Heliyon

Received:
13 February 2017
Revised:
7 August 2017
Accepted:
12 October 2017

Cite as:
Philemon N. Wambura, Alexanda Mzula. A novel rapid direct haemagglutination-inhibition assay for measurements of humoral immune response against non-haemagglutinating *Fowlpox virus* strains in vaccinated chickens.
Heliyon 3 (2017) e00428.
doi: 10.1016/j.heliyon.2017.e00428

A novel rapid direct haemagglutination-inhibition assay for measurements of humoral immune response against non-haemagglutinating *Fowlpox virus* strains in vaccinated chickens

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Abstract

Fowlpox (FP) is a serious disease in chickens caused by *Fowlpox virus* (FPV). One method currently used to control FPV is vaccination followed by confirmation that antibody titres are protective using the indirect haemagglutination assay (IHA). The direct haemagglutination inhibition (HI) assay is not done because most FPV strains do not agglutinate chicken red blood cells (RBCs). A novel FPV strain TPV-1 which agglutinates chicken RBCs was discovered recently and enabled a direct HI assay to be conducted using homologous sera. This study is therefore aimed at assessing the direct HI assay using a recently discovered novel haemagglutinating FPV strain TPV-1 in chickens vaccinated with a commercial vaccine containing a non-haemagglutinating FPV.

Chicks vaccinated with FPV at 1 day-old had antibody geometric mean titres (GMT) of $\log_2$ 3.7 at 7 days after vaccination and $\log_2$ 8.0 at 28 days after vaccination when tested in the direct HI. Chickens vaccinated at 6 weeks-old had

http://dx.doi.org/10.1016/j.heliyon.2017.e00428

antibody geometric mean titres (GMT) of $\log_2$ 5.0 at 7 days after vaccination and $\log_2$ 8.4 at 28 days after vaccination when tested in the direct HI. The GMT recorded 28 days after vaccination was slightly higher in chickens vaccinated at 6-week-old than in chicks vaccinated at one-day-old. However, this difference was not significant ($P > 0.05$). All vaccinated chickens showed "takes". No antibody response to FPV and "takes" were detected in unvaccinated chickens (GMT < 1). There was a slightly higher GMT in chickens of all ages throughout the observation period when the standard assay, the passive (indirect) haemagglutination was used (Overall GMT reached $\log_2$ 9.3 $\pm$.0.3 on day 28). However, the difference between the two assays was not significant ($P > 0.05$).

Conclusion: These findings indicate that a simple and rapid direct HI assay using the FPV TPV-1 strain as antigen may be used to measure antibody levels in chickens vaccinated with non-haemagglutinating strains of FPV, and that the titres are comparable to those obtained by indirect IHA.

Keywords: Veterinary medicine, Veterinary science, Vaccines, Immunology, Zoology

1. Background

Fowlpox virus (FPV) is a member of the *Avipoxvirus* in the *Poxviridae* family that infects many bird species and is an important pathogen of the poultry industry which causes Fowlpox (FP) in chickens [1, 2]. Fowlpox occurs in three forms in affected chickens; dry form with cutaneous, wartlike nodules on unfeathered skin around the eyes, beak and feet, comb and wattles transmitted by mosquitoes [3], a diphtheritic/wet form of infection on mucous membranes of the mouth and upper respiratory tract due to inhalation of viral particles forming a false membrane of coagulated necrosis in the mouth, pharynx, larynx, and trachea, and a rare systemic form that may occur throughout tissues of the infected host [4]. Chickens can be affected with any or all forms of FP.

Fowlpox remains a serious disease in chickens of all ages but it usually causes mortalities of up to 60% in chicks infected with the wet type of FP where the respiratory tract is affected. One method of controlling FP is through vaccination. Commercial vaccination for FP is available and has been used effectively to control the disease in chickens [5, 6]. The indirect (passive) haemagglutination (IHA) assay, virus neutralization (VN) assay, agar gel immunodiffusion (AGID) test and enzyme linked immunosorbent assay (ELISA) are commonly used to measure the immune response against FP in vaccinated chickens, some of these serological assays are time consuming and require sophisticated laboratory equipment [7].

Therefore, serological assays which are simple and rapid to perform would be useful for measurement of immune response to FP in vaccinated chickens in

http://dx.doi.org/10.1016/j.heliyon.2017.e00428

laboratories with no sophisticated laboratory equipment. One of the main limiting factors of using a direct HI assay is that most FPV strains do not agglutinate chicken RBCs thus passive or indirect HA assay has been used for several years in determining antibody response [8]. Recently Wambura and Godfrey [9] discovered a novel FPV strain TPV-1 (Accession no. KF032407) which has the ability to agglutinate chicken RBCs, thus enabled a direct HI assay to be done on sera collected from chickens vaccinated with the homologous strain FPV TPV-1 strain.

The objective of this study was to use the FPV TPV-1 strain as an antigen to develop a novel direct HI assay as a test for measurement of antibody responses in chickens vaccinated with heterologous FPV strains which are not capable of agglutinating chicken RBCs.

2. Materials and methods

2.1. Experimental chickens

One-day-old chicks (n = 120) were purchased from a commercial hatchery in Dar es Salaam, Tanzania. The chicks were either used immediately or kept for 6 weeks prior to use. In this experiment, two age groups of chickens, 1-day-old (n = 30) and 6-week-old (n = 30) were used, the remaining chickens (n = 60 were divided into two groups of 30 chickens each) and were mock vaccinated and served as negative controls. All the chickens in both groups were seronegative to FPV antibody before vaccination.

2.2. Vaccination of chickens against FP

A commercially available live attenuated FP vaccine (BIOVAC VIR 102^{TM} BIOVAC LTD, ISRAEL at a titre of $\geq 10^3 EID_{50}$) was used for vaccination of chickens. The vaccine was inoculated subcutaneously into the wing web of experimental chickens at 1-day-old and 6-week-old, respectively. The negative control chickens were injected with uninfected chorioallantoic membrane (CAM) suspension in PBS. The chickens were checked for pox lesions around the inoculation sites for 7 days after vaccination. Observed pox lesions were considered as evidence of infection. All chickens were kept for 5 weeks following vaccination.

2.3. Direct hemagglutination-inhibition assay

Blood samples were collected aseptically from brachial vein of each chicken on days 0, 7, 14, 21 and 28 days after vaccination. The blood was allowed to clot to extract serum. Thereafter serum samples were stored frozen at −20 °C until they were tested to measure levels of antibody specific to FPV. The direct HI assay was carried out by the procedure described by Allan and Gough [10] with the

http://dx.doi.org/10.1016/j.heliyon.2017.e00428

modification of using microplates with "V" bottomed wells. Briefly, the test sera were inactivated at 56 °C for 10 min to destroy complement and HI inhibitors. Twenty-five microlitres of each test sera was serially diluted two-fold in wells containing 25 μL phosphate buffered saline (PBS, followed by the addition of 25 μL of four hemagglutinating units (HAU) of FPV strain TPV-1. After incubation for 15 min at room temperature. Fifty microlitre of 0.5% chicken red blood cells (RBCs) was added to each well. Plates were then incubated at room temperature for 15 min. The results were recorded as $\log_2$ of the reciprocal of the highest serum dilution that completely inhibited hemagglutination of the chicken RBCs. Geometric mean titres (GMT) were calculated from each experimental group.

2.4. Indirect (passive) haemagglutination assay

To prepare soluble FPV antigen the homogenised CAMs from the FPV-infected SPF eggs were centrifuged at 13000 rpm for 20 min, three times to concentrate the virus. The sensitization of Sheep Red Blood Cells (SRBCs) with a non-hemagglutinating FPV vaccine strain antigen (BIOVAC VIR 102^{TM} BIOVAC LTD, ISRAEL) was done according to the method described by Sadawa et al. with modifications. Briefly, 100 ml of a suspension of fresh sheep RBC was washed three times in phosphate buffered saline (PBS) by centrifugation at 3000 rpm for 10 min. A 20% (v/v) suspension of washed SRBCs was prepared in PBS and stored at 4 °C. Equal volumes of SRBCs and 0.2% (v/v) formalin solution and incubated at 37 °C for 15 min. The formalin fixed SRBCs were washed three times by centrifugation at 3000 rpm for 4 min in sterile PBS containing 0.1% sodium azide and finally diluted in 0.01 M PBS to give a 20% (v/v) suspension. The 20% suspension of formalin fixed SRBCs and the soluble FPV antigen were mixed in equal volumes and then incubated at 37 °C overnight with occasional agitation. Finally, the suspension was washed three times by centrifugation and a 2% suspension of sensitized SRBCs was prepared for use in the passive or indirect haemaglutination assay (IHA). The IHA assay was done in microtitre plates by the procedures described by Sawada et al. (1982). Briefly, two-fold serial dilutions of antiserum from the vaccinated chickens were made in PBS and 25 μl of sensitized SRBCs was added to 25 μl of antiserum dilution in U-bottomed plate. The IHA titre was expressed as the reciprocal of the highest dilution of serum showing a definite positive pattern (flat sediment) as compared with the pattern of negative control (smooth dot in the centre of the well) and recorded as GMT.

2.5. Examination of "takes" in vaccinated and unvaccinated chickens

Chickens were observed for presence or absence of "takes" to assess success or failure of vaccination programme against FP from 1 day to 7 days after vaccination according to the procedure described previously by OIE [11]. A scab or swelling at

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the site of vaccine application is considered a positive reaction ("takes"). A "take" occurrence in at least 80% of vaccinated chickens is considered a successful vaccination.

2.6. Analysis and statistics used to interpret data

Each treatment group was tested for FPV-specific antibody levels for a 4-week period post-vaccination. The analysis of variance (one-way ANOVA) was done using the Tukey-Kramer multiple comparison test.

2.7. Ethics approval

All authors hereby declare that "Principles of laboratory animal care" (National Institutes of Health [NIH] publication No. 85-23, revised 1985) were followed, and the Tanzania Animal Welfare Act of 2008 was complied. All experiments were approved by the Research, Publication and Ethics Committee of the FVM, SUA, and Tanzania. The reference number for the ethical approval is SUA/VET/012/03.

3. Results

The HI antibody responses to FPV and the overall results are shown in Table 1. All chickens inoculated with the BIOVAC VIR 102 vaccine developed antibody against FPV when tested by both the direct HI and indirect HA methods. Chicks vaccinated with FPV at 1 day-old had antibody geometric mean titres (GMT) of $\log_2$ 3.7 at 7 days after vaccination and $\log_2$ 8.0 at 28 days after vaccination when tested in the direct HI (Table 2). Chickens vaccinated at 6 weeks-old had antibody GMT of $\log_2$ 5.0 at 7 days after vaccination and $\log_2$ 8.4 at 28 days after vaccination when tested in the direct HI (Table 3). However, the differences between the different age groups were not significant ($P > 0.05$). All vaccinated chickens showed "takes". No antibody response to FPV or "takes" were detected in the mock-vaccinated chickens ($n = 60$) (GMT < 1). There was a slightly higher antibody GMT in chickens of all age throughout the observation period when the standard assay, the IHA was used, where the overall GMT reached a maximum of

Table 1. Overall antibody response in chickens vaccinated with Fowlpox live vaccine (BIOVAC VIR 102) through wing web.

Method used	Geometric Mean titre antibody response at days after vaccination				
	0	7	14	21	28
Novel Direct HI	<1	6.7 ± 0.2	7.1 ± 0.1	8.2 ± 0.2	8.2 ± 0.2
Indirect HA	<1	5.9 ± 0.1	7.8 ± 0.1	9.2 ± 0.1	8.8±.0.3

Number of chickens (n = 60).

http://dx.doi.org/10.1016/j.heliyon.2017.e00428

Table 2. Antibody response in day-old chickens vaccinated with Fowlpox live vaccine (BIOVAC VIR 102) through wing web.

Method used	Geometric Mean titre antibody response at days after vaccination				
	0	7	14	21	28
Novel Direct HI	<1	3.7 ± 0.6	6.5 ± 0.2	7.6 ± 0.1	8.0 ± 0.2
Indirect HA	<1	3.9 ± 0.2	7.9 ± 0.3	7.9 ± 0.2	8.3±.0.1

Number of chickens (n = 30).

Table 3. Antibody response in 6-week-old chickens vaccinated with Fowlpox live vaccine (BIOVAC VIR 102) through wing web.

Method used	Geometric Mean titre antibody response at days after vaccination				
	0	7	14	21	28
Novel Direct HI	<1	5.0 ± 0.4	6.9 ± 0.2	7.6 ± 0.1	8.4 ± 0.2
Indirect HA	<1	5.4 ± 0.1	7.0 ± 0.1	7.4 ± 0.1	9.3±.0.3

$\log_2$ 9.3 ± 0.0.3 on day 28. Statistical analysis showed that the difference between the two assays was not significant ($P > 0.05$). The results indicated that 100% of all vaccinated chickens (n = 60) showed "takes" demonstrating that the vaccination was successful. However, no "takes" were observed in mock-vaccinated chickens (n = 60).

4. Discussion

The success of a vaccination campaign against FPV is indicated by a presence of a protective antibody response in greater than 80% of chickens in vaccinated flocks [12]. The presence or absence of "takes" has been used to measure vaccination success or failure and is indicative of level of protection against field challenge by virulent FPV [11]. These findings correlate well with the results from the present study indicating that all vaccinated chickens (100%) have shown vaccine "takes".

This report describes a direct simple, inexpensive, and rapid HI assay which has been developed and evaluated. The assay will enable measurements and quantification of antibody response to different FP vaccine strains in vaccinated chickens without using complicated assays such as AGID, IHA, VN test and ELISA which take a long time to achieve comparable results.

Previous studies have shown that vaccinated chickens with a titre of GMT of $\log_2$ 3.0 are protected and survive when challenged with virulent FPV or after field infection [9].

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Furthermore, other studies have shown that IHA assays would detect serum antibodies against FPV earlier than the AGID test as early as 7 days after vaccination [8, 13]. Results from the present study indicated similarly that FPV-specific antibodies were detected at 7 days after vaccination in all ages of experimental chickens. The antibody responses were slightly higher in older chickens vaccinated at 6 weeks-old than those in chicks vaccinated at one-day old. These findings correlate well with the previous findings by Wang et al. [14] who reported higher antibody responses for chickens vaccinated at 6 weeks-old than those vaccinated at one day-old.

The development of a direct HI test for detecting antibody responses to FPV opens up a new, potentially valuable general test that could be used in determining immune responses in chickens vaccinated with non-haemagglutinating strains of FPV. Prior to this unique development there were no general tests which could directly use chicken red blood cells for HA and HI tests in quantifying antibody response to FPV.

The development of this test was made possible by the discovery of a novel FPV strain TPV-1 which agglutinates chicken red blood cells [9], paving the way for its use in a direct HI for the determination of antibody responses in chickens vaccinated against FPV.

Previous attempts to use HA test by the use of some commercial vaccine strains of FPV have been generally unsuccessful. There is only one study which resulted in partial haemagglutination of red blood cells but the titre was very low to be considered positive [15].

5. Conclusions

Avipoxviruses are antigenically and immunologically distinguishable from each other, but varying degrees of cross relationships do exist [2, 16]. The direct HI assay developed in the present study has been used successfully to detect antibodies against heterologous FPV vaccine strain antigen (BIOVAC VIR 102^{TM} BIOVAC LTD, ISRAEL). The assay may now be used to detect and quantify antibodies in chickens vaccinated with other non-haemagglutinating strains of FPV.

To the best of our knowledge this is the first report to describe the use of a direct HI assay to detect and quantify antibody response provoked by non-haemagglutinating strains of FPV.

Declarations

Author contribution statement

Philemon N. Wambura: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

http://dx.doi.org/10.1016/j.heliyon.2017.e00428

Alexanda Mzula: Performed the experiments; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Funding statement

This work was supported by COSTECH funded under Kilimo Kwanza Initiative in the project titled "Increasing poultry productivity through thermostable vaccines for control of respiratory diseases".

Additional information

No additional information is available for this paper.

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