

Comparative study of haemagglutination inhibition, Agar gel precipitation test, Serum neutralization and Enzyme linked immunosorbent assay for detection to avian influenza viruses

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ABSTRACT

The sensitivity, specificity and reproducibility of the serological tests for detection of avian influenza viruses were carried-out by using Hamagglutination inhibition (HI), Agar gel precipitation test (AGPT), and Enzyme linked immunosorbent assay (ELISA) and Serum neutralization test. The geometric mean titre (GMT) of haemagglutination inhibition antibodies recorded as \log_2 indicated that the post vaccination titres in the field were on higher side i.e., 7.9 for H7 and 5.9 for H9. The correlation between HI titre and AGPT affirmed that for the AGPT test need high antibody titre for positive reaction. The pooled sera were also used to correlate the serum neutralization test and enzyme linked immunosorbent assay. The serial two fold dilutions were tested for the serum neutralization activity and concluded that the HI titre \log_2 4 provided 100% protection, than 52% and 45% protection in 1:2 and 1:4 dilution was recorded, respectively. Similarly, the ELISA test showed positive results up to 1:16 HI titre, i.e. \log_2 4 and confirmed the linear relation between these two serological tests. In HI test, the concentration of antigen can influence the result. It also needs careful preparation of concentration of erythrocyte suspension. Agar Gel immuno-diffusion is basically a qualitative test as it can not determine the quantity of antigen or antibody with the help of this test. It lacks the level of sensitivity as offered by other test. If serum neutralization test is performed on a pooled serum samples, then it could lead to a false conclusion on antibodies status. ELISA is most sensitive, spe-

cific and accurate as compare to all other serological tests.

Keywords: Serology; HI; ELISA and Avian Influenza Virus.

1. INTRODUCTION

Infectious viral diseases are a major threat to poultry. Avian influenza is one of most important among them which inflicts heavy economical losses. It is caused by a virus that belongs to family Orthomixoviridae, genus influenza A virus [1].

Avian influenza virus is classified into subtypes on the basis of antigenic differences in their surface glycoprotein hemagglutinin (HA; H) and neuraminidase, (NA; N). To date 16H subtypes (H1-H16) and 9N subtypes (N1-N9) have been recognized [2]. It rapidly infects the poultry when heavy outbreak occurs. Many species of birds are found to be susceptible to avian influenza virus.

Avian influenza viruses are circulating periodically among the domestic poultry over 100 years [3]. Wild waterfowl and shorebirds are considered to be reservoir of influenza A virus because the species harbor all 16 HA subtypes [4]. Although chicken and turkeys are not natural host species for avian influenza but these viruses routinely cross over from wild bird's reservoir to infect poultry birds [5].

During 2003 and 2004, there were quite a few outbreaks of avian influenza throughout the Asia, including South Korea, Japan, Indonesia, Vietnam, Thailand and China. The outbreak resulted not only tremendous economic losses in the poultry industry but also claimed death in human in Vietnam and Thailand in 2004.

Table 1. Calculation of the 50% endpoint of a neutralization test by the method of Reed and Muench.

Serum Dilution		Response			Accumulated Values		Ratio Infected / Total	% Infected Embryos
Numerical Value	Log	Infection Ratio	Infected embryos	Non Infected embryos	Infected embryos	Non Infected Embryos		
Undiluted	-	0/5	-	-	-	-	-	-
1:2	10 ^{-0.3}	0/5	5	0	18	0	18/18	100%
1:4	10 ^{-0.6}	1/5	4	1	13	12	13/25	52%
1:8	10 ^{-0.9}	2/5	3	2	9	11	9/20	45%
1:16	10 ^{-1.2}	3/5	3	2	6	9	6/15	40%
1:32	10 ^{-1.5}	3/5	2	3	3	7	3/10	30%
1:64	10 ^{-1.8}	4/5	1	4	1	4	1/5	20%
Negative Control Sera	-	05	-	-	-	-	-	-
Positive Control Sera	-	00	-	-	-	-	-	-

Table 2. Correlation among HI (log₂) AGP and ELISA.

H7			H9		
HI	AGPT	ELISA	HI	AGPT	ELISA
00	00	-	2	-	0.0
00	00	-	4	-	0.1
00	00	-	8	-	0.2
16	-	0.4	16	-	0.5
32	-	0.8	32	-	1.01
64	-	0.9	64	-	1.32
128	+	1.02	128	-	1.28
256	+	1.43	256	+	1.42
512	++	1.72	512	++	1.59
1024	++	1.78	1024	++	1.62
2048	++	1.82	2048	++	1.80

Shows positive and negative Pattern reactivity of AGPT

- Negative

+ Positive

++ Strong Positive

Avian Influenza viruses have been reported to cause high mortality in Pakistan. The first outbreak of avian influenza virus in Pakistan was recorded in 1995 when highly pathogenic influenza of H7 subtype was identified Naeem and Hussain [6]. In 1998 Avian Influenza subtype H9N2 was isolated from breeder flock showed a reduction in egg production along with respiratory infection Naeem *at el.* [7]. The H7 and H9 strains were still being isolated in Karachi in 2004.

New diagnostic test for emerging avian diseases are developed using molecular biology techniques. These mainly rely on detection of nucleic acid (either RNA or

DNA) unique to that pathogen. Analysis is being used to identify the species, subspecies, type and subtype and even in some cases individual strains. These tests are based on molecular techniques such as Restriction fragment length polymorphism (RFLP), Polymerase chain reaction (PCR) and nucleic acid sequencing. The serological detection of antibodies for avian Influenza virus in the poultry birds is of the great importance in preventing and controlling the avian influenza. This study revealed standard sero-diagnostic techniques used to evaluate sensitivity, specificity and accuracy of the techniques in identification of avian influenza in birds.

2. MATERIALS AND METHODS

Day-old broiler chicks were purchased and used for the experiment. The broiler chickens were divided into three groups A, B and C. Group A and B were vaccinated with H7N3 and H9N2, respectively, while group C was kept as control. Before vaccination, the blood samples were collected randomly from 5% birds prior to vaccination at 3rd week of age to determine the status of maternal antibodies in the sera of chicks.

2.1. Serum Samples

To assess the susceptibility of chickens the serum samples were checked prior to vaccinate and found zero titre against avian influenza. Blood samples were allowed to coagulate than serum was collected, marked, centrifuged and stored at 20 °C prior to use.

2.2. Source Virus

A local virus isolate chicken/Pakistan/23/99 (H9N2) virus was used with ELD 50 of $10^{9.26}/0.1\text{ml}$. Another virus isolate H7N3 was also received from Sindh Poultry Vaccine Centre (SPVC) with ELD 50 of $10^{7.56}/0.1\text{ml}$. Both serotypes were confirmed from southeast poultry research institute Georgia.

2.3. Haemagglutination Inhibition Test

The (HI) test was performed by adopting the technique described in (OIE) manual of diagnostic tests using 4HA units of H7 and H9 viruses with 1% suspension of washed chicken RBCs. Serum was diluted while antigen was constant.

2.4. Agar Gel Precipitation (AGP) Test

This test was performed to adopt the method described by Beard in 1998. Agar was prepared in borate buffer, than allowed to solidify. Cylinder of gel were cut with a gel cutter, than antigen was placed in the central wells while the sera were in the peripheral wells than the Petri plates were kept in moist chamber to avoid drying of gel.

2.5. Virus Neutralization Test

The virus neutralization test was performed to titrate the antibodies against avian influenza virus; both strains were checked and confirmed for sterility. Test was done by using the embryo inoculation, β -method (constant virus diluted serum) and followed the technique described by Beard [8], while endpoint titre was calculated as \log_2 exponent that was 50% neutralization endpoint or PD50 by the formula of Reed and Munch.

2.6. ELISA Test

The ELISA test was run in accordance as described by

Terry and Tony in 1991 [9] and as described in the monograph of Australian Centre for International Agricultural Research (ACIAR 1995), in which plates were read at 405nm absorbancy in a microplate ELISA reader.

3. RESULTS

The geometric mean titer (GMT) in \log_2 of serotype H7 was recorded as 7.9 ± 0.23 (66) and for H9 was calculated as 5.7 ± 0.29 (66). The mean H7 titre was significantly ($p < 0.05$) higher than H9.

3.1. Agar Gel Precipitation Test

The pattern of reactivity of both strains is similar and no remarkable difference has been observed. However, the precipitation line can be seen in those samples which have HI titres more than 7 \log_2 in H9 subtype. Moreover, there is a linear relation between precipitates of antibodies and antigen. Results also showed that H7 subtype was not positive till the HI titre was 9 \log_2 .

3.2. Virus Neutralization Test

Before the using of strains for serum Neutralization Test, the ELD 50 was determined and recorded as $10^{11.26}/\text{ml}$ for H9 and $10^{8.31}/\text{ml}$ for H7 subtype. The results in (Table 1) indicated that undiluted and 1:2 dilution of serum showed 100% protection and than gradually decreased i-e 52% and 45% protection in 1:4 and 1:8 dilution respectively. However, negative control sera showed no protection while positive showed 100%.

3.3. ELISA Test

The average optical density value (OD405nm) of positive sera showed 0.4 OD up to 1:16 dilution, i.e, 4 on \log_2 . Both serotypes showed positive correlation between HI titre and ELISA at GMT $\log_2 4$. However there is a linear relation between HI titre, ELISA and AGPT shown in **Table 2**.

4. DISCUSSION

Present study revealed that ELISA is better than HI in identifying sera with low antibody titer. HI titer within range of 1:2 to 1:4 are considered as suspected whereas 1:8 may be considered as positive and according to the manufacturer of ELISA kit, 0.2 value considered as a suspected positive value if value was recorded more than 0.6 considered as positive Ewing *et al.* [10].

Meilinjin *et al.* [11] conducted the comparative study on newly developed ELISA technique, in which they used nucleoprotein as antigen for detecting the antibodies to Avian Influenza Virus. They compared this technique with (HI) and ELISA test. Comparative study indicated that these two tests had a high agreement ratio and

no statistically significant difference.

Haem-agglutination inhibition test and titration of antibodies by geometric mean titer (GMT) is the convenient and best technique to measure the level of protection in vaccinated chickens as well as to check the efficacy of vaccine, such types of results were also reported by Meulemans, *et al.* [12] who carried out a study to run HI, AGP and ELISA for measuring the antibodies against avian influenza virus infection. His studies showed a linear relation among these three techniques, when the chickens were exposed with Avian Influenza Virus the antibody status was measured 157 day post infection. It was suggested that AGP is a type-specific; the HI detects only haemagglutinin subtypes, however only ELISA is most sensitive test to detect the antibodies.

The findings of present study showed a degree of correlation between HI, AGP, ELISA and virus Neutralization test, however the degree of accuracy depends on the standardization of reagents, buffers and all other parameters related to the standard procedures of the test. This statement correlates with the availability of equipments and expertise in different laboratories as they perform the same test. That's why the repeatability and reproducibility are two key features that a test must have to be accepted for different laboratories.

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