280 ratio of ~1.41 with 163.2 ng/µL concentration, RNA of sample 2 from company B yielded 260/280 ratio of~ 1.47 with 297.5ng/µL concentration, RNA of sample 3 from company C yielded 260/280 ratio of~ 1.37 with 446.8ng/µL concentration and RNA of sample 4 from company D yielded 260/280 ratio of~ 1.03 with 1195.5ng/µL concentration. cDNA was synthesized using eluted RNA with transcriptor first strand cDNA synthesis Kit® (Fermantas, USA) according to manufacturer's instructions.

Table 1. Samples type and numbers.

Company Name	Local Manufacturer/Importer	Vaccine	No. of Samples
Company A	Local Manufacturer	ND LaSota strain	4
Company B	Local Manufacturer	ND LaSota strain	4
Company C	Importer	ND LaSota strain	4
Company D	Importer	ND LaSota strain	4

Ethical Approval statement

This study protocol was approved by the institutional ethic committee of University of Veterinary & Animal Sciences, Lahore-Pakistan

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Synthetized cDNA was amplified using H9 gene primers set (Table. 2) for confirmation of challenge virus. A total of 25µL reaction mixture was prepared by using 5µL cDNA, 1µL each of forward and reverse primers for H9 genes, 10 µL of 2X Mastermix (Invitrogen, USA) and 8 µL of Nuclease free water (DEPC, Invitrogen, USA) (Table 2).The amplification of H9 gene was carried out according to the following program, initial denaturation at 94 ºC for 4 minutes, denaturation at 94 ºC for 30 sec, annealing at 60ºC (H9) for 30 sec, extension at 68 ºC for 1 mint and last extension at 68 ºC for 7 minutes (Table. 3). RT-PCR products were detected by Agarose Gel Electrophoresis. Agarose gel 1.5% was prepared (Annexure-1) and placed in the electrophoresis tank filled with 1X TAE buffer containing 3µL ethidium bromide. PCR products (5 µL) with loading dye (3 µL) (6X) was loaded in the wells. Molecular weight marker (2 µL) (DNA Ladder of 100bp) was added to one of the wells. A charge of 110 volts/300 Amp was passed through the gel for 30 minutes. The gel was viewed under UV light in Gel documentation system (Bio-Rad, USA), as shown in Figure 1 and Table 4.

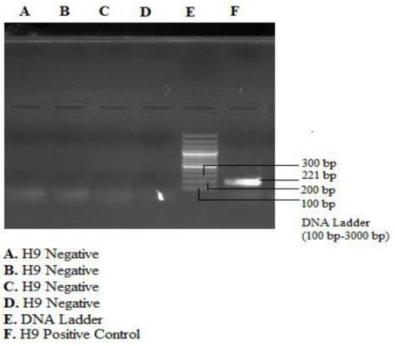

Figure 1. Showing ND live virus vaccine samples negative for H9 virus, DNA Ladder and H9 positive control.

Table 2. Primer sequences for H9 genes amplification.

Primer Name	Sequence (5'---------3')	Primer length	Product size
F-H9	ATCGGCTGTTAATGGAATGTGTT	23bp	221bp
R-H9	TGGGCGTCTTGAATAGGGTAA	21bp	

Table 3. Thermo cycler conditions for H9 gene amplification.

Stages	PCR Conditions	Cycles
Initial Denaturation	94^{o}C for 4 min	1
Denaturation	94^{o}C for 30sec	40
Annealing	60^{o}C for 30sec	
Extension	68^{o}C for 1 min	
Final Extension	68^{o}C for 7min	1

Table 4. No H9 contamination in Newcastle Disease live virus vaccine.

Vaccines/DNA Ladder/Positive Control	Samples	No. of samples examined	No. of positive samples
ND LaSota (Local)	A	2	0
ND LaSota (Local)	B	2	0
ND LaSota (Imported)	C	2	0
ND LaSota (Imported)	D	2	0
DNA Ladder	E	2	2
Avian Influenza A virus (H9) +ve Control	F	2	2

Gross and histopathology of samples

Gross pathological examination was done to get diagnostically critical information from presented birds. An adequate data was collected with help of such diagnostic tool. After every 10 days, 5 birds from each broiler sub-group and 2 birds from each Desi sub-group were slaughtered and gross pathology was performed. The following organs including trachea, lungs, liver and spleen were examined for gross lesions. Birds of each group were slaughtered at age of 10, 20, 30 and 40 for postmortem. From the excised tissues gross lesions were observed on visceral organs. Tissue samples of liver, spleen, lungs and trachea were collected after postmortem and preserved in 10% buffered formalin (Formalin 100ml, Sodium dihydrogen phosphate 4 gm, Disodium hydrogen phosphate 6.5 gm and Dis. water 900 ml). For histopathological study tissues samples were processed by techniques described by [10]. From each group of experimental birds a complete record of mortality was noted on daily basis. In order to identify the cause of death, postmortem of each dead bird was done according to [11, 12, 13].

3. RESULTS

The present study was carried to detect the contamination of Influenza A virus in Newcastle Disease live virus vaccine and their pathological effects on visceral organs. A total of 8 samples, out of the 4 samples A, B, C, D of Newcastle Disease live virus vaccine (both local & imported), followed by other 4 samples and 1 sample F of Influenza A virus (H9) which is used as positive control, were examined by RT-PCR. All 8 samples A, B, C and D samples were found to be negative for Avian Influenza A virus (H9) contamination, while sample F was found to be positive for H9. Regarding research design, RT-PCR was conducted for possible contamination of Avian Influenza A virus in Newcastle Disease live virus vaccine. The results on the basis of RT-PCR test indicate that both the local and imported ND LaSota strain live virus vaccines were free of Avian Influenza A virus (H9) contamination.

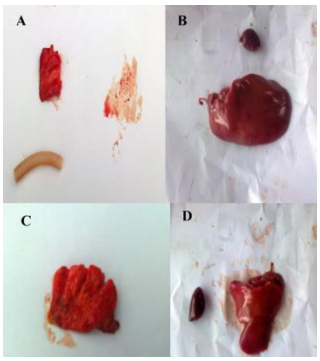

Figure 2. Gross lesions. A. Normal Trachea and Lungs without any gross lesions (Sub-group –A), B: Normal liver and spleen without any gross lesions (Sub-group-B). C: Lungs did not show.

The gross pathology of the organs including trachea, lungs, liver and spleen were examined for gross lesions. All groups were absent for any specific gross lesions against avian influenza (H9) (Figure 2, 3, 4).The histopathology including liver, spleen, trachea and lungs were studied. The groups A and B showed accumulation of RBCs in bronchioles and degeneration of some hepatocytes in liver, respectively. The group C showed no lesions but D group exhibited lungs with mild congestion and edema. The group E exhibited no lesion and F showed lungs with mild congestion and edema (Figure 5). The group G and H exhibited degeneration and necrosis of splenic cells and normal liver without any histopathological lesions, respectively. The group 4 showed coagulative necrosis in spleen and accumulation of RBCs in bronchioles against the avian influenza (H9) (Figure 6; Table 5).

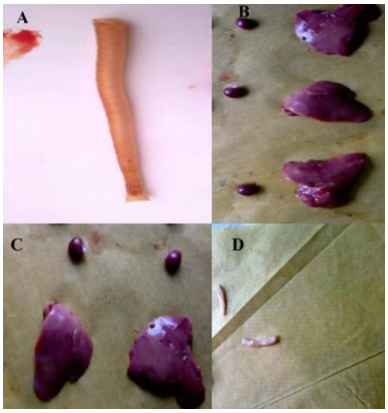

Figure 3. Gross lesions. A: No specific gross lesions were observed in trachea (Sub-group-E). B: No specific gross lesions were observed in liver and spleen (Sub-group-F) 3: Gross lesions were not observed in liver and spleen (Sub- group-G), D: Gross lesions were not observed in trachea (Sub-group-H).

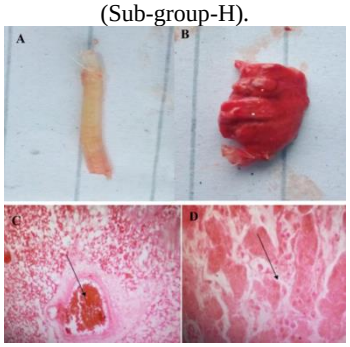

Figure 4. Gross lesions. A: Normal trachea without any gross lesions (Group-4), B: Normal lungs without any gross lesions (Group-4), C: Arrow showing accumulation of RBCs in bronchioles of lungs (Sub-.

The present study was conducted to assess the assumption regarding the Avian Influenza virus serotype (H9) that might be the causative agent of Avian Influenza through Newcastle Disease virus vaccine. To investigate this perception that usage of the NDV live virus vaccine could be the source of Avian Influenza A virus (H9) contamination, sixteen samples of ND live virus vaccines were purchased from the local market. These samples prior to use in chickens were tested for traces of Avian Influenza A virus through RT-PCR. Moreover, the live birds used in this study were checked for any gross pathological and histopathological changes against Avian Influenza. There were no macroscopic and microscopic lesions observed in visceral organs like liver, trachea, lungs and spleen for Avian Influenza. A study was conducted on positive identification of Newcastle disease

virus vaccine strains and detection of contamination in vaccine batches by restriction site analysis of the matrix protein gene [14, 15]. Farsang et al. 2003 [16] prepared twelve vaccine batches from avirulent vaccine strains of Newcastle disease virus produced by seven manufacturers were identified by analysis of the matrix protein gene with restriction enzymes. The analyses revealed the presence of the strain indicated by the manufacturers (namely B-1, LaSota or Ulster 2C), except in one case when the vaccine contained strain V4 Queensland instead of VGGA as indicated. In addition, several batches of both monovalent and combined vaccines containing strain LaSota of the same company consistently disclosed contamination with strain B-1 that's why our results are controversial to their work. Amer et al. 2011 [17] studied that use of embryonated chicken eggs in preparation of avian virus vaccines is the principal cause for contamination with *Chicken Anemia Virus* (CAV). Identification of CAV in contaminated vaccines relies on the expensive, tedious, and time-consuming practice of virus isolation in lymphoblastoid cell lines. The experience of the last 2 decades indicates that polymerase chain reaction is extending to replace most of the classic methods for detection of infectious agents. In the present report, a simple, rapid, and accurate polymerase chain reaction method for detection of CAV in poultry vaccines are described. Bruhn et al. 2005 [18] carried out studied regarding *Avian Reovirus* (ARV) contamination in different poultry live virus vaccines through RT-PCR and he was able to find ARV contamination in these vaccines, so contrast with our results. PCR for *mycoplasmal* contamination in poultry live virus vaccines to find this bacterial contamination [19] which makes the contrasting results with our findings. So, on the basis of RT-PCR test, ND live virus vaccine samples did not show evidence of the traces of Avian Influenza (H9). The results of study using RT-PCR indicate that the ND live virus vaccine both (local and imported) were free of *Avian* Influenza A virus (H9). There was a perception among some technical persons that some Avian Influenza outbreaks in the field might be through the source of Newcastle Disease live virus vaccine. This theory regarding contamination of Avian Influenza (A) in Newcastle disease live virus vaccines found to be wrong on the basis of this test and these commercial vaccines placed in the market are safe to use against Newcastle disease and are not the source of Avian Influenza outbreaks in the field.

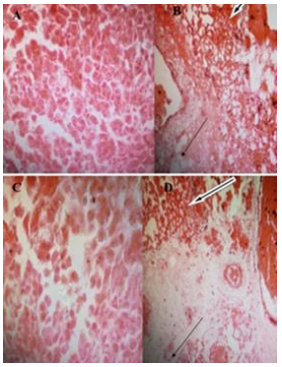

Figure 5. Histopathological changes. A: Normal spleen without any histo-pathological changes (Sub-group-C), **B:** Arrows indicating lungs with mild congestion and edema (Sub-group-D), **C:** No histopathological changes were observed in spleen (Sub-group-E), **D:** Lungs with mild congestion and edema.

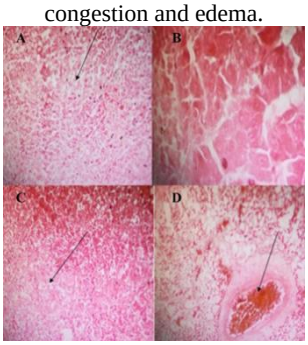

Figure 6. Histopathological changes. A: Arrow showing degeneration and necrosis of splenic cells (Sub-group-G), **B:** Normal liver without any histopathological changes (Sub-group-H), **C:** Arrow indicating coagulative necrosis, degeneration and presence of pink color debris in spleen (Group-4), D: Arrow showing accumulation of RBCs in bronchioles of lungs (Group-4).

Table 5. Gross pathology and Histopathology results for Avian Influenza (H9) at different age.

Groups	No. of Birds	Vaccination (Age in days)	Post mortem performed (Age in days)	Gross Pathology against Avian Influenza (H9)	Histopathology against Avian Influenza (H9)
A	20	5 & 21	10,20,30 & 40	No specific lesions were observed	Accumulation of RBCs in bronchioles
B	20	5 & 21	10,20,30 & 40	No specific lesions were observed	Degeneration of some hepatocytes in liver
C	20	5 & 21	10,20,30 & 40	No specific lesions were observed	Normal spleen without any histopathological changes in any bird
D	20	5 & 21	10,20,30 & 40	No specific lesions were observed	Lungs with mild congestion and edema
E	10	5 & 21	10,20,30 & 40	No specific lesions were observed	Normal spleen without any histopathological changes in any bird
F	10	5 & 21	10,20,30 & 40	No specific lesions were observed	Lungs with mild congestion and edema
G	10	5 & 21	10,20,30 & 40	No specific lesions were observed	Degeneration and necrosis of splenic cells
H	10	5 & 21	10,20,30 & 40	No specific lesions were observed	Normal liver without any histopathological changes in any bird
4	40	No vaccination	10,20,30 & 40	No specific lesions were observed	Coagulative necrosis in spleen and accumulation of RBCs in bronchioles

4. CONCLUSIONS

The results on the basis of RT-PCR and vaccination in birds indicated that the ND live virus vaccine either local or imported was free of Avian Influenza A virus (H9) contamination. There was a perception among some technical persons that some Avian Influenza outbreaks in the field might be through the source of Newcastle Disease live virus vaccine, found to be wrong with confirmation of RT-PCR test. So, these commercial vaccines placed in the market are safe to use against Newcastle Disease and are not the source for Avian Influenza outbreaks in the field.

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