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Research Article

Development and optimization of virus neutralization test in chicken embryonated eggs for indirect identification of avian influenza and Newcastle disease virus

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ABSTRACT

Avian viral problems have been consistently reported in commercial poultry of Pakistan causing heavy economic losses to the poultry farmers. Authentic identification and confirmation of the causative agent is always been question mark for the selection of vaccinal strain in this regard. Current study was therefore undertaken to optimize the virus neutralization test for the serological survey of vaccinated poultry particularly for avian influenza virus's subtypes and Newcastle disease virus. Various physiochemical factors such as concentration of antigen and antibody, Incubation temperature and incubation period for in vitro and in-vivo reaction of antigen and antibody were optimized in chicken embryonated eggs. Serum samples were obtained from vaccinated breeder birds of five commercial poultry breeder companies and subjected for VNT using different concentration of three antigen and their respective homologous antibodies under optimized conditions. AIV H9 (EID₅₀-1×10^{9.0}/ml) and NDV (EID₅₀-1×10^{8.2}/ml) having biological titer of 10⁻⁷/50ul HA units were neutralize with 10⁻²/50ul HIU of antibody and incubated at 37°C for 30 minutes was injected subsequently into 10 day old chicken embryo followed by incubation at 37°C for 38 hours showed ≥90% neutralizing specificity. Furthermore, sera obtained from five AIV-H9, AIV-H5 and NDV exposed commercial poultry farms revealed that Big bird broiler, Big bird breeders and A&S chicks are 100% sensitive and specific whereas, Gateway chicks and Waqas poultry breeders showed 100% homology for AIV-H5 virus but do not confers similarity with prevailing AIV-H9 and NDV field strains. Therefore, high sensitivity, reproducibility and specificity VNT, it could be a tool for indirect detection of homology between vaccinal strain and wild virus antigen using known antisera. Particularly, for those organisms possess natural ability to mutate in the adverse climatic conditions.

Keywords: Virus neutralization test, Avian Influenza Virus, Newcastle Disease Virus, Sensitivity, Specificity



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INTRODUCTION

Pakistan is an agriculture country and the major sub division of the agriculture has become the poultry production¹ (Mushtaq, 1994). In Pakistan poultry sector is the feasible source of animal protein in term of egg and meat production. The enormous growth of the division is distinctively influenced by numerous viral, bacterial, protozoan and metabolic afflictions² (Amare *et al.*, 2012). Avian influenza (AI) is most infectious disease which is caused by several serotypes of influenza virus among all viral diseases. The first epidemic of avian influenza virus was in 1994. The subtype H5N1 of Avian influenza virus causes huge economical losses in Pakistan^{3,4} (Muneer *et al.*, 1995, Nauta *et al.*, 2005). The low pathogenic subtype of

avian influenza is H9N2 which is still present in avian species. In Pakistan first outbreak of avian influenza virus subtype H9N2 was reported in 1999 which was also found in young and lying birds^{5,6} (Naeem *et al.*, 1999; Bano *et al.*, 2003). Newcastle disease (ND) is a lethal viral disorder first originated in England in 1926 (Barbezange and Jestin, 2005)⁷.

Virus neutralization test (VNT) is one of the biological tests used for the semi quantitative detection of antigen and antibody. It has been also used in variety of epidemiological studies to carry out the seroprevalence. The development of VNT is described, evaluating the diagnostic specificity and sensitivity, repeatability and reproducibility⁸ (Loeffen *et al.*, 2012). VNT is also known as micro neutralization assay which measures the

concentration of antibodies present in the serum to block the receptors of the virus to penetrate into the cell during *in vitro* trans infection. Although, neutralization is intuitively more appealing because it is more closely mirrors the disease process *in vivo*, it considered harder to standardize across laboratories^{9, 10} (Stephenson *et al.*, 2007; Wood *et al.*, 2012).

In influenza virus for the detection of specific neutralizing antibodies microneutralization test is used which is highly specific and sensitive assay in human and animal sera. Furthermore, it is also used for the detection of human antibodies to avian subtypes (WHO, 2013). Neutralizing antibodies play an important role in defense against influenza virus^{11, 12} (Burton *et al.*, 2012, Gerhard, 2001). Neutralizing antibodies chiefly affect the haemagglutinin protein and glycoprotein. Glycoprotein is the main envelop of the influenza virus¹³ (Bertram *et al.*, 2010). So, micro neutralization test is used as Gold standard for sub typing of influenza virus now days (Kwang *et al.*, 2013).

Research has shown that virus neutralization test is used with monoclonal antibodies to determine the antigenic difference between the strains of Newcastle disease virus. It is also used to evaluate the sequences of neutralizing epitopes¹⁴ (Panshin *et al.*, 2002). Two serotypes of the virus have been identified which are serotype 1 and serotype 2. Serotype 1 virus is very dangerous to chicken and also it is further classified into two types which are classic and variant. Serotype 2 virus is nonpathogenic to the chicken. To differentiate between the elicited antibodies by the two serotypes of serotype 1 virus the virus neutralization test is used (Ashraf *et al.*, 2005).

The application of virus neutralization assay is very simple and easy when a significant concentration of antibody is present; the antibody is identified by adding undiluted fluid such as blood serum in which suspected antibody is also present for the suspension and it also contains a known concentration of antigen. It is used to observe the geographic diffusion of its antigen and the titer of antibody in an anti-serum. The immunological factors of any disease are underlying to these principles¹⁵ (peter *et al.*, 1947).

MATERIALS AND METHOD

Source of virus:

Five ml of each characterized Avian influenza H5N1, H9N2 (Tahir *et al.*, 2016)¹⁷ and ND live viruses (Vaccinal strains) were officially obtained from Ottoman Pharma (Immuno division) licensed veterinary vaccine manufacturing company located at 10-km Raiwind road, Lahore Pakistan on the written request of director IMBB, The University of Lahore.

Source of antibody:

Two milliliter of monoclonal antisera against each strain of AIV-H5N1, AIV-H9N2 and ND virus was purchased from GD- diagnostic Holland. Furthermore, total of 25 blood samples were collected from 5 commercial breeders farms located in the periphery of Lahore, Pakistan and were also stored at -80°C till further use.

Source of embryos:

Ten day old chicken embryonated eggs were purchased from big bird poultry breeders located at Raiwind road, Lahore. External surface of the eggs were disinfected with 2% pyodine, ethanol solution and transferred to the incubator at 37°C till inoculation of virus.

Optimization of VNT:

Many variables were optimized during the study for the best neutralization of homologous antigen and antibody under controlled condition. However each of the following parameters were tested for five times in every experiment:

1. Concentration of antigen
2. Concentration of antibody
3. *In vitro* Incubation temperature
4. *In vitro* incubation period
5. *In vivo* Incubation Temperature
6. *In vivo* incubation Period

Final Protocol:

Inoculation:

Antigen and antisera suspension was centrifuged at 3600g for five minutes. 0.1ml of supernatant was inoculated through the air sac opening with one ml disposable syringe by inserting the needle (25G) into the allantoic sac of the egg. The opening of the each egg was sealed with sterilized molten wax and the eggs were incubated along with non-inoculated control eggs at 37°C for 48 hours till the cultivation of the virus.

Harvesting of antigen:

The eggs were chilled at -10°C for 30 minutes. The shell was cut above the air sac and carefully removed. The chorioallantoic membrane (CAM) and the amniotic membrane were turned with the help of sterile forceps, and the allantoic fluid was collected.

Control groups:

Five serum samples were obtained from non-exposed AIV and ND vaccinates. The samples were evaluated for relevant virus exposure through PCR and HI test using homologous antigens.

Spot Haemagglutination:

Sterile glass slide was labeled with respect to the marked eggs and test tubes containing allantoic harvest. Equal quantity (200ul) of each 25% washed chicken RBCs and harvested allantoic fluid were mixed on a clean glass slide allowed to react with each other for 2 minutes and results were recorded.

Haemagglutination of allantoic fluid¹⁷:

The HA test was performed using chicken erythrocytes and harvested allantoic fluid according to the method described by Allan *et al.*, 1978.

Specificity, sensitivity and repeatability:

Each of the factor optimized in the study was run five times and data was evaluated through mean standard deviation (M±SD) to get p-value on the basis of Bonferroni analysis. Moreover, 25 virus free serum samples were tested in the final version of the assay. They were used in the test according to the cut off value in the assay to estimate the specificity, sensitivity and reproducibility.

RESULTS

Several parameters were optimized for virus neutralization test using different concentrations of antigen and antibody for AIV-H5, AIV-H9 and NDV in the study. It was recorded that AIV-H5 antigen best neutralize at concentration of 1×10^{-7} with antibody concentration of 1×10^{-2} . *In Vitro* optimum incubation temperature was 37°C for 60 minutes as compare to *in-vivo* chicken embryonated

eggs temperature 37°C for 38 hours. Similarly, AIV-H9 antigen finest neutralization occurred at concentration of 1×10^{-7} with antibody concentration of 1×10^{-2} . In Vitro optimum incubation temperature was 37°C for 30 minutes as compare to in-vivo chicken embryonated eggs temperature that was 37°C for 38 hours. Whereas, NDV antigen and antibody greatest neutralization observed at antigen concentration of 1×10^{-7} with antibody titer of 1×10^{-2} . In Vitro optimum incubation temperature was 37°C for 30 minutes as compare to in-vivo chicken embryonated eggs temperature was 37°C for 38 hours (Fig. 1,2,3,4,5).

Antigen and antibody concentration was considered as one of the critical variable in neutralization process and it was observed that increase in antigen or antibody count may adversely affect the sensitivity of the assay. At highest antigen 1×10^{-8} with lowest antibody level 1×10^{-0} did not show significant neutralization indicated by HA titer after incubation at optimized incubation temperature (37°C) and time period (38 hours).

Serum samples collected from AIV and NDV confirmed infected birds showed higher anti-HI titers (64 HIU) from non-infected flocks. On the basis of these results the cut off value was set to 64 HAU. Serum samples from five poultry breeder companies were test through VNT using five mentioned optimized conditions for three different viruses. It was found that Big Bird Poultry breeder and big bird foods showed 12 ± 4.38 , 41 ± 21.46 and 32 ± 19.59 mean standard deviation (M+SD) status of allantoic fluid harvested post in vivo neutralization HA for H5, H9 and NDV respectively. Serum samples from A&S chicks showed 32 ± 19.59 , 14 ± 3.57 and 17 ± 4.38 (M+SD) status of allantoic fluid harvested post in vivo neutralization HA for H5, H9 and NDV respectively. Whereas, Gateway chicks showed 17 ± 4.38 , 102 ± 35.05 , 256 ± 156.76 (M+SD) status of allantoic fluid harvested post *in-vivo* neutralization HA for H5, H9 and NDV respectively. Similarly, Waqas poultry revealed 41 ± 21.46 , 819 ± 280 and 716 ± 280.43 (M+SD) status of allantoic fluid harvested post *in-vivo* neutralization HA for H5, H9 and NDV respectively as shown in Fig. 6.

DISCUSSION

Wide ranges of biochemical and serological tests have been used for the detection of animal and human disease causing pathogen. It is imperative to have authentic method to evaluate and confirm the organism. Various serological tests are being used for the confirmation of isolates and antibodies actively raised against vaccines.

In current studies virus neutralization test was developed showing high specificity and sensitivity for avian influenza viruses subtype AIV-H5, AIV-H9 and Newcastle disease virus. The assay was reproduced and repeated for antisera collected from different vicinities of Lahore and displayed high level of homology to immunoglobulins actively raised against vaccination in commercial poultry flocks. However, sera obtained from the flocks were hit by ND and AIV-H9 at different age did not show any significant resemblance to the antigen. VNT for embryonated egg adapted viruses is easy to perform and can be evaluated by spot hemagglutination using 25% washed chicken RBCs. Despite of excessive vaccination in commercial poultry viral problems has been reported with massive economical

losses. In most of the cases researcher believed that the inefficiency of vaccines against their specific wild antigen is due to change in genotype often posed by pre disposing factors. Furthermore, influenza viruses are remained focused in research due its unique nature of nucleic acid which is prone to rapid mutation facilitated by the process of reassortment. Fusion protein (F) controlled by F gene of Newcastle disease virus is another unique phenomenon supporting the virus in attachment and penetration in to the cell. Mutation in F gene results in alteration of expressed proteins which cannot neutralize native antigen. Therefore, in order to rule out the cause of vaccine failure, VNT is the most effective technique could be used to investigate the homology between vicinal strain and wild type antigen. It is imperative to have authentic technique for evaluation of immunogenicity. Neutralizing antibody response to the viruses is serotype specific and VNT is considered as gold standard to detect avian influenza and Newcastle disease virus neutralizing antibodies.

In current study it was observed factors such as amount of antigen and antibody, temperature and incubation time affected the neutralization reaction *in-vitro* and *in-vivo* in chicken embryonated eggs. Each of the factors has critical significance in antigen antibody neutralization reaction. The optimum neutralization reaction set for AIV-H7, AIV-H9 and NDV were antigen (512 HAU) 1×10^{-7} versus 1×10^{-2} of antibody (512 HIU), 37°C for 38 hours of incubation. However, AIV-H9 was significantly has better neutralization results when incubated at 37°C for 38 hours. Olitsky and Harford 1938, Morgan, 1945 are in partial agreement with data obtained in current studies that the degree of neutralization in extra neural test is maximal and was not influenced by incubation²³.

It is evident that temperature has significant role in the neutralization of specific antigen and antibody under specified physiochemical conditions. Whereas, *In vitro* antigen antibody neutralization results revealed that AIV-H9 and NDV are more susceptible for neutralization reaction at 37°C if incubated for 30 minutes in contrast to AIV-H5 which showed better neutralization at 37°C for 60 minutes. So, Li *et al.*, 1994 collaborates that immuno precipitation and neutralization reaction are thermal dependent and require co incubation of virus and antisera particularly at 37°C which can act as catalyst for antisera against peptides corresponding to normal internal portions of VP1 that have the ability to precipitate virus.

Andrewes and Elford, 1933 described that antigen and antibody neutralization reaction, antibody neutralize the relative proportion of specific antigen on the basis of percentage law. Dilution factor of antigen such as AIV-H5, AIV-H9 and NDV showed significant neutralization at 10^{-6} , 10^{-4} and 10^{-1} respectively²¹. Diluted Antiserum reacts at its best at the level of 10^{-2} against 10^{-7} dilution of AIV-H5, AIV-H9 and NDV antigen titer. However, Klein *et al.*, 1999 partial agreed that if concentration of human cytomegalovirus was increased up to 100 fold does not affect the titer of neutralizing sera. Further explanation was made on the basis of observation that assay performed under ordinary conditions relative occupancy of viral epitopes is masked by antibody titer and its functional affinity for antigenic determinant²⁰ (Klasse, 1996).

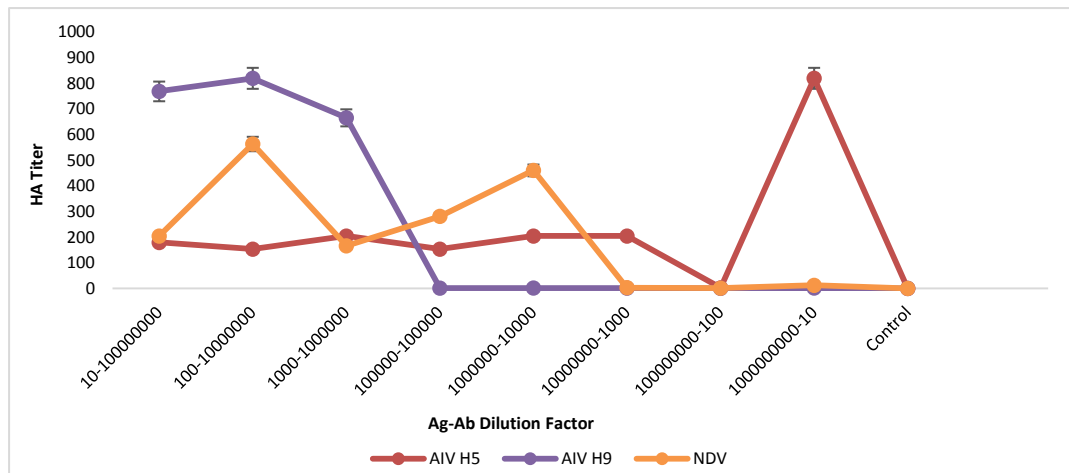


Figure 1: Effect of antigen and antibody dilution factor on virus neutralization test

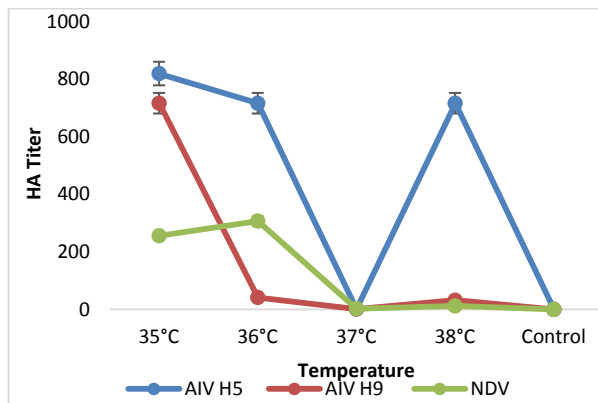


Figure 2: In vitro effect of temperature on the antigen and antibody neutralization reaction

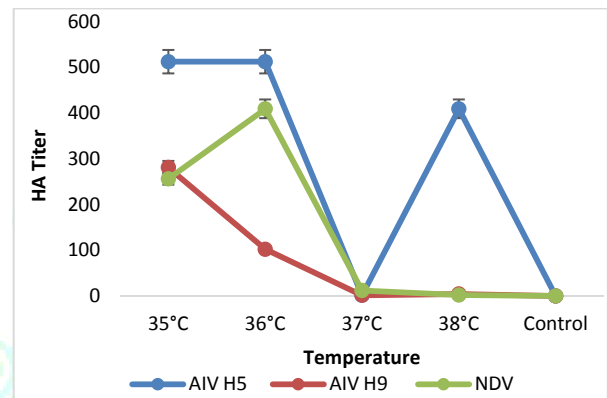


Figure 4: In vivo effect of temperature on the inoculated eggs for virus neutralization test

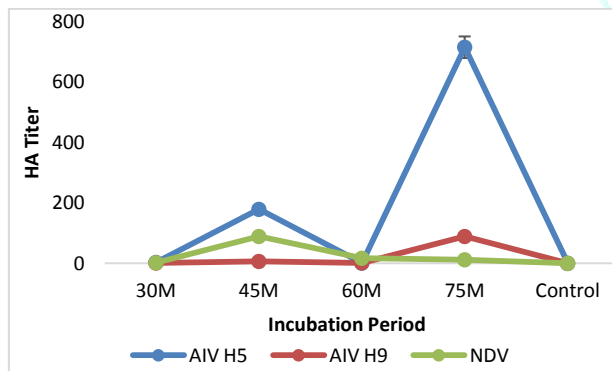


Figure 3: In vitro effect of incubation period on the antigen and antibody neutralization reaction

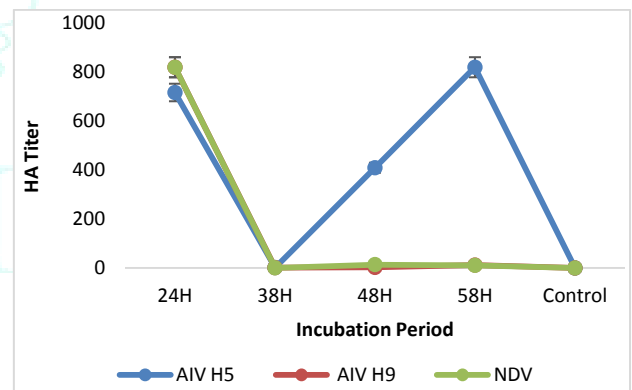


Figure 5: In vivo effect of incubation period on the inoculated eggs for virus neutralization test

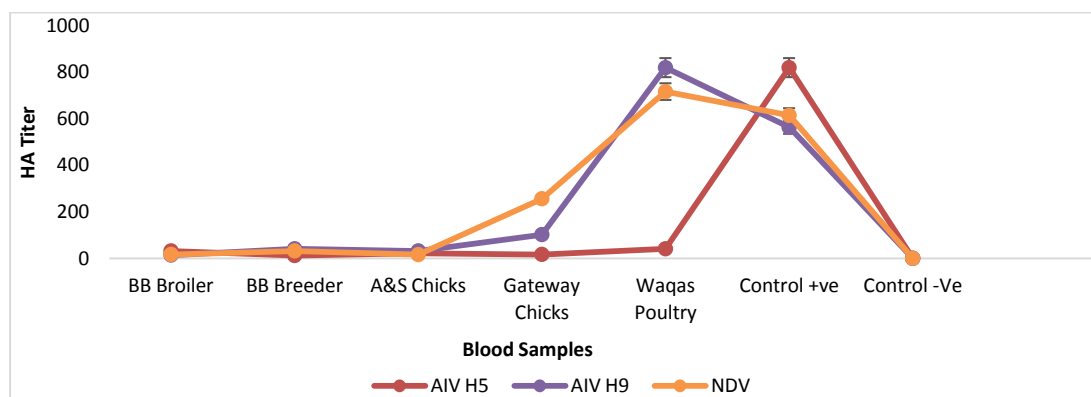


Figure 6: Serum neutralization of samples collected from birds naturally exposed with AI and NDV

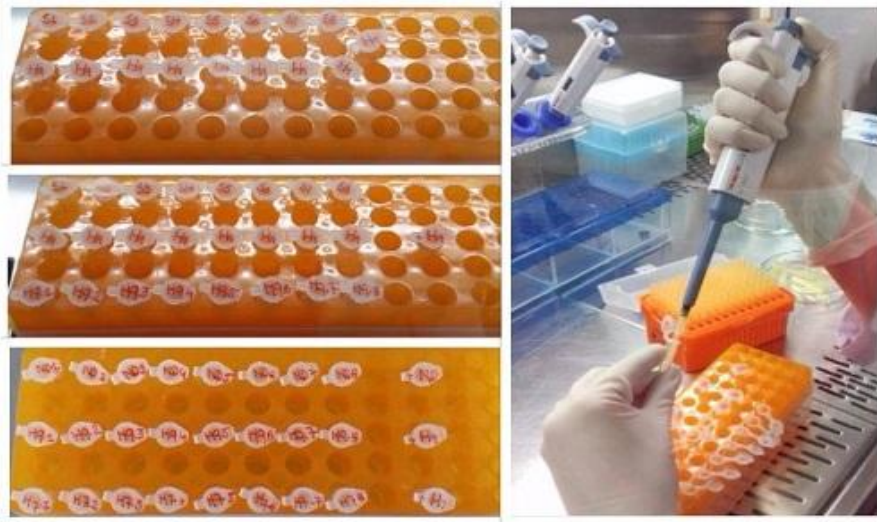


Figure 7: Ag, Ab dilution and mixing



Figure 8: Steps involved in virus inoculation into embryonated chicken eggs

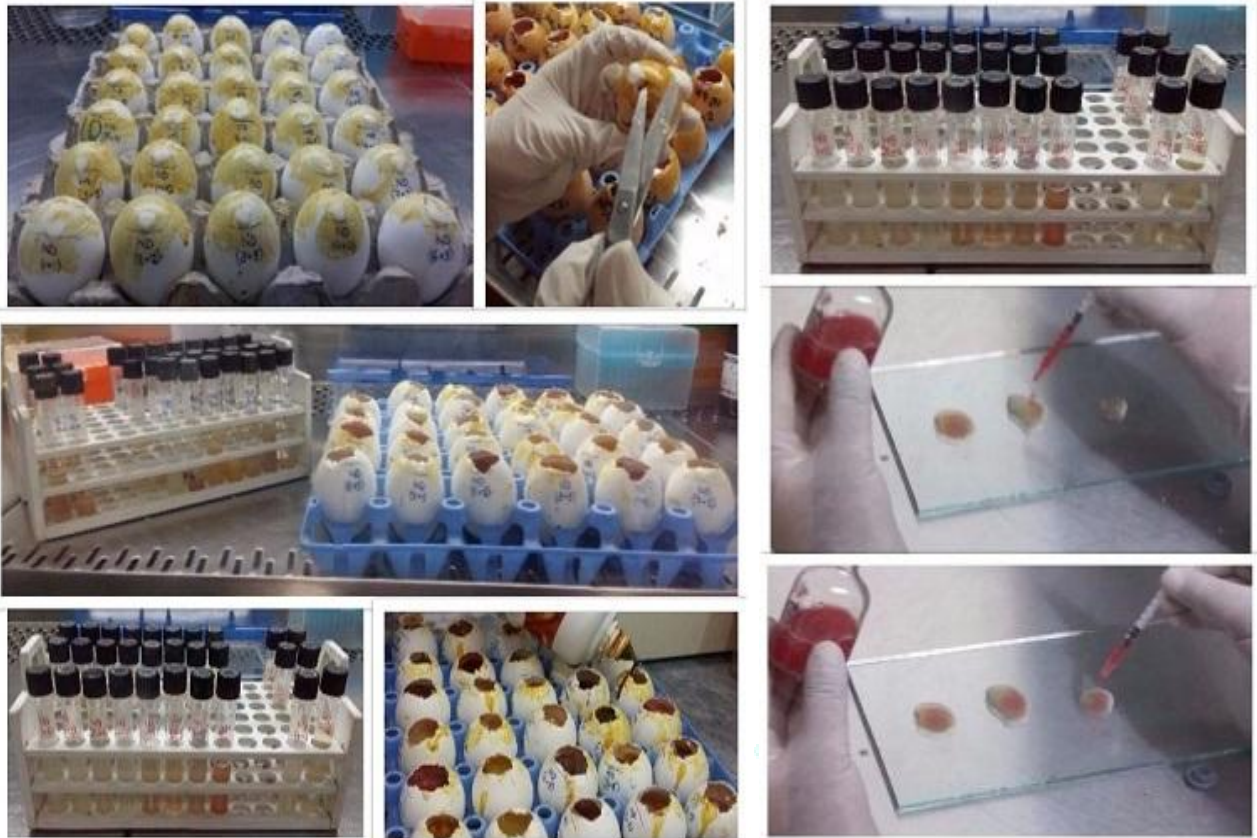


Figure 9: Harvesting of antigen

Figure 10: Spot agglutination of allantoic harvest



Figure 11: Haemagglutination of allantoic harvest

CONCLUSION

In antigen antibody reactions, both of the participants shall be highly specific and require certain optimal physiochemical factors for their neutralization. It is evident based on the results of current study that for in vitro neutralization reaction two phases must be considered to get reliable results. Firstly in vitro antigen and antibody reaction is predominantly influenced by biological titer of virus and antibody concentration, temperature and incubation period followed by in vivo elution of antigen and antibody association is greatly affected by temperature

and incubation period. The optimized technique of virus neutralization could be used for mass scale screening of Avian influenza and Newcastle disease virus infected birds. Moreover, the test can be best applied for the detection of different serotypes prevalent in the environment has been considered as the cause of vaccine failure. Confirmation of prevalent wild strain makes scientists able to design strategy of vaccination for effective immune-prophylaxis measures against natural outbreaks of new castle disease and avian influenza virus.

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