A study on the sterility, safety, potency and purity of hydropericardium hepatitis syndrome (Angara disease) vaccines

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The present study was conducted in order to determine the quality of commercially available Angara disease vaccines. For the experiment, 200 broiler chicks of one day old were reared at National Veterinary Laboratories, Islamabad in isolation units. Chicks were divided into three groups that is, 1, 2, 3 and having 25, 25 and 150 chicks, respectively. Group 1 was used to determine LD₅₀ of the virus in chicks, whereas the chicks of group 2 and 3 were used to assess the safety and potency of the vaccines. The LD₅₀ (lethal dose 50) of the virus was determined in 26 days old broiler chickens divided into sub-groups a, b, c, d and e. The LD₅₀ titre of the (10%) viral suspension was prepared from liver extract and determined as 10⁻².₄ per ml. During investigation before use, the sterility of the vaccines was carried out by culturing on microbiological media. All four vaccines were found free from any contamination. The safety standard of the vaccines was also tested by inoculating in 25 chicks of sub-groups F, G, H, I and J, each contained 5 chicks. The chicks were kept under observation for five days post-vaccination. All four vaccines used in chicks were found safe. During study, the potency of the vaccines was determined by vaccinating 150 chicks of sub-group K, L, M, N and O with dose of 0.2 ml. The vaccinated and non-vaccinated chicks of sub-groups were challenged with viral dose of 2 ml at day 17 post-vaccination to know the protection potency of the vaccines. No any chick showed clinical manifestation of disease up to five days post challenge. On post-mortem examination, lesions of hydropericardium syndrome such as straw colour like fluid was observed around the heart. Other lesions recorded were hydronephrosis and hepatitis. Sera of different groups were checked for demonstration of antibody titration. A higher antibody titre was observed at day 21 as compared to 7 and 14 days post vaccination. Purity of vaccines was also determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Multiple proteins bands were seen however, a band at the position of 18.6 Kda was observed in all four vaccines and positive control as well, which was detected as viral protein. All four vaccines showed similar viral protein profile by SDS-PAGE.

Key words: Quality, antibody, potency, Angara disease, titre.

INTRODUCTION

Fowl adenoviruses are very heterogenous viruses with numerous serotypes (FAV 1-12). Avian adenovirus serotype-iv (Benko et al., 2005) is prevalent in Pakistan for causation of hydropericardium syndrome (HPS), which is also known as Angara disease was first recognized in broiler flocks in Angara Goth near Karachi.
Metropolitan City of Pakistan, in late 1987 (Jaffery, 1988). Because of the emergence of disease in Angara Goth, the disease was initially referred to as “Angara disease”. This syndrome initially causes havoc in thickly populated broiler growing areas and then in breeding and laying flocks (Jaffery, 1988). The most important gross lesion of Angara disease is filling of pericardial sac with straw coloured fluid measuring up to 13-15 cc or even more, which is crystal clear, colorless, sometimes yellowish, greenish or amber, colored. Pericardial sac, itself is membranous transparent and the heart is mis-shaped and flabby. Other most commonly encountered lesions include necrotic foci on the liver, with mottling appearance and hepatomegaly (Rabbani., 1997; Ganesh, 1998).

The consistency of liver is usually friable. In some of the affected birds, lungs show congestion, edema and exudation of frothy material from the bronchi. Sometimes hemorrhages on the muscles of thigh and breast are also observed (Niazi, 1989). Both types of aqua based and oil based vaccines are used for the control of disease in Pakistan which provides 100% protection (Mehmood et al., 2011). Therefore the present study was designed to determine the quality of commercially available Angara disease vaccines, which are useful in controlling the Angara disease, the quality of vaccines is directly involved in the immunization of birds.

MATERIALS AND METHODS

Rearing of chicks

For the present study, 200 a day old quality broiler chicks were purchased from the market and were reared at Poultry Research Institute (PRI), Rawalpindi and National Veterinary Laboratories (NVL), Islamabad in poultry rearing units. The chicks were further divided into groups 1, 2 and 3 containing 25, 25 and 150 chicks, respectively. Twenty-five chicks of group 1 were reared at PRI, Rawalpindi and chicks of group 2 and 3 were reared at NVL, Islamabad.

Feed and water were provided ad-libitum. Twenty-five chicks of group 1 were divided into five subgroups named group A, B, C, D and E having five chicks in each group. These chicks were used to determine the biological titre that is, LD₅₀ of the virus suspension to use further in this study. Twenty-five chicks of group 2 were divided into five subgroups named as sub group F, G, H, I and J with five chicks in each sub group. These sub groups were used for vaccine safety test. 150 chicks of group 3 were divided into five sub groups named as K, L, M, N and O with 30 birds in each sub group. These sub groups were used for vaccine potency testing.

Procurement of Angara disease vaccines

Four Commercially available Angara Disease vaccines were selected and purchased from the market for quality testing. Their brand names were kept confidential. These vaccines were granted codes as AD Vac-I, AD Vac-II, AD Vac-III and AD Vac-IV for study purpose. These vaccines were subjected to sterility, safety, potency and purity testing in the study.

Procurement of antigen

10% infectious Angara disease liver suspension was obtained from the Angara Disease vaccine production laboratory, Disease Section, Poultry Research Institute, Murree Road, Rawalpindi. This virus suspension was used to determined the biological titre that is lethal Dose for 50%, challenge protection test and indirect haemagglutination test.

Determination of biological titre (LD₅₀) of Angara disease (AD) virus

The biological titre that is: LD₅₀ was calculated by the method described by Reed and Muench (1938).

Sterility test of vaccines

Sterility of vaccines were checked on bacteriological and fungal media. The sterility of the vaccines was checked on bacteriological synthetic media including brain heart infusion agar (BHIA), reinforcement clostridial medium (RCM), blood agar (BA), mycoplasma agar (MA) and sabouraud agar (SA).

Safety test of vaccines

Five times higher from recommended dose of vaccines ADVac-I, ADVac-II, ADVac-III and ADVac-IV were administered through recommended route to five chicks of each subgroup named as F, G, H and I, respectively.

Potency test

200 a day old broiler chicks were housed at Animal House, National Veterinary Laboratory (NVL), Islamabad under standard husbandry conditions. Vaccine potency was tested by challenge protection and serology. 10 chicks of subgroup K, L, M, N and O were subjected to challenge protection by 2 ml of 10% infectious virus suspension. These chicks were observed for 5 days post challenge. The serum samples from subgroup K, L, M, N and O were also obtained at day 7, 14 and 21-post vaccination for serology.

Serology

Antibody response of the experimental Angara disease vaccines were determined by indirect haemagglutination test as described (Rehman et al., 1989).

Purity of vaccines

Purity of the experimental vaccines under study were determined by the protein analysis on sodium dodecyl sulphate-polyacrylamide* Corresponding author. E-mail: mughal_161@yahoo.com.

Abbreviations: BHIA, Brain heart infusion agar, RCM, reinforcement clostridial medium; BA, blood agar; MA, mycoplasma agar; SA, sabouraud agar; GMT, geometric mean titre.
Table 1. Determination of lethal dose (LD₅₀) of Angara disease (AD) virus.

<table>
<thead>
<tr>
<th>Sub-groups of chicks</th>
<th>Dilution</th>
<th>Number of chicks inoculated</th>
<th>Number of chicks died</th>
<th>Number of chicks survived</th>
<th>Cumulative proportion death rate</th>
<th>Percentage (%) Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10⁻¹</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>12/12</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>10⁻²</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>7/9</td>
<td>60</td>
</tr>
<tr>
<td>C</td>
<td>10⁻³</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>4/7</td>
<td>40</td>
</tr>
<tr>
<td>D</td>
<td>10⁻⁴</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>2/6</td>
<td>20</td>
</tr>
<tr>
<td>E</td>
<td>10⁻⁵</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>1/5</td>
<td>20</td>
</tr>
</tbody>
</table>

RESULT

The present study was successfully completed at the National Veterinary Laboratories (NVL), Islamabad. Most of the techniques applied throughout the research were directly adopted without any modification. Utmost care was taken in recording the results.

Determination of biological titre (LD₅₀) of Angara disease virus suspension

From subgroups A, B, C, D, and E, respectively, 5, 3, 2, 1, and 1 out of 5 chicks died (Table 1). Biological titre (LD₅₀) of the viral suspension was calculated by the method described by Reed and Muench 1938 (Table 1). The LD₅₀ titre of the 10 percent viral suspension prepared from liver filtrate was found to be 10⁻².⁴ per ml in 26 days old broiler chicks. Lesions recorded in the dead chicks were representative of typical field cases of Angara disease (hydropericardium syndrome). The proportionate distance obtained was corrected by the dilution factor, which was the logarithm of the dilution step employed. Then 50% end point dilution was calculated as,

Negative logarithm of LD₅₀ titre = (Negative logarithm of the next dilution above 50% mortality + PD) x dilution factor = (-2.0.4) x 1 = - 2.4 Log of LD₅₀ titre =10⁻².⁴

Sterility test

Four inactivated vaccines under study were checked for sterility by inoculating each vaccine on synthetic medias (BHIA, BA, RCMA, SA and MA). No growth was observed on BHIA and BA after incubation of vaccine samples aerobically for 24 h at 37°C. Similarly no growth was observed on RCMA after anaerobically incubating for 24 h at 37°C. Furthermore no growth was observed on Sabouraud agar after incubating for 14 days at 25°C. Similarly no growth was observed on mycoplasma agar after aerobically incubating for 24 h at 37°C. All the four vaccines complied with the sterility test.

Vaccine safety testing

Five chicks of each sub group F, G, H AND I were injected by five times higher dose from recommended level with AD Vac-I, AD Vac-II, AD Vac-III and ADVac-IV, respectively, whereas sub-group J was kept for negative control. These sub-groups were observed for five days post inoculation. No bird showed clinical sign of the disease or died from subgroups F, G, H, I and J in the observed period of five days. On termination of this period, all survived chicks of these subgroups were slaughtered and no lesion of Angara disease (hydropericardium syndrome) was found in any bird. All the four experimental vaccines ADVac-I, ADVac-II, ADVac-III and ADVac-IV were found safe for use in the chicks.

Vaccine potency testing

Vaccine potency testing were performed by challenge protection test and antibody titration after vaccination of chicks of sub-groups K, L, M and N. Chicks of sub-group O were kept as non-vaccinated control. The chicks of vaccinated sub groups K, L, M and N were subjected to challenge protection test 17 days post vaccination. As well as chicks of non-vaccinated group O were also challenged on the same day. No bird died or showed clinical manifestation of disease up to five days post challenge in any sub group even sub-group O. On the 6th day post challenge, all chicks of sub groups K, L, M, N and O were slaughtered and subjected to post mortem and the lesions on liver, hydro nephrosis and water around heart (yellow colour) were noted.

Serology

Serum samples collected from chicks of sub group K, L, M, N and O, were subjected to indirect haemagglutination test for antibodies tiration against Angara disease virus. Serum samples were collected from chicks at 7, 14 and 21 days post vaccination. Seven days post vaccination geometric mean titre (GMT) was 1.87, 1.37, 1.75, 1.25 and 0.5 in chicks of sub groups K, L, M, N and O, respectively. Fourteen days post vaccination, GMT were 2.25, 2.25, 3.12, 2.25 and 1.75 in chicks of sub-group K, L, M, N and O, respectively. 21 days post vaccination,
Table 2. Geometric mean titre of antibodies in the sera of chicks 7, 14 and 21 days post vaccination.

<table>
<thead>
<tr>
<th>Experimental subgroup</th>
<th>Vaccines administered</th>
<th>Mean antibody at day 07 post vaccination</th>
<th>Mean antibody at day 14 post vaccination</th>
<th>Mean antibody at day 21 post vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>AD Vac-I</td>
<td>1.87</td>
<td>2.25</td>
<td>2.62</td>
</tr>
<tr>
<td>L</td>
<td>AD Vac-II</td>
<td>1.37</td>
<td>2.25</td>
<td>3.12</td>
</tr>
<tr>
<td>M</td>
<td>AD Vac-III</td>
<td>1.75</td>
<td>3.12</td>
<td>2.75</td>
</tr>
<tr>
<td>N</td>
<td>AD Vac-IV</td>
<td>1.25</td>
<td>2.25</td>
<td>2.87</td>
</tr>
<tr>
<td>O</td>
<td>Unvaccinated</td>
<td>0.50</td>
<td>1.75</td>
<td>2.16</td>
</tr>
</tbody>
</table>

Figure 1. Showing SDS-PAGE analysis of liver homogenate containing HPS adenovirus. Lane 1, Positive control; lane 2, negative control; lane 3, AD Vac-1; lane 4, AD Vac-2; lane 5, AD Vac-3; lane 6, AD Vac-4; lane 7, molecular weight marker.

GMT were 2.62, 3.12, 2.75, 2.87 and 2.11 in chicks of sub-group K, L, M, N and O. The cumulative results of antibody profile at 7, 14 and 21 days post vaccination in chicks of sub-groups K, L, M, N and O are given in Table 2. Statistically, there was no significant difference among the titre after 7, 14 and 21 days of post vaccination. Also there was no significant difference among the titres within the groups, however numerically; there was difference in the mean values within the titres where post vaccination gave increased antibody titres as compared to non-vaccinated control.

Purity of vaccines
The vaccines containing the hydropericardium syndrome viral contents were separated by high-speed centrifugation and was subjected to SDS-PAGE. The multiple bands were received and side by side negative control (liver homogenate) and positive control (liver homogenate plus virus) were run. The bands appearing for liver proteins were neglected and the bands only present in the vaccines and the positive control was considered. A band at the position of 18.6 kda was commonly observed in all the four vaccines and in the positive control, which appears to be the viral protein observed in SDS-PAGE. All four vaccines had similar SDS-PAGE profile (Figure 1).

DISCUSSION
During this study, the biological titre that is, LD<sub>50</sub> of the 10% viral suspension was found to be 1x10<sup>2.4</sup>/ml. This finding is in close agreement to previously reported findings which were from 1x10<sup>4</sup>LD<sub>50</sub>/ml and 1x10<sup>5</sup>LD<sub>50</sub>/ml Ahmed et al. (1989) and 4x10<sup>4</sup>LD<sub>50</sub>/ml Ahmed (1989).
while it is quite different from the findings of Azhar et al. (2012) who find LD$_{50}$ as 4x10$^{-5}$. The reductions in the virulence of the virus may be due to extensive vaccination in the field. As Ahmed et al. (2004) determined the biological titre LD$_{50}$ of the 20% viral filtrate as 1x10$^{-1}$/ml inoculated subcutaneously in 25 days old broiler chicks. Ahmed (1999) conducted a study to determine the biological titre, LD$_{50}$ of the viral suspension as 1x10$^{-1}$/ml inoculated sub-cutaneously in 28 days old broiler chicks. However, similar study was conducted by Mashkoor et al. (1994) and determined the LD$_{50}$/ml as 2.5x10$^{6}$/LD$_{50}$/ml. In brief, it is clear from the present study that the results are in close agreement to the previous results. Vaccine should be free from any contamination such as bacteria, fungus and mycoplasma (OIE, 2000). The vaccines included in this study were found free from contamination and are in agreement with the previously reported findings. Therefore the vaccine, under study fulfills the requirement. The vaccines used during the present study were found to be safe for vaccination in the chicks. No previous work reported in literature on the sterility and safety of Angara

**Disease vaccines**

During the present work, the challenge protection was performed in the chicks at the age of 37 days, none of the chick in any group showed any mortality. In the previously reported experiments, the challenge protection test was performed in the chicks aged different 25-30 days (Ahmed, 1989; Ahmed et al., 1999; Mashkoor et al., 1994) but in this study, the chicks were aged 37 days at the time of challenge protection. Moreover the biological titre LD$_{50}$ of the viral suspension was determined in chicks at the age of 25 days in this study. By using these liver homogenate, there is great risk of secondary bacterial infections (Khan et al., 2005). Oil adjuvanted vaccines however provides good protection against challenge (Sahidullah et al., 2008). This may be one of the reason of failure of mortality in the control group O as well as in vaccinated groups K, L, M and N. In the present study, it was found that the antibody titres of vaccinated sub-group were higher as compare to non-vaccinated sub-group O which indicate that vaccination of chicks leads to an increase in antibody level. The results are comparable with the previous study of Rehman et al. (1989).

In this study, SDS-PAGE was run for normal liver homogenate as negative control, infected liver homogenate as positive control and centrifuged liver homogenate vaccines 18.6 kda protein was found of viral protein and not present in the negative control, which was assumed to be a viral protein. Earlier study by Rabbaneri and Naeem (1998) has observed similarity of SDS-PAGE profile of various isolates of hydropericardium syndrome agent. Balamurugan et al., (2002) characterized the polypeptides of three fowl adenovirus-4 (FAV-4) field isolates of hydropericardium syndrome. These isolates were subjected to SDS-PAGE. Protein profile analysis of FAV-4 isolates revealed similarity of polypeptides with molecular weight ranging from 20-107 Kda. Therefore the results regarding the protein profile of adenovirus vaccines during the present study are very much similar to Balamurugan et al. (2002) who recorded 20 kda protein band in the adenovirus isolates. For ascertaining the full profile of hydropericardium syndrome viral proteins, it is suggested that such profiles should be observed after purification of virus and viral contents from the different vaccines.

**Conclusion**

It is concluded from the present study that the Angara disease vaccines commercially available are pure and safe to use against the disease in poultry birds in field conditions. It was also observed that vaccines are less immunogenic and cannot provide 100% protection against the disease. It is clear from the present study that the chicks vaccinated with Angara disease vaccines when challenged with viral dose (2.0 ml) developed lesions in heart, kidney and liver. It is further observed that virus produced inclusion bodies in the hepatocytes in all chicks those received vaccines and all others who did not receive vaccines (control). Generally, it is concluded that vaccines are ineffective to control the disease in poultry birds.

**REFERENCES**


