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## Research Paper

# Production of avian immunoglobulin Y (IgY) against the linear epitope E2EP3 of chikungunya virus

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## Abstract

Utilization of Immunoglobulin Y (IgY), instead of mammalian antibodies, in diagnostics has become increasingly popular due to non-invasiveness, non-reactiveness to rheumatoid factors and human anti-mammalian antibody, simple isolation process, and large yield. This study produced Immunoglobulin Y antibody against the synthetic linear epitope E2EP3 of Chikungunya virus and evaluated its specificity against the antigen. Twenty-eight-week-old White Leghorn hens were immunized with E2EP3 synthetic peptide by intramuscular injection followed by two boosters. Eggs were collected 1 week before and 13 weeks after primary immunization. IgY was isolated from egg yolks by delipidation and sodium sulfate precipitation and then pooled on a weekly basis. Pooled IgY isolates were characterized by native and Sodium Dodecyl Sulfate (SDS) - Polyacrylamide Gel Electrophoresis (PAGE) for presence and purity and by Radial Immunodiffusion Assay (RIA) and indirect ELISA for binding activity. E2EP3-specific IgY was purified by affinity chromatography and its binding affinity and specificity were determined by indirect ELISA. Native PAGE confirmed that the molecular weight of the isolated IgY was 165 kDa, while SDS-PAGE showed the presence of the heavy (60 kDa) and light (28 kDa) chains of IgY. In RIA, precipitin lines were observed between the antigen (E2EP3) and pooled IgY isolates from 4 – 11 weeks after primary immunization, which indicate antigen-IgY binding. In a series of E2EP3 antigen dilution, indirect ELISA showed that the putative E2EP3-specific IgY was sensitive to the E2EP3 antigen solution at 10 µg/ml. The putative E2EP3-specific IgY from 4 – 13 weeks after primary immunization did not exhibit cross-reactivity against the Dengue virus specific DEN-1 synthetic peptide. Therefore, the Chikungunya virus E2EP3-specific IgY obtained in this study, with no cross-reactivity against a Dengue virus synthetic peptide, can be further developed into a cheap and simple diagnostic tool for Chikungunya infection at onset.

**Keywords:** Chikungunya virus, E2EP3 epitope, IgY-based diagnostic tool, Immunoglobulin Y, synthetic peptide

## Introduction

Dengue infection had been on the rise in the past few years. However, more recently, Chikungunya virus (CHIKV) infection became another public health problem to contend with. In 2007, the first Chikungunya outbreak ever occurring in a temperate area of the Northern hemisphere was reported in Emilia-Romagna, Italy<sup>[1]</sup>. Active circulation of CHIKV in the region of Kédougou, Senegal was reported between 2009 to 2010 and re-emerged in August 2015, wherein 10 confirmed cases were

reported<sup>[2]</sup>. In Argentina, 55 cases of Chikungunya infection were reported from January to February 2016, being the first outbreak of the disease in the country<sup>[3]</sup>. In Chennai, India, it has been reported that 44% of the participants were seropositive to CHIKV in June to July 2011 and the age-specific seroprevalence was suggestive of epidemic chikungunya transmission<sup>[4]</sup>. As of April 15, 2016, 220 confirmed cases of CHIKV in North America had been reported to the Pan American Health Organization, 310 in Central American Isthmus, 1021 in Bolivia, and 174 in Ecuador<sup>[5]</sup>. To date, no vaccines are available for the prevention of CHIKV infections. However, various vaccines are being developed including live CHIKV vaccine (CHIKV/IRES)<sup>[6]</sup>, a plasmid coding for the CHIK-capsid E1 and E2<sup>[7]</sup>, a recombinant live-attenuated measles vaccine expressing CHIKV-like particles comprising capsid and envelope structural proteins from a CHIKV strain La Reunion<sup>[8]</sup>, chikungunya vaccine strain 181/clone 25, and vaccines employing recombinant E2 protein and formalin- and betapropiolactone-inactivated whole virus<sup>[9]</sup>, all causing immunity of mice to CHIKV. Recently, a live recombinant measles virus-based chikungunya vaccine raised neutralizing antibodies of the participants with a generally acceptable tolerability profile<sup>[10]</sup>.

The signs and symptoms of Dengue and Chikungunya infections are similar, with the exception of severe joint pains for the latter. Common manifestations of the two infections are fever, headache, rash, nausea, vomiting, fatigue, depression, and disabling joint and muscular pain<sup>[11]</sup>. In addition to this, CHIKV is transmitted by *Aedes* mosquitoes and shares the same geographical distribution as the Dengue virus (DENV)<sup>[12]</sup>. Thus, CHIKV infection is often mistaken as dengue infection. Laboratory diagnosis of Chikungunya is generally accomplished by testing serum or plasma to detect virus-specific immunoglobulin M (IgM) and neutralizing immunoglobulin G (IgG) antibodies by ELISA. The virus may be isolated from the blood during the first few days of infection and tested by reverse transcriptase-polymerase chain reaction (PCR) methods that are, however, of variable sensitivity. Recently, a single-reaction, multiplex molecular diagnostics for DENV and CHIKV was developed and showed good agreement with individual molecular comparators for DENV and CHIKV<sup>[13]</sup>. However, PCR techniques require costly equipment and trained technical support.

Chikungunya infections are usually associated with a low mortality rate; however, severe manifestations such as meningoencephalitis, fulminant hepatitis, and bleeding may be life-threatening<sup>[14]</sup>. There are no antiviral drugs available for the treatment of CHIKV infections. However, there are molecules that have been reported to inhibit CHIKV entry such as Chloroquine, Arbidol, and Flavaglines; those that inhibit viral protein synthesis such as Harringtonine and homoharringtonines; and those that inhibit viral genome replication such as Ribavirin, 6-Azauridine, and Favipiravir<sup>[15]</sup>.

It has been established by the study of Kam et al.<sup>[16]</sup> that the naturally-acquired early IgG3 response against CHIKV in 30 infected patients is strongly focused on the E2E3 epitope. From their study, scanning of a peptide library showed that the IgG response was most pronounced against the N'-terminal part of the E2 glycoprotein (pool P1) and further assay with the complete set of single peptide from this active pool showed that the antibodies strongly recognized the first two peptides of pool 1 (P1-1 and P1-2) suggesting that the E2E3 epitope should be present within the overlapping part of peptides P1-1 and P1-2. The overlap is located to the site required for the proteolytic generation of E2 and E3 glycoproteins from the common precursor protein<sup>[17]</sup>. The E2E3 epitope is located at the N-terminus of the E2 glycoprotein proximal to a furin E2/E3 cleavage site that is conserved in many alphaviruses.

Several studies showed therapeutic ability of CHIKV Ig. CHIKV Ig, isolated from plasma samples from donors in convalescent phase of CHIKV infection, completely protected mice against CHIKV infection, inhibited viral amplification in tissue, prevented viremia, and completely protected neonates against the infection<sup>[18]</sup>. E2E3 vaccination was able to induce the natural IgG3 protective response in preclinical models with significant reduction in viral counts and joint inflammation<sup>[19]</sup>.

Immunoglobulin Y (IgY), the avian counterpart of the mammalian IgG, is exclusively transferred from hens to the egg yolks to confer passive immunity to the embryo. Utilization of IgY instead of mammalian antibodies in therapeutics and diagnostics has become increasingly popular due to cost effectiveness, non-invasiveness, non-reactiveness to rheumatoid factors and human anti-mammalian antibody<sup>[20]</sup>, simple and economical isolation process, large yield, and scalable production<sup>[21]</sup>. Tan et al.<sup>[22]</sup> stated that their procedure is cost-effective (\$5 USD per egg), rapid (within 5 hours), high yielding (60 mg), and of high purity (~80% purity). Another advantage of using IgY is the non-invasive, non-stressful collection of antibodies as no bleeding and only egg collection from immunized hens is required, thus favoring animal welfare<sup>[23]</sup>.

Since E2EP3 can induce the natural IgG3 protective response in preclinical model and several studies support that IgY is functionally similar to IgG and that chicken antibodies have many advantages to the traditional mammalian antibodies, this study determined if the E2EP3 epitope can elicit a specific IgY for CHIKV infection. Initial studies on IgY-based diagnostic tool for DENV infection had been done, hence, we would also like to develop a comparable test for CHIKV infection in the hope of having a cheaper yet specific diagnostic tool for the two similar infections at the onset.

This study aimed to produce IgY antibodies against the E2EP3 peptide of the CHIKV, characterize the crude and affinity-purified IgY, and determine its binding affinity against the E2EP3 peptide and cross-reactivity against an epitope-based dengue peptide antigen.

## Materials and Methods

### Animals

White Leghorn hens (vaccinated against Marek's, Infectious Bursal Disease, Newcastle Disease, Infectious Bronchitis, Fowl Pox, *Mycoplasma gallisepticum*, *Coryza*, Fowl cholera, and Egg-drop Syndrome) were bought from Renell M. Lantin Poultry Farm in Lipa, Batangas, Philippines. They were acclimatized for two weeks in the Animal Facility of the Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila before experiments were performed. Chickens were housed in a well-ventilated and air conditioned room under recommended temperature (16 – 27 °C) and light-dark cycle (16 hours light/8 hours dark) for egg laying. They were given laying mash and mineral water *ad libitum*.

### Experimental design

Eight White Leghorns were randomly divided into two groups: experimental and control. Chickens in the experimental group were injected with 1:1 (v/v) synthetic peptide-adjuvant emulsion [100 µg of bovine serum albumin (BSA) conjugated E2EP3 peptide (STKDNFNVYKATRPY) dissolved in 0.01 M PBS pH 7.4, Complete Freund's Adjuvant (primary immunization)/Incomplete Freund's Adjuvant (booster immunization)] into four sites on the breast tissue. Chickens in the control group, on the other hand, were injected with 250 µl 0.01 M PBS pH 7.4 into four sites on the breast tissue. Primary immunization was done when all eight chickens have lain at least one egg. Two booster doses were administered two and four weeks after the primary immunization. Eggs were collected daily, 1 week before to 13 weeks after primary immunization. All experimental procedures were in accordance with the animal care guidelines of the Institutional Animal Care and Use Committee of the University of the Philippines Manila.

### IgY isolation

IgY isolation was performed as described by Guevarra *et al.* [20]. For each egg collected, the egg yolk was separated from the egg white, washed with distilled water and then diluted with equal volume of distilled water. A 1:1 volume of 0.15% λ-carrageenan was added and the suspension was centrifuged at 3200 x g at 20 °C for 30 minutes. Sodium sulfate was added until a total of 19% salt concentration was achieved. The solution was centrifuged at 3200 x g at room temperature for 30 minutes. The resulting precipitate, which is the isolated IgY, was dissolved in 0.01 M PBS pH 7.4. The IgY isolates were pooled per week (from 1 week before to 13 weeks after primary immunization) by combining equal volumes of the isolated IgY solution from all collected eggs per week.

### Purification of pooled IgY isolates

Pooled IgY isolates were purified using affinity chromatography following the method of Camenisch [24]. The coupling solution was prepared by first dissolving 2.5 mg of the E2EP3 synthetic peptide in 50 µL distilled water and incubating the solution at room temperature for 2 hours. Then, the solution was brought to 2.5 mL with coupling buffer (0.1M NaHCO<sub>3</sub> with 0.5 M NaCl, pH 8.3).

The medium was prepared by combining one gram of freeze-dried CNBr-activated Sepharose 4 Fast Flow with 5 mL of 1 mM HCl. The resin was washed with 60 mL of cold 1 mM HCl. The swollen Sepharose was then combined with the previously prepared coupling solution and incubated at room temperature for 3 hours with gentle agitation. Afterwards, the gel was transferred to the Econo-column chromatography column (Bio-Rad). Unbound ligands were removed by washing the gel with at least 5 ml volumes of coupling buffer. Excess CNBr-active groups that were not conjugated with the peptide were blocked using 0.1M Tris-HCl, pH 8.0 for 2 hours. The gel was then washed with acetate buffered saline (0.1M NaCH<sub>3</sub>COO containing 0.5 M NaCl, pH 4.0) and Tris buffered saline (0.1M Tris-HCl

containing 0.5 M NaCl, pH 8.0) three times. After washing, the column was rinsed with 50 ml 0.1 M PBS pH 7.4.

Before loading into the column, pooled IgY isolates were dialyzed against the coupling buffer for 1 hour using a membrane with a 14 kDa molecular cut-off. Five milliliters of the dialyzed pooled IgY isolates were loaded into the column and then washed with 50 mL of 0.1 M PBS (pH 7.4). Fractions were collected at 5 mL intervals. Afterwards, elution buffer (0.2 M glycine, pH 2.2, containing 0.15 M NaCl) was introduced to the column and each fraction was collected in 2 mL intervals. Collected fractions were neutralized immediately by adding 0.8 mL 1 M Tris buffered saline (pH 8.0).

Affinity chromatography was repeated using BSA as ligand to remove potential BSA-specific IgY due to the BSA conjugated to the E2EP3 synthetic peptide that was immunized to the experimental group of chickens.

#### **Determination of Total Protein and IgY Concentration**

Total protein and IgY concentrations were determined using the Bio-Rad Protein Assay, which is based on the Bradford method, for microplates. Absorbance was measured at 595 nm using a microplate reader (Model 550, Bio-Rad). BSA was used as the protein standard.

#### **Assessment of IgY Purity**

Native and SDS-PAGE were performed to assess the purity and determine the molecular weight of pooled IgY isolates using the Mini-PROTEAN system (Bio-Rad) following the manufacturer's instructions. For native PAGE, 10% resolving gel and 4% stacking gel was used. For SDS-PAGE, 12% resolving gel and 4% stacking gel was used. Pooled IgY isolates and the chicken IgY standards were run under the same conditions at 180 volts. Gels were stained in Coomassie Brilliant Blue R-250 staining solution (0.025% in 40% methanol containing 7% acetic acid) and then destained in 40% methanol with 7% acetic acid.

#### **Determination of IgY Binding Affinity and Specificity**

##### **Radial Immunodiffusion Assay**

The method of immunodiffusion developed by Ouchterlony and done by Waggett<sup>[25]</sup> was employed. Briefly, 1.2% agarose gels in 0.07 M PBS was prepared in 9 cm petri plates. Wells measuring 0.7 centimeters in diameter were cut, one at the center for the E2EP3 synthetic peptide solution and six outer wells for the pooled IgY isolates. The outer wells were 0.5 centimeters away from the center. The pooled IgY isolates and E2EP3 synthetic peptide solution were left to diffuse for 3-10 days at 25-30°C. Agarose gels were stained by soaking in 0.1 M NaCl three times for 15 minutes at a time and then immersing the gel in the Coomassie Blue staining solution for 5 minutes with gentle shaking. Afterwards, the agarose gels were soaked in distilled water for 10 minutes.

##### **Indirect Enzyme-linked Immunosorbent Assay (ELISA)**

Indirect ELISA was used to determine peptide-antipeptide binding activity. For the binding affinity assay, four-fold serial dilution (10 µg/ml, 2.5 µg/ml, 0.625 µg/ml, 0.156 µg/ml, 0.039 µg/ml, 0.0098 µg/ml) of the E2EP3 synthetic peptide in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) was dispensed in a 96-well plate and incubated for 16-18 hours at 4°C. Wells were washed with washing buffer (0.01 M PBS containing 0.05% Tween 20) three times. Then, 100 µl of blocking buffer (1% ovalbumin in washing buffer) was dispensed to the wells and incubated for 2 hours at 37°C, followed by three times washing with washing buffer. Pooled IgY isolates (50 µl) was added to each well and incubated for 1 hour at 37°C. After washing three times, 50 µl of HRP-conjugated anti chicken IgY (1:30,000) was added to each well and incubated for 1 hour at 37°C. Wells were again washed with washing buffer three times. Afterwards, 100 µl of TMB substrate solution (Sigma) was added to wells and incubated for 30 minutes at room temperature. The reactions were stopped with 50 µl of 2 M H<sub>2</sub>SO<sub>4</sub> solution and read at 450 nm in a microplate reader (Model 550, Bio-Rad) after 2 minutes. For the binding specificity assay, affinity-purified pooled IgY isolates were tested against the dengue-1 virus envelope protein synthetic peptide (FKLEKEVAETQHGT).

#### **Statistical Analysis**

Data are expressed as means of triplicate absorbance readings. Total protein concentrations of samples were calculated using linear regression analysis. The statistical significance of the differences between the mean values of control and experimental groups was evaluated through one-way analysis of variance (ANOVA).

For the binding affinity assay, cut off values were determined using the formula of Singh [26]. For tests with known negative samples, the cut off point is calculated as follows:

**Cut off value = Mean + 2SD**

Where SD = standard deviation of the absorbance of the blank.

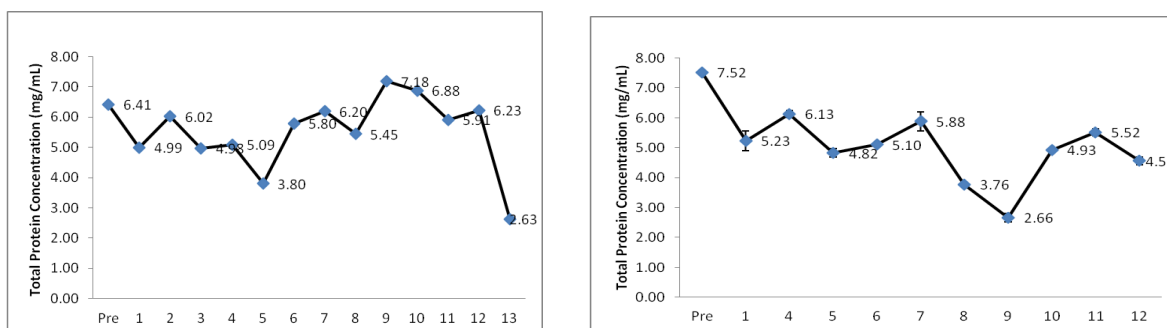
For binding specificity assay, a statistically valid method devised by Frey et al. [27] was used for calculating cutoff values based on the absorbance due to background. The cutoff point is calculated as follows:

**Cut off value = Mean + SD\*f**

Where SD = standard deviation of the absorbance of the blank, and f = standard deviation multiplier which is dependent on confidence interval and number of replicates. Samples with absorbance greater than the cut off value were considered positive with peptide-antipeptide binding. The tests were done at 95% confidence interval.

**Total Protein Concentration of IgY Isolates**

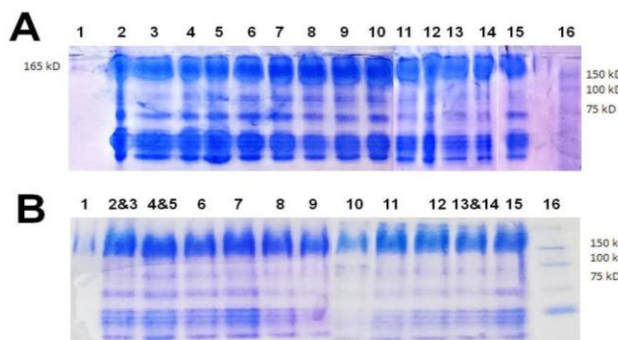
Figure 1 shows the total protein concentration of pooled IgY isolates from 1 week before up to 13 weeks after primary immunization. The mean total protein concentration of pooled IgY isolates of experimental and control groups from 1 week before up to 13 weeks after primary immunization were 5.54 and 5.10 mg/ml, respectively. There is no statistically significant difference in the mean total protein concentration of pooled IgY isolates from the two groups based on ANOVA.



**Figure 1: Total protein concentration of pooled IgY isolates from the experimental group (left) immunized with the E2EP3 synthetic peptide and control group (right) as determined by the Bio-Rad Protein Assay**

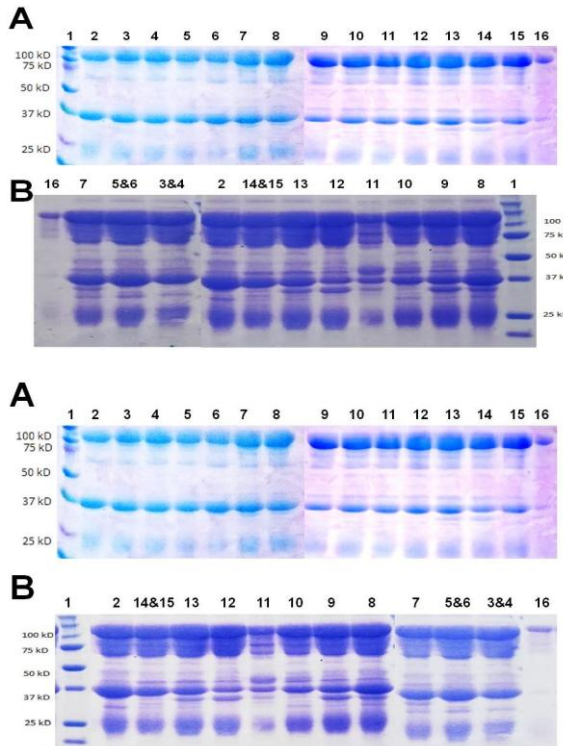
**Purity of IgY Isolates by Native and SDS-PAGE**

Native PAGE of the pooled IgY isolates showed the presence of bands that have the same electrophoretic mobility and molecular weight as the standard IgY, which is 165 kilodaltons (Figure 2).



**Figure 2: Native PAGE of pooled IgY isolates from the (A) experimental group immunized with the E2EP3 synthetic peptide and (B) control group. 1: chicken IgY standard; 2 – 14: 1 to 13 weeks after primary immunization; 15: 1 week before primary immunization; 16: Precision Plus Protein Standards (Bio-Rad)**

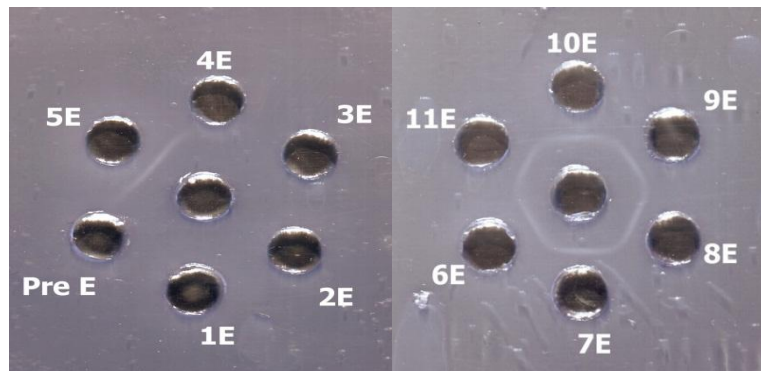
Both the heavy (67 – 70 kDa) and light chains (25 kDa) of IgY were shown to be present in pooled IgY isolates from 1 week before up to 13 weeks after primary immunization using SDS-PAGE (Figure 3). Other bands present between the 100 – 150 kDa mark could possibly represent not fully denatured IgY, while other bands may be attributed to other impurities such as  $\beta$ -livetins<sup>[28]</sup>.



**Figure 3: SDS-PAGE of pooled IgY isolates from the (A) experimental group immunized with the E2EP3 synthetic peptide and (B) control group. 1: Precision Plus Protein Standards (Bio-rad); 2: 1 week before primary immunization; 3 – 15: 1 to 13 weeks after primary immunization; 16: chicken IgY standard**

**Binding Affinity of IgY Isolates**

As seen in Figure 4, white precipitation lines developed in the gel between the E2EP3 peptide solution (0.05 mg/mL) and pooled IgY isolates from 5 weeks until 11 weeks after primary immunization. The RIA results provide information that the pooled IgY isolates from 5 weeks up to 11 weeks after primary immunization contains E2EP3 peptide-specific IgY.



**Figure 4: Radial immunodiffusion assay reactions between 0.05 mg/mL of the E2EP3 synthetic peptide and pooled IgY isolates from the experimental group from pre-immunization (Pre E) up to 11 weeks after primary immunization (1E – 11E)**

Indirect ELISA further confirmed the presence of E2EP3 peptide-specific IgY in the pooled IgY isolates (Table 1). E2EP3 peptide specific IgY was detected as early as 4 weeks after primary

immunization. This could be attributed to higher sensitivity range of ELISA (0.01 µg/ml) compared to RIA (20 – 200 µg/ml) [29].

**Table 1: ELISA results of pooled IgY isolates from 1 week before to 13 weeks after primary immunization against the E2EP3 synthetic peptide (10 µg/ml)**

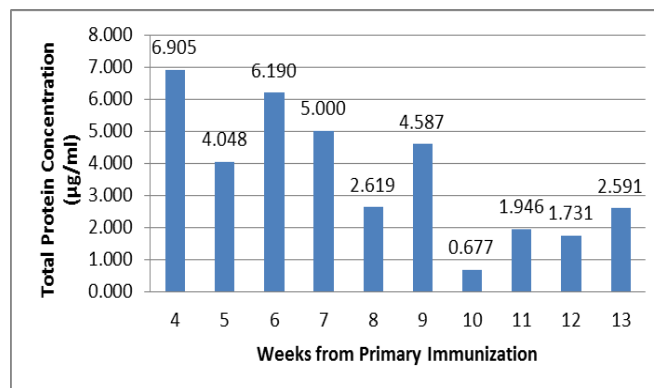
	Pre-immunization	Weeks after Primary Immunization												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Mean Absorbance	0.0823	0.13	0.14	0.14	0.17	0.25	0.28	0.17	0.26	0.17	0.16	0.134	0.15	0.17
Cut off	0.0844	0.36	0.30	0.16	0.11	0.14	0.15	0.10	0.17	0.11	0.11	0.0620	0.13	0.13
Interpretation	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)

(=) no E2EP3 peptide specific IgY; (+) presence of E2EP3 peptide specific IgY

**Purification and Characterization of IgY Isolates**

Based on the ELISA results (Table 1), pooled IgY isolates from 4 up to 13 weeks after primary immunization were selected for purification of E2EP3 specific IgY by affinity chromatography.

Figure 5 shows the protein concentrations of the putative E2EP3-specific IgY. The peak total IgY protein concentration, 6.905 µg/ml, was observed at 4 weeks after primary immunization. However, a decrease in the total protein concentration was observed from 10 to 13 weeks after primary immunization.



**Figure 5: Total protein concentration of putative E2EP3-specific IgY from 4 weeks to 13 weeks after primary immunization**

In the studies by Chui *et al* [30], Lee *et al* [31], Rahimi *et al* [32], and Guevarra [20], IgY with activity against the target antigen was detected starting the first two weeks after primary immunization. However, based on RIA and ELISA results presented herein, IgY with specific activity against the target antigen was detected starting 4 weeks after the first immunization. The peptide-specific IgY in this study peaked 4 and 6 weeks after first immunization. However, Guevarra [20], Lee *et al* [31], Li *et al* [33] and Chui *et al* [30], and Rahimi *et al* [32] detected the optimum peptide-specific IgY at weeks 5 – 6, 7, 8, and 9, respectively. It should be noted that the yolk IgY concentration may vary among individuals and genetic lines or breeds [34]. In a study by Sudjarwo *et al* [35], anti-dengue IgY were detected at 2 weeks after immunization and peaked 4 weeks after immunization.

Indirect ELISA of affinity-purified pooled IgY isolates from 6 and 11 weeks after primary immunization, as representatives of weeks with high and low putative E2EP3-specific IgY concentrations, respectively, showed that the minimum E2EP3 peptide concentration that can be detected by the purified pooled IgY isolates is 10 µg/ml (Table 2).

**Table 2: Binding affinity of the putative E2EP3-specific IgY from 6 and 11 weeks after primary immunization against different concentrations of the E2EP3 synthetic peptide**

Peptide Concentration ( $\mu\text{g/ml}$ )	6 Weeks After Primary Immunization		11 Weeks After Primary Immunization	
	Mean Absorbance	Interpretation	Mean Absorbance	Interpretation
10.000	0.213	(+)	0.173	(+)
2.500	0.097	(-)	0.101	(-)
0.625	0.132	(-)	0.092	(-)
0.156	0.093	(-)	0.066	(-)
0.0391	0.111	(-)	0.080	(-)
0.0098	0.162	(-)	0.094	(-)
Cut off Value*	0.157		0.157	

\*Cut off Value was computed at 95% confidence interval. (+) E2EP3 detected by IgY; (=) no detection

Binding specificity assay results showed that the mean absorbance readings of IgY isolates from 4 to 13 weeks after primary immunization for the DEN-1 synthetic peptide was lower compared to the E2EP3 synthetic peptide (Table 3). In effect, only the mean absorbance reading for the E2EP3 peptide was higher than the cut off value for all samples as seen in Table 3. This indicates that the IgY isolates only exhibits positive binding affinity to the E2EP3 peptide, confirming that the IgY purified through affinity chromatography is specific for E2EP3.

**Table 3: Binding specificity of putative E2EP3-specific IgY from 4 to 13 weeks after primary immunization against the Chikungunya E2EP3 synthetic peptide and Dengue DEN-1 synthetic peptide**

Affinity-purified pooled IgY isolates per week	Mean Absorbance Reading		Cut off Value
	Chikungunya E2EP3 synthetic peptide (10 $\mu\text{g/ml}$ )	Dengue DEN-1 synthetic peptide (10 $\mu\text{g/ml}$ )	
Week 4	2.356 (+)	1.048 (-)	1.184
Week 5	0.115 (+)	0.095 (-)	0.114
Week 6	0.087 (+)	0.085 (-)	0.086
Week 7	0.136 (+)	0.111 (-)	0.130
Week 8	0.156 (+)	0.144 (-)	0.155
Week 9	0.127 (+)	0.097 (-)	0.113
Week 10	0.109 (+)	0.090 (-)	0.100
Week 11	0.116 (+)	0.094 (-)	0.095
Week 12	0.126 (+)	0.098 (-)	0.106
Week 13	0.143 (+)	0.118 (-)	0.137

The production of antibodies in chicken is simple and non-invasive with large yield [20]. In this study, IgY was successfully elicited by immunizing the hens through the intramuscular route with the synthetic CHIKV E2EP3 peptide emulsified in Complete/Incomplete Freund's Adjuvant. We adopted a carrageenan extraction method followed by sodium sulfate precipitation. According to Ren *et al* [36], antibodies purified with carrageenan exhibited high purity in comparison to caprylic acid extraction. Furthermore, the method is safe making it appropriate for use in pharmaceutical production. The IgY concentration in egg yolk peaked at 4 weeks after immunization and decreased starting 10 weeks after primary immunization. The general structure of the IgY molecule is the same as the IgG molecule. However, the heavy chain in IgY is larger than that of mammalian IgG (67 – 70 vs. 50 kD), which is due to an increased number of heavy chain constant domains and carbohydrate chains [37]. Both the heavy (67 – 70 kDa) and light chains (25 kDa) of IgY were shown to be present in pooled IgY isolates from 1 week before up to 13 weeks after primary immunization using SDS-PAGE.

Affinity purification with immobilized antigen as affinity ligand is widely used due to specific antigen recognition by antibodies. This is used if a specific antibody must be obtained from a mixture of immunoglobulins with different specificities. In this study, the IgY isolate was applied to the E2EP3-coupled sepharose under conditions that favored specific binding. After washing the unbound



materials through the column, the target protein was recovered by changing the pH. Based on the ELISA results presented, the affinity-purified IgY exhibited activity against the target E2EP3 antigen with no cross reactivity with the dengue DEN-1 synthetic peptide, indicating specificity. The putative E2EP3-specific IgY was detected as early as 4 weeks after primary immunization; showing that White Leghorn hens require about 4 weeks for antibody production and can efficiently provide specific IgY in a non-invasive way.

Advantages of the use of IgY in immunological tests include cost effectiveness, non-invasiveness, and non-reactiveness to rheumatoid factors and human anti-mammalian antibody<sup>[20]</sup>. Another advantage is the phylogenetic distance and genetic background that distinguishes birds from mammals improving the likelihood that an immune response will be elicited against antigens or epitopes that may be non-immunogenic in mammals<sup>[38]</sup>.

### Conclusion

In this study, immunoglobulin Y (IgY) that is specific for the synthetic linear E2EP3 peptide from the E2 envelope protein of the Chikungunya virus was successfully extracted, isolated, and partially purified from the yolk of eggs laid by White Leghorn hens immunized with the peptide. The presence and purity of isolated IgY was revealed using SDS-PAGE, with the heavy chains having a molecular weight of approximately 67 kDa and light chains of 25 kDa. Total protein concentration of the isolates range from 4 – 7 mg/mL and 0.677 – 6.905 µg/ml for the pooled IgY isolates and putative E2EP3-specific IgY, respectively. It was demonstrated that there is peptide-antipeptide binding of E2EP3 synthetic peptide and pooled IgY isolates as early as 4 weeks after primary immunization based on indirect ELISA. Affinity purified IgY was found to be sensitive to antigen concentrations starting at 10 µg/mL. Putative E2EP3-specific IgY from 4 to 13 weeks after primary immunization did not show cross reactivity with the dengue specific DEN-1 synthetic peptide indicating that the affinity purified IgY is specific for E2EP3. Additional studies using the Chikungunya virus and serum of patients with Chikungunya infection should be done to further confirm the specificity of the putative E2EP3-specific IgY. Furthermore, the isolated specific IgY should also be tested against the dengue virus and serum of patients with dengue fever to test its efficiency in differentiating the two virus infection. Data from this study can now be used as supporting information for further development of IgY-based diagnostic tools for Chikungunya and dengue virus infection.

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