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Raising of polyclonal hyperimmune sera in broilers against avian influenza virus subtypes H5N1, H7N3, H9N2 and Newcastle disease virus for diagnostics and therapeutics

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Abstract
Antibody production in chicken is comparatively beneficial with respect to easy availability of birds and their management, relaxed handling in natural host. The study was attempted to raise polyclonal antibodies in broilers to examine their diagnostic and therapeutic potency simultaneously against avian Influenza virus subtypes and Newcastle disease virus. Healthy broilers were divided into 4 groups A, B, C and D. Birds in group A, B, C and D showed 1024, 512, 1024 and 1024 peak anti-influenza H5N1-HI antibody, anti-influenza H7N3-HI antibody, anti-influenza H9N2-HI antibody and anti NDV-HI antibody titer respectively on 42 day post vaccination. Whereas, therapeutic studies indicates 100% protection in naturally infected laboratory confirmed NDV commercial broilers in contrast to AIV H9N2 infected broilers where 80% protection is achieved. Polyclonal antiserum can be used for large-scale screening of AI and NDV carrier commercial and wild birds. Moreover, current study suggests that purified polyclonal antiserum could be used in successful treatment of avian Influenza virus infected broilers.

Keywords: Avian influenza virus, Newcastle disease virus, therapeutics, hyperimmune serum, polyclonal antibodies

Introduction
Maternal derived antibodies (MDA) provides passive protection against pathogens early in life [1, 2]. Avoiding the energy required to combat infection with an immature immune system and retaining this saved energy for growth and further development of the immune system [3]. However, MDA is also responsible for some more adverse effects, *inter alia* interference with the induction of a specific active immune response by antigen capture, reducing antigen presentation, increasing clearance or reducing the immunological memory [4, 5, 6]. The inhibition of the vaccine-induced immune response by MDA has been described for avian pathogens such as Newcastle disease virus (NDV) [7]. H5 avian influenza virus (AI-H5) [8, 9]. Infectious bursal disease virus [10, 11]. And is of great concern to the poultry industry.

Poultry sector generates income and direct and indirect employment for about 1.5 million people till 2012 [12]. Its contribution in agriculture is 6.40% and in livestock 11.50%. In total, meat production of country and poultry meat contributes 25.8%. Poultry sector has rapid growth of about 8-10% every year, which shows its inherent potential. According to currently conducted survey, the present investment in the Pakistan poultry industry is about Rs. 200.00 billion. ND and avian influenza (AI) are major concerns of animal husbandry due to hazardous infections [13]. All over the world, poultry industry is facing severe economic losses with every passing year [14, 15].

Highly pathogenic Avian Influenza (HPAI) is an extremely contagious, multi-organ system disease of poultry leading to high mortality and is caused by some of the H5 and H7 subtypes of type-A Influenza virus. However, most Avian Influenza virus strains are mildly pathogenic and produce either sub-clinical infections or respiratory or reproductive diseases in a variety of wild and domestic bird species [16, 17, 18, 19, 20].

APMV-1 viruses circulating in poultry flocks are being characterized [21]. ND is fatal and still top ranked poultry disease. Annual losses caused by this disease worldwide are in millions of

dollars [22, 23].

ND is an economically important disease and also a major threat to poultry industry [24]. According to variation in strains of NDV, the rate of mortality and morbidity in a flock [25]. Varies from 90-100% [26]. Along with decrease in egg production [27].

The antigen specific hyperimmune serum can also be used for differential diagnosis during an outbreak of a particular disease. Currently, the imported hyperimmune serum used for diagnostic in poultry is very expensive and has been imported from different countries of the world. Moreover, the imported strains of viruses may differ from indigenous isolates showing non-specificity in diagnosis. Hyperimmune serum is already used for the successful treatment of foot and mouth diseases, tetanus and canine viral diseases [28]. The treatment against avian coccidiosis, rabies, hepatitis B, tetanus toxin and varicella zoster were done through passive immunization i.e. hyperimmune serum antibodies.

With the emergence of antimicrobial resistance against antibiotics and antiviral drugs, the avian diseases became stronger and spread epidemically across the country. Whereas, in viral diseases the vaccinating strategy did not accomplished as effective due to versatility in the nucleic acid of various micro-organism. Influenza viruses and NDV are considered to be the most important re-assortment possessing mechanism in their genomes. In such cases, vaccination is less effective in high exposed areas. The diagnosis of these viruses is also a difficult task due to expensive imported antibodies and versatility in their genomes. Therefore, there is a divine need to develop new and effective strategies to diagnose and control the outbreaks of these viruses. The current study is therefore, undertaken to develop polyclonal antisera against Avian Influenza subtype H5, H7, H9 and Newcastle disease viruses in specific antibody negative broilers for immunotherapeutic and serological diagnosis.

Materials and methods

Research place

The research experiment was conducted at the Institute of Molecular Biology and Biotechnology, University of Lahore, in collaboration with Ottoman Pharma (Immuno Division), Lahore, Pakistan. Study period of research was January 2017 to June 2017.

Experimental broiler chickens

Eight day old 25 broiler used in this research were obtained from commercial hatchery of Big Bird poultry breeders located at Raiwind road, Lahore, Pakistan. The birds were kept at a disinfected and cleaned experimental animal shed of CRIMM, The University of Lahore.

Management of birds

The birds were kept in 5 separate compartments and offered with fresh water and feed adlibitum. The environmental conditions such as temperature and humidity were monitored regularly twice and recorded in log book.

Source of vaccines

In this study four oil based inactivated vaccines were used and was obtained from Ottoman Pharma (Immuno-Division) 10-km raiwand road, Lahore, Pakistan. Influenza virus subtype H5N1, H7N3, H9N2 and Newcastle disease virus were used for raising of hyperimmune serum.

Research design

The research was divided in two experiments. In first experiment, hyperimmune sera was raised in birds. Whereas, in second experiment, the serum was evaluated for its therapeutic effect in naturally infected birds.

Experiment 1: Raising of diagnostic hyperimmune sera:

Experiment design

A total of 25 birds were divided into 5 groups GA, GB, GC, GD and GE containing 5 birds each marked with specific dye mentioned in table.1. Each group was kept separated from other groups and also they were fed separately so that antigen exchange cannot take place between birds of different groups during exposure. Each group was inoculated with specific virus for the production of specific hyperimmune serum.

Vaccine Inoculation

Priming

Chicken at age of 8 days were inoculated with inactivated vaccines. Each group was inoculated with a 0.3ml of inactivated specific virus through sub cutaneous route.

1st booster (2nd dose)

1st booster dose were given at 22 day of age to chicken, after 14 days interval post priming.

2nd booster (3rd dose)

2nd booster dose was injected to each bird of the every group through same routine and dose 36 day post priming.

Blood sampling

Three milliliter of blood was collected from wing vein of each bird of every group with 5ml capacity of sterile syringe on 0, 8, 16, 32 and 42 day post vaccination for evaluation of antibody titer through Haemagglutination inhibition test. Clear serum separated during the period was collected and centrifuged at 3000 rpm for 10 minutes and was packed in 2ml Eppendorf tubes, marked with specific code and kept at -80C^{o} freezer till further processing.

Table 1: Experimental Design

SR. NO	Group (N=05)	Marking	Antigen
1	GA	Blue	H5N1
2	GB	Green	H7N3
3	GC	Yellow	H9N2
4	GD	Black	NDV
5	GE	White	Control

Experiment 2: Therapeutic effect of raised hyperimmune sera

Experimental broiler chickens

15 naturally infected birds of each NDV and AIH9 were collected from commercial poultry farm located at Multan road, Lahore –Pakistan. The birds were divided into four groups GA, GB, GC and GD. Group A and Group B containing 10 birds each of NDV and AIH9 respectively were served for specific antisera injection. While, group C and D each comprised of 5 birds were served as un inoculated antiserum control (Table-2).

Management of birds

The birds were shifted to the control animal shed of Ottoman Pharma to avoid the spreading of viruses. Complete biosafety protocols were followed for safety handling of these chicken. Before shifting to the control animal shed it was fumigated

(2% Formalin+$KmNO_4$) to avoid contamination and disinfection of animal shed.

Source of Hyperimmune sera

Hyperimmune sera that were raised, were used for immune-therapy to birds. The anti-H9N2 and anti-NDV hyperimmune sera HI titer used for therapy was 1024 and 1024 respectively.

Inoculation of Hyperimmune sera

Each bird of group GA and GB was injected with hyperimmune sera raised in the study against H9N2 and NDV intramuscularly with the dose of 1ml per birds (Table-2).

Table 2: Inoculation of HIS for therapeutic efficacy

Sr. no	Groups	Marking	Antigen	Route of Injection 1ml/Bird	Hyperimmune sera HI antibody titer
1	Group A	Yellow	H9N2	IM	1024
2	Group B	Black	NDV	IM	1024
3	Group C	White	-	-	-
4	Group D	Green	-	-	-

Results

Mean Values HI Titer

Experiment 1

Group A

Group A birds containing H5N1 antigen showed mean anti-Avian Influenza virus H5N1-HI antibody titer 4±2.82, 2±0.00, 64±0.00, 307±114.48 and 1024±0.00 at day 0, 8, 16, 32 and 42 respectively.

Group B

Group B birds containing H7N3 antigen showed mean anti-Avian Influenza virus H7N3-HI antibody titer 4±2.68, 2±1.41, 35±17.52, 128±0.00 and 614±228.97 at day 0, 8, 16, 32 and 42 respectively.

Group C

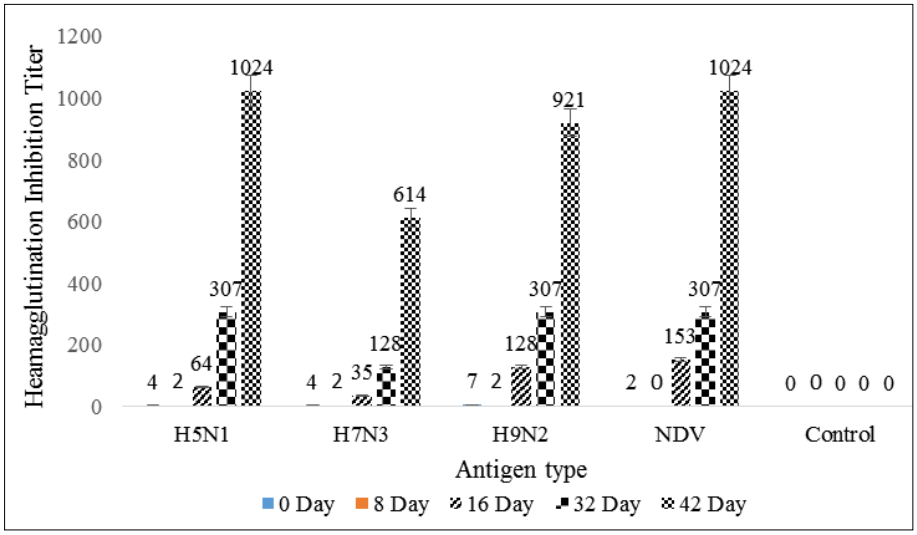
Group C birds containing H9N2 antigen showed mean anti-Avian Influenza virus H9N2-HI antibody titer 7±1.78, 2±1.41, 128±78.38, 307±114.48 and 921±228.97 at day 0, 8, 16, 32 and 42 respectively.

Group D

Group D birds containing NDV antigen showed mean anti-NDV-HI antibody titer 2±0.00, 0±0.00, 153±57.24, 307±114.48 and 1024±0.00 at day 0, 8, 16, 32 and 42 respectively.

Group E

Group E birds served as control showed mean anti-NDV-HI antibody titer 0±0.00, 0±0.00, 0±0.00, 0±0.00 and 0±0.00 at day 0, 8, 16, 32 and 42 respectively.



Experiment 2

Therapeutic efficacy of raised hyperimmune sera

It was observed that all birds of group D showed recovery after 24 hours of injection whereas all birds of control group died during this period. In group C, all birds recovered except 2 birds.

Table 3: Passive immunization

Groups	Marking	Antigen Type	Injection Description 1ml/Bird	Efficacy With Reference to Time				Efficacy Percentage%
				24Hrs		48Hrs		
				Died	Lived	Died	Lived	
Group A	Yellow	H9N2	IM	2	8	-	8	80%
Group B	Black	NDV	IM	-	10	-	10	100%
Group C	White	Control	-	10	-	10	-	0%
Group D	Green	Control	-	10	-	10	-	0%

Discussion

Immunogenesis is a complex process where immune system is stimulated by the invasion of foreign particle of certain size and biochemical composition. The basis of immunity is antigen recognition and specific response to the particular antigen. Higher antibody response is mostly achieved by the

administering series of injection. In our study, series of injection was administered to chicken for raising of hyperimmune sera described by [29].

Inactivated vaccines required repeated doses for achieving required antibodies titer [30]. We use chicken for raising of hyperimmune sera whereas previous researchers used farm animals, rabbits, chicken and laboratory rodents [31]. Protective immune response depends upon various factors like antigenicity of vaccine, maturity in birds, physiological and environmental factors, hormonal factors and stress [32]. Considering all the factors we maintained optimum conditions in our study for authentic results.

Haemagglutination test provide rapid, sensitive, specific and more accurate identification of viruses for confirmatory diagnostics [33]. Initial anti-HI-antibody titer in both groups were zero but gradually increases and reached 512 on day 32 post inoculation. Booster dose of vaccine in both groups triggered the immune response and reached to 1024 peak Anti HI-antibody titer on day 42 post priming. Peak titer was obtained after initial dose at 8 and a booster dose oil based vaccine on 22 day of age. The oil based vaccine injection retained the antigen at injection site prolonging its effect for longer period of time making immune system of the bird to make immunogen availability to antigen processing presenting cells [34].

Results of the current study are in agreement with [35]. Who stated that use of adjuvants in vaccines enhance the effect of immune response by limiting the immunogen count [36]. Booster doses enhance the antibody titer after the priming, when priming fails to raise high antibody titer [37]. Little or too much antigen induce tolerance, series of booster doses is an ideal way to achieve high antibody titer [38].

In current study therapeutic efficacy of the raised serum greatly recovered the naturally infective birds of group B as compared to birds in group A. Since birds were obtained from different source and it may be assumed that Birds in the group A were infected with different wild serotypes which would not be homologus the strain used for the raising of hyperimmune sera.

Anti-NDV-HI antibody titer raised significantly high (P>0.05) with the passage of time for day of priming to 24 day post vaccination. Furthermore, the titer attain maximum peak at 44 days post vaccination which significantly higher (P>0.05) the 24 day post vaccination. The substantial increase in HI antibody body titer on 42 day post vaccination would be due to the booster dose inoculation on 16 day post priming.

Our results are supported by the (Gelb *et al*., 1987) who revealed that the cross reactivity between the antigen and antibody is highly specific but the extent of reaction could not be similar due to the antigenic variation between therapeutic and wild strain. Our diagnostic results of hyperimmune sera are supported by Thangameenakshi and Michael that hyperimmune sera can be used as an alternative and viable replacement of conventional antibodies and can be used in diagnostic assay of viruses [39]. Our therapeutic results are supported by Pansota who claimed that mortality and morbidity of viruses can be decreased by the use of hyperimmune sera [40].

Conclusion

Based on results obtained in current study it is suggested that locally raised polyclonal hyperimmune sera could be used for the detection of wild strains prevalent in the field and to design effective vaccine selection strategies for effective immuno prophylaxis measures. Moreover, the use of pathogen free antiserum in naturally infected animals can be alternative approach to control the naturally occurring avian Influenza and NDV epidemics.

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