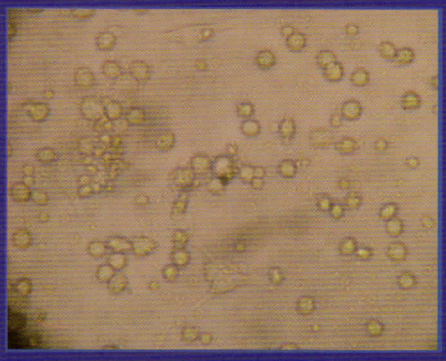
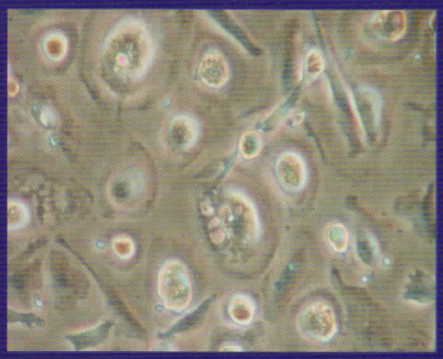
e ISSN 2356 - 0991
p ISSN 2085 - 1103

Indonesian Journal of Tropical and Infectious Disease





e-journal.unair.ac.id/index.php/IJTID

Vol. 6 • No. 2 May – August 2016

IJTID

Indexed by : ISJD IPI Google Scholar DOAJ DIRECTORY OF OPEN ACCESS JOURNALS Crossref

Volume 6 Number 2 May–August 2016

e ISSN 2356 - 0991
p ISSN 2085 - 1103

Indonesian Journal of Tropical and Infectious Disease

EDITORIAL BOARD OF
INDONESIAN JOURNAL OF TROPICAL AND INFECTIOUS DISEASE

Editor in Chief

Prihartini Widiyanti (Universitas Airlangga)

International Editorial Boards

Hak. Hotta (Japan)
Hartmut Kuehn (Germany)
Yoshitake Hayashi (Japan)
Fumihiko Kawamoto (Japan)
Kazufumi Shimizu (Japan)
Masanori Kameoka (Japan)
Takako Utsumi (Japan)
Yoshihiko Yano (Japan)

Editorial Board

Nasronudin (Universitas Airlangga)
Maria Inge Lusida (Universitas Airlangga)
Puruhito (Universitas Airlangga)
Soetjipto (Universitas Airlangga)
Indropo Agusni (Universitas Airlangga)
Retno Handajani (Universitas Airlangga)
Kuntaman (Universitas Airlangga)
Ni Made Mertaniasih (Universitas Airlangga)
Suharto (Universitas Airlangga)
Soegeng Soegianto (Universitas Airlangga)
Bambang Prajogo (Universitas Airlangga)
Ni Nyoman Sri Budayanti (Universitas Udayana)
H. Achmad Fuad H (Universitas Airlangga)
Budiman Bela (Universitas Indonesia)
Fedik A. Rantam (Universitas Airlangga)
Tri Wibawa (Universitas Gadjah Mada)

Associate Editors

Marcellino Rudyanto
Aty Widyawaruyanti
Irwanto
Dwi Wahyu Indriati
Juniastuti
Yulis Setya Dewi
Laura Navika Yamani
Indah S. Tantular
Dadik Rahardjo
E. Bimo Aksono
Ali Rohman
Retno Pudji Rahayu

Secretariat

Zakaria Pamoengkas
Firda Fatma Hamzah

Secretariat Office

Publishing Unit of Indonesian Journal of Tropical and Infectious Disease, Institute of Tropical Disease Universitas Airlangga
Kampus C, Jalan Mulyorejo Surabaya 60115, Jawa Timur – Indonesia. Phone 62-31-5992445-46 Faximile 62-31-5992445
E-mail: ijtid@itd.unair.ac.id Homepage: e-journal.unair.ac.id/index.php/IJTID

Volume 6 Number 2 May–August 2016

e ISSN 2356 - 0991
p ISSN 2085 - 1103

Indonesian Journal of Tropical and Infectious Disease

CONTENTS

Printed by: *Universitas Airlangga Press*. (RK 557/10.16/AUP-457). Kampus C Unair, Mulyorejo Surabaya 60115, Indonesia. Telp. (031) 5992246, 5992247, Fax. (031) 5992248. E-mail: aupsby@rad.net.id; aup.unair@gmail.com

Indonesian Journal of
Tropical and Infectious Disease
Vol. 6. No. 2 Mei–Agustus 2016

Research Report

PRODUCTION AND CHARACTERIZATION OF IMMUNOGLOBULINE YOLK AS ANTI ANTIGEN MEMBRANE *TOXOPLASMA GONDII*

Yuliana Praptiwi[1], Heni Puspitasari[2a], Luciana T Suwanti[3], Mufasirin[3]
[1] Magister of Veterinary, The Faculty of Veterinary Medicine, Universitas Airlangga Surabaya
[2] Toxoplasma Study Group, Instutute of Tropical Disease, Universitas Airlangga Surabaya
[3] Parasitology Departement, The Faculty of Veterinary Medicine, Universitas Airlangga Surabaya
[a] Corresponding author: henipuspitasari486@gmail.com

ABSTRACT

Toxoplasma gondii is an obligate parasite intracellular which can infection human and other mammalian. Immunoglobulin Y technology offers several advantages better than antibody production in mammals. This research is aimed to get immunoglobulin Y from egg yolk, and to find the characterization of immunoglobuline Y according to molecular weight by SDS PAGE and targeted protein with antibodies using Western Blot. This research divided from many step: culture tachyzoites of T. gondii fromintraperitoneal fluid, preparation of membrane antigen tachyzoite of T. gondii, then immunization laying hens with membrane antigen, extraction and purification immunoglobuline Y from egg yolk and then protein analyzed by SDS PAGE and Western Blot. The result of this research showed that immunoglobulin Y from egg yolk can produced antibody against protein membrane of T. gondiiandprofile protein immunoglobuline Y according SDS PAGE has molecular weight 179,8 kDa. Immunoglobuline Y was analyze by Western Blot can recognize antigen epitope of T.gondii on molecular weight 35,7kDa and 78,8 kDa.

Keywords: *Toxoplasma gondii, anti membrane T.gondii, immunoglobulin Y anti membrane*

ABSTRAK

Toxoplasma gondii merupakan parasit obligate intraselluler yang dapat menginfeksi manusia dan mamalia lain. Pemanfaatan immunoglobulin Y memberikan beberapa keuntungan dari pada antibodi yang diproduksi mamalia. Tujuan dari penelitian ini adalah mendapatkan immunoglobulin Y dari kuning telur dan menemukan karakterisasi immunoglobulin Y berdasarkan berat molekul dengan SDS PAGE dan penargetan protein dengan antibody menggunakan WESTERBLOT. Penelitian ini dibagi menjadi beberapa tahapan yaitu kultur takizoit Toxoplasma gondii dari cairan intraperitoneal, preparasi antigen membran Toxoplasma gondii, imunisasi ayam dengan protein membrane Toxoplasma gondii, ekstraksi dan pemurnian kuning telur untuk mendapatkan Ig Y dan karakterisasi Ig Y dengan SDS Page serta penargetan protein westrn blott. Hasil SDS PAGE Ig Y ditemukan pita protein 178 kDa- 7038kDa, kemudian analisa penargetan protein dengan westrn blottdapat mengenali antigen epitop Toxoplasma gondii pada berat molekul 35,7kDa dan 78,8kDa.

Kata kunci: *Toxoplasma gondii, membran anti T. gondii, immunoglobulin Y anti membran*

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that can infect humans. Definitive host of this parasite is the cat, while the intermediary hosts include mammals, birds and reptiles nation even fish. In the life cycle of this parasite can infect a host by 3 ways: through ingestion of tissue cysts by bradizoit, by ingestion of oocysts and congenital infection with tachizoit.[1] Cats infected with *Toxoplasma gondii* in all excretions will spend millions of oocysts. When the oocyst is ingested by an intermediate host such as humans, cows, goats on the various tissues will be established intermediate host groups tropozoit actively dividing to form the rest of the stadium in the form of cysts

(bradizoit) on the network. At the intermediate host is not formed sexual stage but only just asexual stage. When cats eat mice containing cysts are formed in the sexual stage in the cat's intestine.[2]

Humans infected with the *T. gondii* occurs not only on those who keep cats or dogs but can also occur in other people who like to eat the food of undercooked meat containing tissue cysts, drinking fresh milk undercooked, water contaminated with raw vegetables and raw contaminated by disease-causing agents toxoplasmosis.[2] Incidence of toxoplasmosis has not significant changed in recent years, caution and attention to these diseases has increased dramatically. About 30-50% of the world population is estimated have been infected by *Toxoplasma gondii*. According to Chandra, Gandhahusada research that conducted in 1995 showed that prevalence toxoplasmosis in humans ranges between 2–63%, 35–70% on cat, 75% in dogs, 11–61% in goats, 11–36% in pigs, and less than 10% on the cow.[3] Research results from Fitria, showed that 46,66% pork intersection in RPH Surabaya positive toxoplasmosis.[4]

The negative impact on the human is very detrimental to the failure of pregnancy and abortion. In human and animal therapy for this disease is very expensive, the impact of livestock on the economic loss due to a decline in production. The administration of drugs such as pyrimethamine and a sulfonamide can kill tachizoit of stadium *T. gondii*, but these treatments are not effective on stage bradizoite. In addition, these drugs are toxic, so is not recommended for use in the long term. Prevention by vaccination not fully provided protection. Using antibodies for controlling is start assess, one of them is making antibodies from the egg yolk. Antigens which used as the immune system host stimulation can come from different parts of the body *T. gondii*. One of them that can be used as an antigen is membrane of stadium tachizoit *T. gondii*. Specific antigen in tachizoit surface are P30, SAG-1, P22 (SAG-2), P35, while the same protein membrane between tachizoit and bradizoite are P23 and P43 (SAG-3).[5] This membrane has immunogenic protein. Some protein major of tachizoit such as P22, P23, P30 and P45 have molecular weight around 20–43 kDa. Membrane protein is able to provoke both cellular immune response (lymphocyte cell, NK cell) and humoral (immunoglobulin), so that many use as diagnostic kit and vaccine.[6] In chronic toxoplasmosis cases, one of the immune system that had the play role is immunoglobulin G (IgG). Some efforts had done to multiply both polyclonal and monoclonal antibody using animal trial. Serum from this animal should be taken to get its antibody and this animal should be sacrificed. It becomes consideration both animal welfare aspect and economics.

The research of IgY usage as passive immunization had been done by Chalghoumi *et al*., on Wilkie, by using *Salmonella enteridis* that was changed into antigen and given to layer hens.[7] The advantage of IgY is not activated the complement, IgY did not bind A and G protein, IgY did not bind the mammals antibody, such as rheumatoid factor which is similar with auto-antibody that react with Fc receptor in IgG and HAMA (Human Anti-Murine Antibodies), IgY did not bind Fc receptor in the surface. The character of IgY which is similar with IgG in mammals, both from its structure and its function.

MATERIAL & METHOD

This research included in laboratory explorative research. Research design was used descriptive analysis. The animals that were used for collecting IgY were layer hens strain with 20 weeks of age, 5 hens were adapted during 2 weeks. *Toxoplasma gondii* passage and cultivation in *Mus musculus* strain Balb/C with 3 months of age. *Toxoplasma gondii* strain RH was used in this research, from Department of Parasitology, Medical Faculty, Universitas Gajah Mada.

Tachizoit *T. gondii* cultivating and harvesting were done by infecting mice with *T. gondii* RH strain isolation by 10^3 for doses in 50 mice Balb/C through intraperitoneally. Protein membrane tachizoit of *T. gondii* was isolated by sonication and centrifugation. Protein concentration was interpreted using spectrophotometry with 595 nm.[6]

In Vitro cultivating and harvesting of tachizoit *T. gondii* were done by using infected mice with isolate *T. gondii* strain RH through 1 x 10^3 on 50 Balb/C mice by intraperitoneal. Isolation of protein membrane tachizoit *T. gondii* used sonication and centrifugation.[6] Protein concentration reader used spectrophotometry with 595 nm. Protein was aliquot and saved on -20° C until it was used.[6] Protein membrane tachizoit of *T. gondii* was analyzed using SDS PAGE.

The chicken that immunization using antigen from protein membrane tachizoit of *T. gondii* by 50 µg that was diluted on PBS and emulted using *Freund's Complete Adjuvant* through 1:1 of ratio until homogenous. Emulsion injected through intra-muscular (on femur). Immunization was repeated twice with 14-days interval. On Repetition immunization, 50 µg of antigen was emulted using *Freund's Complete Adjuvant* then 14 days after second repetition.

Isolation of anti-toxoplasma antibody on yolk used combination of chloroform and ammonium sulfate precipitate method was the chosen method that produce antibody with high purity level.[8] Purification was done by using precipitation of ammonium sulfate 40% and using ratio between IgY supernatant: ammonium sulfate 40% was 1:1. Solution was precipitated one night on 4° C and was centrifuged with 10,000 rpm for an hour.[9] Precipitate was taken for re-suspension using PBS then it was purified and analyzed. Immunoglobulin Y precipitation was covered with specific plastic for sonication, then added 0.5M PBS and stirred using *magnetic stirrer* for 24 hours in 4° C. Characterization was done by reacted antibody from purification with antigen membrane using ELISA method and *Western Bloth*. IgY antibody titter measurement using ELISA.

Immunoglibulin Y protein analyzed using SDS PAGE. Antigenic membrane protein of *T. gondii* was identified using *Western Bloth* method. IgY antibody titter measurement using ELISA.

RESULT AND DISCUSSION

The result of chicken immunization was read using Optical Density level on indirect ELISA. IgY measurement was done after the third immunization booster. ELISA was done on two samples, blood serum and immunized yolk by membrane antigen. Sample consist of yolk that taken before immunization and on the 7th, 14th and 28th day after immunization. The result of ELISA on yolk between before and after immunization of *T. gondii* membrane antigen shows that OD level increased and it significant difference between before and after immunization ($p < 0.005$). The 7th and 14th day after immunization, there were no differences. Result of ELISA OD level can be seen on Table 1.

Immunoglobulin Y was gotten from egg yolk that was extracted with chloroform and precipitated with ammonium sulfate 40% then the protein was analyzed using SDS PAGE.

Marker used for SDS PAGE of immunoglobulin Y could detect protein with molecular weight between 10 kDa-260kDa. Protein molecular weight assessment had done using regression analysis between Rf and BM log. On this research, protein band on marker had line equation $y= -2.501x^5 + 2.920x^4 – 2.202x^3 + 3.467x^2 – 3.253x + 2.597$. On the 2nd column, immunoglobulin without dilution, showed 6 protein bands with molecular weight 179.8kDa, 130.4kDa, 70.6kDa, 59.1kDa, 38.6kDa and 25 kDa. On the 3rd column, immunoglobulin Y with dilution ration 1:5 showed 4 protein bands, 179.8 kDa, 67.4 kDa, 61.6 kDa and 38.6 kDa. On the 4th column, immunoglobulin Y with dilution ration 1:10 showed 4 protein bands, 179.8 kDa, 67.4 kDa, 61.6 kDa and 38.6 kDa. Column 5th, immunoglobulin Y with dilution ration 1:20, showed protein bands with molecular weight were 179.8 kDa dan 67.8 kDa. Concentration of immunoglobulin Y protein from preparation was 0.16 µg/µl.

The result of *Western Blott*, using antigen that were *T. gondii*, was reacted with polyclonal antibody from egg yolk which were Ig Y. Then, it compared with antibody from rabbit that was already immunized using *Toxoplasma gondii* proteins, IgG. Used marker on SDS PAGE could detect protein with 20 kDa–120 kDa of molecular weight. Molecular weight assessment of immunoglobulin Y had been done using regression analysis between Rf and BM log. On this research, protein band on marker had equation as $y = -3.103x^4 + 5.506x^3 – 2.515x^2 – 0.936x + 2.246$. The 1st column was antigen of *T. gondii* membrane which was reacted with rabbit Ig G. This column showed protein band with molecular weight 35.7 kDa. The 2nd and 3rd column were antigen of *T. gondii* membrane that was reacted with chicken IgY and showed protein band with molecular weight 35.7 kDa and 78.8 kDa.

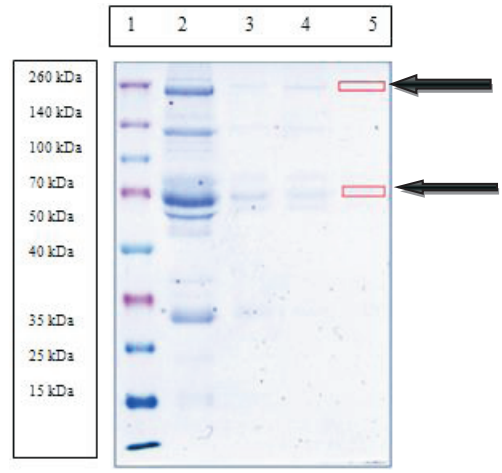


Figure 1. The result of Immunoglobulin Y protein preparation using SDS PAGE

Note: 1st marker
2nd immunoglobulin without dilution
3rd immunoglobulin with dilution ratio 1:5
4th immunoglobulin with dilution ratio 1:10
5th immunoglobulin with dilution ratio 1:20
On the 5th column, there is protein with 67.4kDa and 179.8kDa of weight

Preparation of membrane antigen protein of *Toxoplasma gondii* on tachizoit stadium was continued with characterization using (SDS PAGE) method. This method was the common used of electrophoresis method. Electrophoresis method was used for protein characterization based on molecular weight. The result of SDS PAGE analysis of *T. gondii* membrane protein showed 35.4 kDa, 59.8 kDa; 66.8 kDa; 81.9kDa; 86.8kDa which were more than 118 kDa (Figure 2). Based on Chalghoumi, *et al.,* (2009), 10-100 kDa protein for vaccine were needed

Table 1. Averages of IgY OD level on yolk that was immunized with *T. gondii* membrane antigen

Yolk	OD Level Average	OD Level Deviation
Before Immunization	0.9856[a]	0.5424
7th day after the 3rd immunization	1.5332[b]	0.2201
14th day after the 3rd immunization	1.8873[b]	0.3788
28th day after 3rd immunization	1.8303[b]	0.3640

Different superscript on the same column show significant difference ($p < 0.005$)

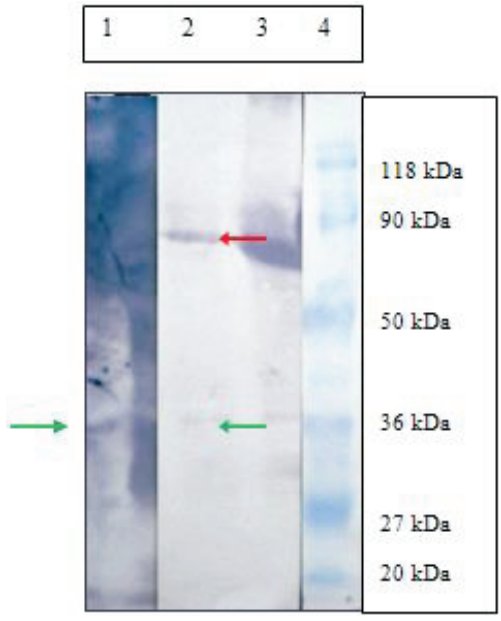


Figure 2. The result of *T. gondii* membrane protein characterization using *Western Blott*. 1st column was *T. gondii* antigen which was reacted with rabbit igG antibody, 2nd and 3rd column were *T. gondii* antigen which were reacted with chicken igY antibody and the 4th column was marker. The red sign was protein with 78.8 kDa of molecular weight, green sign was protein with 35.7 kDa of molecular weight.

to provoke immune response. It fulfill requirement for immunization so hopefully could provoke immune response.[7] From this result was in accordance with the Suwanti's research[6] that major protein on membrane had molecular weight between 60 kDa–200 kDa. 66 kDa–70 kDa of protein also found in membrane and roptry *T. gondii* was proven by Bonhomme et al. (1990) which was citated by Suwanti.[6] It was proven by protein characterization result of P22 recombinant from tachizoit membrane that showed protein band between 35 kDa–40 kDa.[5] Molecular weight of protein band above 118 kDa could not be determined using regression equation between log of molecular weight and Rf from marker, since those protein out of regession line.

Antibody was produced from induced egg yolk with immunization from membrane *T. gondii* protein. On this research, immunization was done via intra-muscular for three times using mix of *Complete Freund Adjuvant* at the 1st immunization and *Incomplete Freud Adjuvant* at the 2nd and 3rd immunization. The aim of CFA and IFA given was for induced the bigger immune response. It caused on CFA consist of protein from death micobactery or component from cell wall of bacteria which had ability to induce both cellular immune response and humoral response against injected protein antigen. Therefore, CFA addition hopefully can form antibody against *T. gondii* antigen membrane.

This research used indirect ELISA model for detected Ig Y, thereby anti-IgY conjugate was needed. The form of IgY similar with IgG was monomer, so the system of IgY also had high affinity against antigen. Fab (antibody fragment) on IgY could recognize antigen epitope more than on IgG. Structure of heavy and light chain of IgG and IgY was relatively similar which was two heavy chains on IgY had molecular weight 67–70 kDa on each chain and two light chain with molecular weight 25 kDa on each. The differences of IgG and IgY only on CH4 chain on Fc.[7,10] OD level on ELISA result both on yolk and on serum which was immunized with antigen of *T. gondii* membrane showed that there was significant different between before immunization and after the 3rd immunization. It showed that antigen of *T. gondii* membrane was immunogenic. Based on Abbas,[10] forming of immunoglobulin antibody would increase on the 2nd antigen exposure with the same antigen type.[11] B cell would produce immunoglobulin after 5 days after antigen exposure and immunoglobulin level would be kept on 23 days.[7] If booster was done every 14 days, the increasing of antibody would occur after the 3rd immunization. On the screening of both yolk and serum on the 7th day and 14th day after the 3rd immunization did not show significantly difference. It indicated that between 7th day and 14th day after the 3rd immunization immunoglobulin Y production was relatively constant on the chicken body. On the 28th day after immunization did not show significantly difference. Antibody testing on chicken serum was done until the 14th day after the 3rd immunization. On this research, protein band with molecular weight 25 kDa and 38.6 kDa might be fragment from Fab IgY on light chain, while protein with molecular weight 59.1 kDa, 61.6 kDa, 67.4 kDa and 70.6 kDa were fragment from Fab IgY on heavy chain. This was in accordance with Michael et al.,[8] who said that two heavy chains on IgY had molecular weight 67–70 kDa on each and two light chain with molecular weight 25 kDa on each.[8] Protein band which had molecular weight 130.4 kDa, 179.8 kDa was probably the fragment from complex bond of Fc receptor Igy (FcR-IgY) and the whole molecule from IgY. This was in accordance with He and Bjorkman, which was said that FcR-IgY complex and whole IgY had molecular weight between 150 kDa–180 kDa.[10] The result of Western Blot using antigen from *T. gondii* membrane which was reacted with polyclonal IgY antibody showed protein band reaction with molecular weight 35.7 kDa and 78.8 kDa (Figure 2). On 35.7 kDa of Western Blot showed that IgY antibody could recognize antigen apitop, that was shown by band reaction between protein from antigen which was 35.7 kDa (SAG 1) *T. gondii* with 38 kDa of IgY molecule (light chain Fab IgY). P30 *T. gondii* (SAG1) was the major protein on RH strain and had molecular weight around 30–38 kDa. Surface antigen (SAG) was protein which took role on attachments. SAG was protein on the tachizoit surface that consist of glicosilphosphatidilinolsitol (GPI) and helpfully gave signal on attachment process between SAG and ligan on the cell surface that would be infected.[12] Western Blott result which had reacted with primary antibody of rabbit (IgG) also showed protein band with molecular weight 82 kDa. It proved that the recognizing epitope of *T. gondii* antigen against Fab IgG of

rabbit was occure. Fab IgG of mammalian on heavy chain had molecular weight 67-70 kDa and light chain 25 kDa.[8] On the result of Western Blott using rabbit IgG antibody showed 35.7 kDa of molecular weight. It meant that Fab IgG could recognize protein of tachizoit membrane and proved there was similarity of Fab structure from IgG and IgY. Fragment from molecular antibody was antigen binding fragment and Fc was crystalizable fragment (constant) as biology effector. On aves, IgY Fc receptor was known as FcRY. In fact, aves FcRY had similarity with FcRn IgG on mammals, whereas FcRn also act as MHC1 which could bind with antigen peptide for T cell. The similarity of FcRY and FcRn was could bind with immunoglobulin molecule on pH ≤ 6 and did not bind on pH ≥ 7. FcRY which bind with the whole IgY molecule had dimer structure with N terminal chain and cyctin receptor for binding peptide from antigen. FcRY bonded on IgY CH4 chain. The differences between FcRY and FcRn were on recognizing ligand receptor of CH3-CH4 IgY and CH2-CH3 IgG whereas IgY ligand had double ability than IgG ligand. Antigen epitope could be recognized by more IgY molecule than mammalian immunoglobulin.[10]

CONCLUSIONS

To sum up briefly, the result from profil analysys of membrane protein of tachizoit *T. gondii* was protein with molecular weight 35.4 kDa, 59.8 kDa, 66 kDa, 81 kDa and 86 kDa. Immunoglobulin Y from egg yolk could produce antibody anti protein of *T. gondii* membrane. Based on Western Blot result, could be concluded that protection mechanism of immunoglobulin Y was on Fab which could recognize epitop of *T. gondii* antigen with molecular weight 35.7 kDa and 78.8kDa

REFERENCES

1. Hanafiah, M., Wisnu N, Mufti K, dan Fadrial K. 2009. Produksi dan Isolasi Protein Membran Stadium Bradizoit *Toxoplasma gondii*: Suatu Usaha untuk Mendapatkan Material Diagnostik dalam Mendiagnosa Toksoplasmosis. Fakultas Kedokteran Hewan Universitas Syiah Kuala. Aceh. Vol. 10 No. 3: 156–164.
2. Hiswani. 2003. Tesis: Toxoplasmosis Penyakit Zoonosis Yang Perlu Diwaspadai Oleh Ibu Hamil. Fakultas Kesehatan Masyarakat. Universitas Sumatera Utara. Chandra, G. 2001. *Toxoplasma gondii*: Aspek Biologi, Epidemiologi, Diagnosis dan penatalaksanaannya.
3. Chandra, G. 2001. *Toxoplasma gondii*: Aspek Biologi, Epidemiologi, Diagnosis dan penatalaksanaannya. http://www.emedice.com. (Juni 2011).
4. Ardhiani, F. 2008. Insidensi Toxoplasmosis pada Babi di RPH Pengirian Surabaya dan RPH Gadang Malang. Fakultas Kedokteran Hewan Universitas Airlangga. Surabaya.
5. Arabpour, M., Mojgan B. Maryam N., Seyyed H.A. 2011. African Journal of Biotechnology Vol. 10(40): Cloning and expression of *Toxoplasma gondii* tachyzoite P22 protein. Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran., pp. 7746–7750.
6. Suwanti, L.T. 1996. Identifikasi dan produksi Antibodi Monoklonal Protein Membran *Toxoplasma gondii* Stadium takizoit. Tesis Pascasarjana Universitas Gadjah Mada. Yogyakarta.
7. Chalghoumi, R., B. Yves, P. Daniel and T. Andre. 2009. Hen Egg Yolk Antibodies (IgY), Production and Use for Passive Immunization Against Bacterial Enteric Infection in Chicken. Gembloux Agriculture University. Belgium. 295–308.
8. Michael, A., S. Meenatchisundaram, G. Parameswari, T. Subbraj, R. Selvakumaran and S. Ramalingam. 2010. Chicken Egg Yolk Antibodies (IgY) as an Alternative to Mammalian Antibodies. Indian J. Science Technology. 3(4): 468–474.
9. Ko K. and Ahn D.U, 2007. Preparation of Immunoglobulin Y from Egg Yolk Using Ammonium Sulfate Precipitation and Ion Exchange Chromatography. Poultry Science. 86: 400–407.
10. He, Y and Pamela J.B. 2011. Strukture of FcRY, an avian immunoglobuli receptor related to mammalian mannose receptor, and its compleks with IgG. California Institute of Technology. USA. Page: 12431–12436.
11. Abbas, A.K., A.H. Lichtman and J.S. Pober. 2000. Cellular and Molecular Immunology. W.B. Saunders Company, Philadelphia. p p. 235–338.
12. Carruthers, V.B. 2002. Host Cell Invasion by the Opportunistic Pathogen *Toxoplasma gondii*. Acta Trop. 81: 111–122.