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TABLE OF CONTENTS

SESSION: INDUSTRY, INNOVATION AND INFRASTRUCTURE ANALYSIS OF FACTORS THAT INFLUENCE THE RISK OF OCCUPATIONAL ACCIDENTS IN TERMS OF BEHAVIORAL ASPECTS (STUDY ON AIRCRAFT PAINT REMOVAL Hesti Fiskalisa Purbayanti, Tri Martiana EARLY STUDY THE POTENCY OF TURMERIC (CURCUMA DOMESTICA VAL.) AS IMMUNOSTIMULATOR FOR LAYERS CHICKENS AGAINST AVIAN INFLUENZA (AI) Dyah Widhowati, Nurul Hidayah, Retina Yunani, Mijania Malia THE USE OF CELL FREE FETAL DNA ICFF-DNAI AS NON-INVASIVE TECHNIQUES ON PATERNITY TEST [FORENSIC IDENTIFICATION] 10 Dimas Agung Trisliatanto, Koko Srimulyo, Helmy Prasetyo Yuwinanto, Mirza Dewi Suntari, Lastika Dimas Agung Trisliatanto, Falih Suaedi, Fahmi Muhammad Az-Zuhri, Teguh Prasetyo, Rizka Pranatasari IDENTIFICATION HUMAN AND ANIMAL BLOOD MIXTURES USING HUMAN Wimbuh Tri Widodo, Abdul Hadi Furqoni, Ahmad Yudianto, Sri Puji Astuti Wahyuningsih ANATOMICAL PATHOLOGY AND RADIOLOGY APPEARANCE OF BALLISTIC WOUND RESULT OF CAL. 177 AIR RIFLE WITH 4,5 MM PELLETS ON EXTRIMITY OF THE DOG B. Putra, J. Rahmahani, E. Aksono, D. Legowo, B. Christoffel JOB SATISFACTION AND JOB MOTIVATION TOWARD PERFORMANCE THROUGH Dimas Agung Trisliatanto, Tan Evan Tandiyono, Dimaz Ganjar Harry Pradana, Pristiandi Teguh Cahya, Nur Abdul Hadi Furgoni, Wimbuh Tri Widodo, A. Yudianto IMMUNOMODULATION EFFECT OF MENIRAN (PHYLLANTHUS NIRURI LINN) ON BLOOD PROFILE OF BROILER CHICKENS INFECTED WITH ENTEROTOXIN OF ANTIBIOTIC-Retno Sri Wahjuni, Emy Koestanti Sabdoningrum, Sri Hidanah, Diyantoro, R. Wahjuni **SESSION: OPENING** ENZOOTIC BOVINE LEUKOSIS: HOW TO PREVENT THE DISEASE AND CONTROL THE SPREAD OF BLV INFECTION. 50 CARBON ACCOUNTING REFLECTION AS A RESPONSE TO FACE THE CLIMATE CHANGE.......52 Sri Iswati DEVELOPMENT OF LEGAL THEORY FOR ENVIRONMENT PROTECTION AND REMEDY Yuzuru Shimada AN OVERVIEW OF THE SEAWEED CULTIVATION IN SEVERAL COUNTRIES:

TECHNOLOGY AND CHALLENGE 62

Mochammad Amin Alamsjah

SESSION: GENDER EQUALITY

COULD THE EXISTENCE OF MICROFINANCE INSTITUTION FOR ALLEVIATION	
POVERTY IN RURAL AREA? A CASE STUDY IN WOMEN'S COOPERATIVE IN MAGERSARI	
VILLAGE, PLUMPANG SUB-DISTRICT, EAST JAVA, INDONESIA	69
Rustinsyah	
WOMEN EMPOWERMENT TO SUPPORT FAMILIES ECONOMY IN SUKAMUKTI CIAMIS	
	7.6
INDONESIA	/6
Nuning Kurniasih, Pawit M. Yusup, Engkus Kuswarno	
THE INFLUENCE OF EMOTIONAL INTELLIGENCE TO THE PERFORMANCE OF FEMALE	
LECTURERS AT SEVERAL PRIVATE COLLEGES IN BALIKPAPAN	79
Mardatillah	
SESSION: RESPONSIBLE CONSUMPTION AND PRODUCTION	
SESSION RESIGNATED TO THE PROPERTY OF THE PROP	
WITH 17 A THON OF PROPERTING A WITHDIFF OF FIFTH BATTONIC CONTAINING BLACK	
UTILIZATION OF DIGESTIBLE NUTRIENTS OF FEED RATIONS CONTAINING BLACK	
GLUTEN AND RED RICE BY MINI REX RABBIT	83
S. Romziah , K. Emy, H Sri	
PRODUCTION OF CARP IMMUNOGLOBULIN M EXPOSED WITH WHOLE PROTEIN FROM	
MYXOBOLUS KOI SPORE THROUGH FEED AS AN IMMUNOSTIMULANT	87
Moch. Saad, Gunanti Mahasri, Woro Hastuti Satyantini	
THE INFLUENCE OF THE DIFFERENT COMMERCIAL PROBIOTIC ON THE BIOFLOC	
NUTRITION	0.4
	94
Endang Dewi Masithah, Fitri Anisha Kurniawati, Azhar Muhammad Helmi	
CECCION, COOD HEALTH AND WELL DRING	
SESSION: GOOD HEALTH AND WELL-BEING	
ANTIGENIC PROTEIN OF LEUCOCYTOZOON CAULLERYI SCHIZONT INDUCING	
CELLULAR IMMUNE RESPONSE: TLR-2 AND CD4 AS MARKER	98
Nunuk Dyah Retno Lastuti, Endang Suprihati, Dony Chrismanto, Anwar Ma'Ruf	
INFECTIOUS RESPIRATORY SUSPECT MALLEUS IN PONY HORSE AT SIDOARJO EAST	
JAVA INDONESIA	102
Arya Pradana Wicaksono, Romziah Sidik	102
	104
CONSUMER PROTECTION ON THE CIRCULATION OF COSMETIC ONLINE	104
Lilik Pudjiastuti, Emanuel Sujatmoko, Indrawati	
THE TRADITIONAL WAY IN PREVENTING AND OVERCOMING HEALTH PROBLEMS	
AMONG SULFUR MINERS IN THE CRATERS OF IJEN	109
Wisnu Setiadji, Myrtati D. Artaria	
SELF INDEPENDENCE OF FAMILY PLANNING IN URBAN AREA GRESIK DISTRICT	114
Yuly Sulistyorini, Nunik Puspitasari, Diah Indriani, Rachmah Indawati	
ANALYSIS OF THE EFFECT OF INDIVIDUAL, FAMILIAL AND ENVIRONMENTAL	
ANALYSIS OF THE EFFECT OF INDIVIDUAL, FAMILIAL AND ENVIRONMENTAL	
FACTORS ON FAMILY STIGMA OF LEPROSY IN JOMBANG REGENCY, EAST JAVA	440
INDONESIA	118
Nasrudin , Tjipto Suwandi, Cholichul Hadi, A. Yusuf, R. Hargono	
THE FREQUENCY OF Y-PATTERN DENTAL TRAITS ON LOWER MOLAR OF JAVANESE	
DEUTROMALAYID	123
Aprian A. Prastya, Myrtati D. Artaria	
ANTIDIABETIC EFFECT ON TEA OF PLUCHEA INDICA LESS AS FUNCTIONAL BEVERAGE	
IN DIABETIC PATIENTS	126
Yesiana Dwi Wahyu Werdani, Paini Sri Widyawati	120
DESIGN OF A WIRELESS TELEMETRIC SENSOR SYSTEM FOR MONITORING THE	120
DEVELOPMENT AND TREATMENT OF CHRONIC DIABETIC FOOT INJURIES	130
Suryani Dyah Astuti, Tri A. Prijo, Wirda A. Ridyananda, I. Suhariningsih	
ACTIVATION OF FAK EXPRESSION IN INFLUENCING BONE DENSITY BY EXERCISE	
TRAINING FOR DECREASIS OSTEOPOROTIC RISK	133
Nurul Mahmudati, Hawin Nurdiana	
ANTIMICROBIAL USAGE SURVEILLANCE OF CATTLE IN INDONESIA TO ADDRESS	
ANTIMICROBIAL RESISTANCE	136
Havan Yusuf, Syafrison Idris, Mathilde Paul, Theera Rukkwamsuk	

EFFECTS OF HEAT EXPOSURE DURATION ON SALIVA TRACES ON CIGARETTE BUTTS	
AS FORENSIC IDENTIFICATION TOOLS	141
THE USAGE OF VISUM ET REPERTUM (VER) AS A SCIENTIFIC EVIDENCE IN ANIMAL	
ABUSE ACCORDING TO THE PERSPECTIVE OF THE PENAL CODE (KUHP) AND THE LAW	
OF CRIMINAL PROCEDURE CODE (KUHAP) IN INDONESIA	144
A. Bilqisthi, N. Ignatius, H. Pudji, Haniyah, Sadjijono	
POLICY INSTRUMENTS ON REPRODUCTIVE HEALTH AS REALIZATION OF CIVIL	
RIGHTS IN GENDER EQUALITY AND JUSTICE	148
Lina Hastuti, Lilik Pudjiastuti, Sukardi	
MODEL OF ADOLESCENT REPRODUCTIVE HEALTH INFORMATION DISSEMINATION IN	
BANDUNG WEST JAVA INDONESIA	153
Nuning Kurniasih	
RELATION EFFECT OF VARIATION TIME STORAGE YAM TUBER (PACHYRRHIZUSEROZUS) TO CHOLESTEROL RATTUSNORVEGICUS	157
Anggi Khairina Hanum Hasibuan, Wa Ode Diana, Yosephin Anis Widiyanti	137
PROTEIN SIGNAL TRANSDUCERS AND ACTIVATORS TRANSCRIPTION (STAT) AS	
GROWTH PROMOTER	162
Anwar Ma'Ruf, Ngakan Made Rw, M. Sukmanadi, Ratna Damayanti	
POTENTIAL PROTEIN GHRELIN ORIGIN OF PLANT AS ENERGY BALANCE SETTINGS	
FOR FEED EFFICIENCY	166
Nove Hidajati, Chairul Anwar, Ratna Damayanti	
ANTIOXIDANT ACTIVITY ASSAY OF ALPHA-MANGOSTIN FOR AMELIORATION OF	
KIDNEY STRUCTURE AND FUNCTION IN DIABETIC MICE	170
Saikhu Akhmad Husen, Firas Khaleyla, Arif Nur Muhammad Ansori, Raden Joko Kuncoroningrat Susilo, Dwi	
Winarni, Salamun RISK ANALYSIS OF OCCUPATIONAL DISEASES IN HARBOR COMMUNITY	175
Martiana Tri, N. Widajati	1/3
THE VASCULAR DISTRIBUSTION USING COLOR DOPPLER SONOGRAPHY IN AXILLARY	
NODES OF BREAST CANCER TO ASSES METASTASIS	179
Lailatul Muqmiroh, Lies Mardiyana, Heru Purwanto, Sri Agustiningsih	
PHOTODYNAMIC INACTIVATION FOR PHATOGENIC BACTERIA: ADDING	
CHLOROPHYLL AND OXYGEN	185
Basitha Febrinda Hidayatulail, Moh. Yasin, Suryani Dyah Astuti	
THE EFFECT OF DAYAK ONION (ELEUTHERINE PALMIFOLIA) TUBER EXTRACT IN	
LIVER MALONDIALDEHYDE (MDA) LEVEL IN MALE WISTAR RATS INDUCED BY	
ALLOXAN	189
Risqia Damayanti, Anwar Ma'Ruf	100
FISH PROTEIN PROFILE SUBMERGE ALUM SOLUTIONBASED ON SDS-PAGE	192
EFFECTIVENESS TEST OF AKAR KUCING PLANT EXTRACT (ACALYPHA INDICA LINN)	
TO LOWER TOTAL CHOLESTEROL LEVELS IN RATS (RATTUS NOVERGICUS) WHICH	
INDUCED HYPERCHOLESTEROLEMIA DIET	195
Retno Sri Wahyuni, Fitria Agung Nugrahaningtyas, Nove Hidayati, Rochmah Kurnijasanti	
SESSION: DECENT WORK AND ECONOMIC GROWTH	
INDICATOR OF ZAKAT OVER MUZAKKI AN EXPLANATORY STUDY ON BAZNAS OF	100
CENTRAL JAVA PROVINCE	198
Suraji, Sri Iswati	202
KAILI WOMEN'S EMPOWERMENT IN INDONESIA	202
THE ROLE OF MUHAMMADIYAH IN THE DEVELOPMENT OF SOCIAL CAPITAL	
COMMUNITY	206
Sri Iswati, Sri Herianingrum, Muslich Anshori, H. Effendie, Tika Widiastuti, Ririn Tri Ratnasari	200
ROLE OF SOCIAL EUNTERPRENEURSHIP ON POVERTY REDUCTION AND ECONOMIC	
GROWTH IN INDONESIA	211
Risma Ayu Kinanti, Sri Iswati, Tjiptohadi Sawarjuwono, Ririn Tri Ratnasari	
WAQF PRODUCTIVE EFFICIENCY: EVIDENCE FROM YAYASAN BADAN WAKAF SULTAN	
AGUNG, SEMARANG	217
Tika Widiastuti, Wahyuningsih	

EFFECT OF THE FINANCING OF SHARIA BANK ON THE INFLATION IN INDONESIA	225
Anas Alhifni, Rully Trihantana Rully, Maya Apriyana	
EMPOWERING COMMUNITY INFORMATION GROUP: STRATEGIC COMMUNICATION PLAN IN COMMUNICATION AND INFORMATICS OFFICE OF MALANG CITY	230
Dani Maroe Beni	
EVALUATION OF INVESTMENT POLICY IN THE FORM OF TAX HOLIDAY IN ORDER TO INCREASE INDONESIAN ECONOMIC GROWTH	240
Resha Dwiayu Pangesti Mulyono, Elia Mustikasari	210
THE EXTERNAL AND INTERNAL FACTORS ON MICRO, SMALL AND MEDIUM	
ENTERPRISE (SME) FINANCING IN ISLAMIC BANK	244
Fira Nurafini, Raditya Sukmana, Sri Herianingrum	
ANALYSIS OF THE ROLE SHARIA FINANCING AND CHARACTERISTICS OF BUSINESS	
INSTITUTIONS TO MICRO, SMALL AND MEDIUM ENTERPRISE (MSMES) DEVELOPMENT	
IN SOLOK CITY OF WEST SUMATERA	249
Neng Kamarni, Muslich Anshori	217
THE LINKAGE BETWEEN ECONOMIC GROWTH AND DEFORESTATION IN OIC (THE	
ORGANIZATION OF ISLAMIC COOPERATION) COUNTRIES	253
Rani Puspitaningrum, Raditya Sukmana, Imron Mawardi	233
EMPLOYEES' COMMITMENT BUILDING THROUGH SOCIAL ESTEEM AT THE	
REMUNERATION'S INCREASEMENT OF RECOGNITION AN SELF-ACTUALIZATION NEED	
IN MASLOW THEORY	250
Djoko Soelistva	239
THE DYNAMIC ANALYSIS ON IMPACTS OF EDUCATION AGAINST POVERTY	
	262
REDUCTION	202
THIRD PARTY FUNDS, NUMBER OF CAPITAL, AND NON PERFORMING FINANCING TO	266
THE NUMBER OF MUDHARABAH FINANCING IN INDONESIA'S SHARIA BANKING	200
Muhammad Iqbal Surya Pratikto, Ririn Tri Ratnasari	
ISLAMIC WORK ETHIC AND SATISFACTION WITH INTRINSIC MOTIVATION AS	272
MEDIATOR VARIABLE	272
Rio Eriawan Putra Tohari, Ririn Tri Ratnasari	
BUILDING SOFT SKILLS AS THE PEOPLE-JOB FIT TO OVERCOME	
COUNTERPRODUCTIVE WORK BEHAVIOR IN CREDIT ASSESSMENT BANKING SECTOR:	
A LITERATURE REVIEW	276
Dewi Khrisna Sawitri	
SESSION: CLEAN WATER AND SANITATION	
SESSION. CLEAN WATER AND SANITATION	
THE MANAGEMENT MODEL ON INTEGRATED SETTLEMENT WASTEWATER	
TREATMENT SYSTEM (SPAL) IN SUPPORTING HEALTH DEVELOPMENT	201
Lilik Pudjiastuti	201
WATER TRADE IN ISLAMIC BUSINESS ETHICS PERSPECTIVE: EVIDENCE FROM	
INDONESIA	205
Bahrina Almas, Tjiptohadi Sawarjuwono, Sri Iswati	283
Bunrina Aimas, 15ptonaar Sawarjawono, 3ri 15waa	
SESSION: ZERO HUNGER	
<u></u>	
OPTIMIZATION OF FOOD ESTATE PROGRAM THROUGH CASH WAQF TO ACHIEVE	
FOOD SOVEREIGNTY OF INDONESIA	200
Denizar Abdurrahman Miraj, Ummi Muthia Fathy, Muhammad Nafik Hadi Ryandono, Tjiptohadi Sawarjuwono	230
Denibus Abaus rannan Maray, Omini Mainia Lainy, Mananinaa Rajik Haat Ryanaono, Typionaat Sawarjawono	
SESSION: CLIMATE ACTION	
BLUE CARBON: ROLE OF SEA TO THE BALANCE OF CLIMATE WITHIN THE	
MITIGATION FRAME OF CLIMATE CHANGE	294
Sunyowati Dina, Ria Tri Vinata	234

LOCAL CONCERN ON PLASTIC BAG CHARGE IN INDONESIA: DO WE REALLY CARE?	298
MAKING MODEL OF VILLAGE REGULATION BASED ON GOOD VILLAGE GOVERNANCE	
IN INDONESIA	302
Suparto Wijoyo, Radian Salman, Bagus Oktafian Abrianto	
STRIP INTERCROPPING PRODUCTIVITY OF MODERN MAIZE HYBRID VARIETIES WITH	
PULSE CROPS ON A DRYLAND	309
I Komang Damar Jaya, Sudirman, Rosmilawati	
ANISAKIDAE AS A BIOINDICATOR CANDIDATE IN RESPONSE OF ENVIRONMENTAL	
DAMAGE.	313
Hartanto M. Raharjo, Setiawan Koesdarto, Qabilah C. K. N. Sumarsono, Febrina D. Permatasari, Zafitri N. Wastomi, Nurul S. A. Sari	
ANALYSIS OF THE DISCLOSURE OF GREENHOUSE GAS EMISSIONS AND	
ENVIRONMENTAL PERFORMANCE IN LISTED FIRMS AT JAKARTA ISLAMIC INDEX (JII)	316
Dwi Swasana Ramadhan, Azizah Anshori, Sri Iswati, Sri Herianingrum	
MARINE ENVIRONMENT AND CLIMATE CHANGE: LEGAL ASPECTS OF PROTECTION	
AND PREVENTION AGAINST CORAL REEFS DEGRADATION IN INDONESIA	321
Dina Sunyowati, Annisa Firdhausy	
Sing Suryowai, Illinisa I illanaisy	
SESSION: ZERO POVERTY	
ISLAMIC CONCEPTS AS EFFORT TO UTILIZE WASTELAND OF PUBLIC REVENUE	
DISTRIBUTION	325
Ridan Muhtadi, Sri Iswati, A. Rohman	
INFLUENCE OF INFLATION ON POVERTY IN SURABAYA AND ISLAMIC SOLUTIONS IN	
ERADICATING POVERTY	331
Amaliah Al Azmi, S. Iswati, R. Sukmana, R. Ratnasari	
ECONOMIC DEVELOPMENT IN INDONESIA: INTEGRATED MODEL OF ISLAMIC	
FINANCIAL INCLUSION	335
Laila Masruro Pimada, N. Firdaus	
POVERTY ALLEVIATION: AN ECONOMIC PRACTICE STUDY OF ISLAM IN CULTURE	341
Renny Oktafia, M. Anshori, I. Mawardi	
DETERMINANTS OF SUCCESSS IN VENTURE CAPITAL ASSISTANCE RECIPIENTS IN	
YAYASAN DANA SOSIAL AL-FALAH (YDSF) SURABAYA	2.15
Doddy Koesnadhi, Tika Widiastuti, Sri Herianingrum	545
OPTIMIZING FUND MANAGEMENT OF MOSQUE CASH FOR ECONOMIC EMPOWERMENT	
OF PEOPLE	250
Sri Wulandari, T. Sawarjuwow, S. Iswati	330
	255
ENHANCING FARMER'S INDEPENDENCE BY BAITUL MAAL WAT TAMWIL CONCEPT	333
Vina Septiana Permatasari, T. Sawarjuwono, S. Iswati	250
THE MOVING OUT OF POVERTY OF MUSTAHIQ PRODUCTIVE ZAKAT IN INDONESIA	339
Imron Mawardi, Tika Widiastuti, Puji Sukmaningrum	
ISLAMIC FINANCIAL DEVELOPMENT AS EFFORTS TO ACCELERATE ECONOMIC	265
DEVELOPMENT AND POVERTY ALLEVIATION	365
Elsi Mersilia Hanesti, Sri Herianingrum, Raditya Sukmana	
HADD AL- KIFAYAH (SUBSISTENCE CRITERIA) AS A MEASUREMENT OF ISLAMIC	
SOCIO-ECONOMIC SECURITY	372
Imron Mawardi, Sri Herianingrum, T. Widiastuti	
Author Index	



Implementation of Climate Change Agreement to Meet Sustainable Development Goals (ICPSUAS 2017)

Antigenic Protein of *Leucocytozoon caulleryi* schizont Inducing Cellular Immune Response: TLR-2 and CD4 as Marker

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Abstract: Leucocytozoonosis is caused by Leucocytozoon caulleryi and is responsible for death in chickens by bleeding. Leucocytozoonosis is an endemic disease in Indonesia and incidences have been reported in several regions in East and Central Java. The financial losses caused by this disease include growth disorders in chick, decreased egg production, higher mortality rate and also a higher production cost. This research aims to detect TLR-2 and CD4 expression as a cellular immune response in rabbits immunised by the protein of L.caulleryi schizont. It is needed as a preliminary research study for molecular vaccine development which is considerably effective when it comes to preventing leucocytozoonosis occurance in Indonesia. This research study was performed in several stages i.e. the isolation of L.caulleryi schizont from a chicken liver infected with leucocytozoonosis to be based on the clinical signs observed, microscopic examination, and the pathological changes in the other chicken organs. The purification of the soluble protein of L.caulleryi schizont including the immunisation of the rabbits. Each of the experimental rabbits was injected with 500 µg of L.caulleryi schizont protein and added adjuvant complete with a ratio of 1:1. Every two weeks the injection was performed with the same protein with a dosage of 500 µg each and an added adjuvant that was incomplete (the booster was performed 5 times in 2 weeks). The examination of the cellular immune response of CD4 and TLR-2 expression in the rabbits' T cells using an immunocytochemistry method visualised by fluorescein isothiocyanate. The examination of the results was done by immunocytochemistry showing TLR-2 and CD4 expression as yellow to green fluorescent colour, mainly in the 5th booster where the activation of the CD4 coreceptor and TLR-2 occurred. The conclusion shows that the antigenic protein of L.caulleryi schizont has the ability to induce a cellular immune response through the co-receptors CD4 and TLR-2 in the rabbits' T cells as the preliminary

research in the sub-unit vaccine development for leucocytozoonosis in chickens.

KEY WORD: antigenic protein, Leucocytozoon caulleryi, schizont, TLR-2, CD4.

I. INTRODUCTION

Leucocytozoonosis is one of the diseases caused by Leucocytozoon caulleryi in poultry, which is transmitted by flies Culicoides sp. or Simulium sp. Leucocytozoonosis is an endemic disease in Indonesia and incidences have been reported in several regions in East and Central Java. Leucocytozoon parasites infect a large number of avian hosts, including the domestic chicken, and causes a significant economical loss to the poultry industry (1). The financial losses impacted by this disease include growth disorders in chicks, decreased egg production, higher mortality rate and also a higher production cost (2, 3, 4, 6). The occurrence of leucocytozoonosis in broiler is between 7-40%, while the mortality rate in chicks is about 7-50% and in layer is about 2-60% respectively (7). The clinical signs observed in chickens are green faeces, depression, a loss of appetite, vomiting blood, paralysis and death due to bleeding (8). In order to overcome leucocytozoonosis in chickens, farmers have carried out the dispensing of medications, eradicating flies using insecticides, and improving the water irrigation in the area around the henhouse, but the latter method is less efficient due to the rapid growth of flies. Based on the vaccine developed by Onaga et al (1999), chickens can be protected from L.caulleryi infection by giving them a second generation schizont extract and blood serum containing an antigen (5). The weakness of this live vaccine administration is due to the possibility of infection because the parasites may become pathogens when the host's condition is weakened. Molecular vaccine development is considerably more effective when it comes to preventing leucocytozoonosis occurance in



Indonesia. Based on the phylogenetic analysis of Cytochrom B *Leucocytozoon* spp in broilers, it was shown that the *L.caulleryi* from various endemic regions is highly homologous (>95%) (6). Referring to the problems above, a preliminary research about the rabbits' immune response induced by *L. caulleryi* schizont protein with TLR-2 and CD4 as marker to explore the cellular immune response is necessary.

TLR is a membrane protein that helps receptor recognition patterns in response to various molecule derivatives from microbes and stimulated innate immunity due to microbe molecule exposure. TLR is known to be a recognition receptor which is involved in pathogen-associated molecular patterns (PAMP) recognised by pattern recognition molecules (PRMs). A phagocytes development system in recognising pathogens can be stimulated any time to respond as an inflammatory system. TLR stimulation through microbial product initiates the signalling pathways which activate not only the innate immunity but also adaptive immunity (9, 10). CD4+T cells play a central role in immune protection and the B cells to produce antibodies, to induce the macrophages to develop enhanced microbicidal activity, to recruit neutrophils, eosinophils, and basophils to the sites of infection and inflammation, and through their production of cytokines and chemokines (11). As a vaccine kit candidate, it is required to know whether or not the *L.caulleryi* protein can induce either humoral or cellular immune response, because then a favourable immune response and immunogenic protein can be explored. Immunogenic protein has main characteristics such as a heavy molecule weight, homogeny and a complex chemical structure and alienation (13, 15). It is necessary to study whether the results of the immunogenic proteins from a liver containing L. caulleryi can be developed for use in a vaccine in the effort to overcome Leucocytozoonosis, for example, with a vaccination program for chickens with a vaccine sub-unit that is safe in its use.

II. METHODS

A. Isolation and identification of L.caulleryi schizont

Schizont *L.caulleryi* isolated from chicken liver infected by leucocytozoonosis is based on the clinical signs observed, microscopic examination and the pathological changes of other chicken organs. Microscopic examination was performed to detect any gametosit stadium developed in eritrosit. Then, further assessment was done on several other organs such as the liver, spleen and intestine to detect the schizont stadium by crushing the organ and a pathological examination. The chicken liver and spleen containing schizont *L.caulleryi* was isolated in 50-100 mg or 0.05 ml cultured wet pellets, with a 2-3 ml 2-Drehydration solution/sample buffer added. Next, the sample was put on ice, sonicated for 30 seconds and cooled down to -80°C for 5 minutes. This treatment was repeated four times. The sample was centrifuged using a microcentrifuge (16.000 x g) for 20-30

minutes at 18-20 $^{\circ}$ C, and was then taken out from the centrifuge, Supernatan was put into a clean tube and the sample was stored at -80 $^{\circ}$ C.

B. Immunization of rabbits

This research used five rabbits treated as per the animal welfare concept. They were given a health examination based on both clinical symptoms and laboratory tests. All of the animals were handled in strict accordance with Ethical Clearance and the experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, of the Universitas Airlangga, No: 630-KE. Four-month old naïve rabbits were prepared for the immunisation trial at the laboratory of experimental animals, in the Faculty of Veterinary Medicine at the Universitas Airlangga. Each of the experimental rabbits were injected with 500 µg L.caulleryii schizont protein (0.3 ml) with an added Freund adjuvant complete (Sigma, USA) with a ratio of 1:1. The injection was performed every two weeks with the same protein with a dosage of 500 µg each and with the added adjuvant incomplete (Sigma, USA). The immunisation (booster) was performed 5 times in 2 weeks. Prior to the first injection, about 10 ml of rabbit blood was taken for TLR-2 and CD4 examination as preliminary data (control) and whole blood examination was conducted at the end of the first booster until the fifth booster (13, 15).

C. Examination of TLR 2 and CD4 expression using Immunocytochemistry

The principle of immunocytochemistry examination is that it is an immunology technique used to visualise specific proteins or antigens in the cells using the first antibody (www.abcam.com/index html). Several stages of the examination will be explained: 1) the blood sample is washed using 10% PBS-T20 five times, and the sample is fixated with 100% methanol (10 minutes) or with paraformaldehyde in PBS pH 7,4 for 15 minutes at room temperature, 2) the sample is washed twice using cold PBS, the sample is incubated for 10 minutes in PBS consisting of 0.1% Triton X-100 or 100 mM digitonin, and then the cells are washed in PBS three times for 5 minutes, 3) the cells are incubated with 1% BSA in PBS-T20 for 30 minutes, and incubated in conjugated antibody TLR 2-FITC labelled (Abcam's RabMab, USA) and CD4-FITC labeled (Abcam's RabMab, USA) and diluted in 1% BSA in PBS-T20 at room temperature for an hour or at night at a temperature of 4°C, 4) the cells are washed three times in PBS (5 minutes for each washing). The results were examined by using a fluorescent microscope using a magnification of 200 times, to find out whether the yellow to green fluorescent colour from the T cells expresses TLR-2 and CD4.



III. RESULTS AND DISCUSSION

The cellular immune response was shown by the expression of TLR-2 and CD4 in the rabbit T cells marked by the yellow to green fluorescent colour after the rabbit immunisation (Figure 1 and Figure 2).

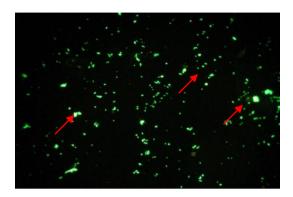


Figure 1. TLR2 expression in rabbit T cells visualized by Fluorescein Isothiocyanate (FITC) (200x magnification).

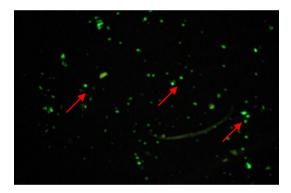


Figure 2. CD4 expression in rabbit T cells visualized by Fluorescein Isothiocyanate (FITC) (200x magnification).

It has been known that the toll-like receptor (TLR) is a receptor that can recognise the ligand from microbes or parasites which are involved in innate immunity. Along with new developments, TLR not only plays a role in innate immunity but also in adaptive immunity (12, 13). The examination results from the immunocytochemistry shows that there is a yellow to green fluorescent colour visualised by fluorescein isothiocyanate (FITC). It shows that the stadium schizont L.caulleryi protein has a ligand that is recognised by TLR 2 as a form of signal transduction which activates and induces a cellular immune response such as the lympocyte T of the rabbits after immunisation. In accordance with the principles of the immunisation method, which is an increasing degree of immunity, it provides protective immunity by inducing a memory response towards a specific pathogen with a non-virulent or non-toxic antigen (14).

According the results of the research, it is shown that the antibody TLR-2 can recognise the ligand from the protein antigen L.caullervi schizont by stimulating T cell activation, marked by the presence of yellow to green fluorescent colour which increased in accordance with the treatment from the various boosters. When the antigen of L.caulleryi schizont enters the body, it will be caught by macrophage or dendritic cells and the phagocytes cells will be activated by TLR as a signal transducer. L.caullervi schizont possess a ligand or pathogen-associated molecular pattern (PAMP) which is recognised by TLR-2. Ligands that are recognised by TLR-2 consist of lipoprotein/lipopolypeptide, flagelin, ssRNA and CpG DNA. The schizont of L.caulleryi is an intracellular microorganism which contains antigens. When the antigen enters the body, it will be caught by a macrophage and the phagocytes cells will be activated by the TLR as a signalling pathway. The signal produced by TLR will activate the transcription factor NFkB which stimulates cytokine production (10, 16). NFkB activation is initiated by a signal which recruits MyD88 and interacts with the IL-1 receptor associated kinase (IRAK). Autophosphorylation then occurs, separating MyD88 and activating the TNF receptor associated factor 6 (TRAF-6) to activate the IkB kinase (IKK). Activated IKK will activate NFkB to transcript gene IL-12, IL-10, IL-4, TNF-α, IFN-γ. IL-2 roles will increase the cytolytic activity from the cytolytic T lymphocytes. This will also promote Th1 cells development together with CD8 activation in order to produce IL-2 which stimulates the proliferation and differentiation of B cells that will produce antibodies. IL-4 is a cytokine which is produced by subset Th2 from Th cells CD4 that function to induce Th2 cells differentiation and stimulate IgE production. (17, 18).

The main function of CD4 is acting as a transduction signal in antigen recognition and to strengthen the bond between T cells and antigen-presenting cells (APC). APC produces IFN-y and IL-12 that stimulate the differentiation of CD4+ cells into Th1 which plays a major role in delayed hypersensitivity reactions. CD4+ T-cells produce a protein named IL-4 cytokine which helps B lymphocytes in antibody production and phagocytosis to destroy ingested microbes (11, 17). CD4 molecules as a co-receptor are a surface cell molecule which are expressed by various types of cells in the immune system which were formed by cluster differentiation. The accessory molecule is used as a marker of Th cell activation with B cells and cytotoxic T cells maturation, which is responsible in regulating chronic inflammatory reactions towards antigens through macrophage stimulation. Lymphocytes B activation is marked by a significant increase (p<0,05) in the antibody titer of rabbits injected with the protein of L.caulleryi schizont (15, 19). As a vaccine kit candidate, the schizont L.caulleryi antigen injected in to rabbits needs to induce a cellular immune response, marked by T cell lymphocte activation which expresses TLR 2 in accordance with humoral immune response. There was an antibody (IgG) titre enhancement produced by B lymphoctes (14, 15).



Conclusion

The antigenic protein of *L.caulleryi* schizont has the ability to induce a cellular immune response through the expression of TLR-2 and CD4 in the rabbits' T cells. The TLR-2 signal plays a role in innate immunity, but also in adaptive immunity. The antigenic protein of *L.caulleryi* schizont may contain ligand which acts as a receptor that is involved in pathogen-associated molecular patterns (PAMP). This study is a preliminary research to explore the immunogenic protein which plays a role in immune system activation for future studies in vaccine development to overcome leucocytozoonosis in chickens.

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Pangkat / Golongan : Pembina TK I / (Gol. IV/b)

Jabatan : Lektor Kepala

Telah melaksanakan penelitian dengan judul sebagai berikut:

No.	Judul Karya Ilmiah	Tahun pelaksanaan Penelitian
1.	Morphological Detection of The Intestinal Parasite Blastocystis sp. In Fresh and Cultured Feces of Pet Sugar Glider (Petaurus breviceps)	2018
	(Mammalia: Petauridae) In Surabaya, Indonesia.	2016
2.	Identification of Active Compounds of Ethanol Extract of Citrus amblycarpa leaves by Analysis of Thin-layer Chromatography and Gas Chromatography-Mass Spectrometry as Bioinsecticide Candidates for Mosquitoes	2020
3.	Histopathological studies on <i>Leucocytozoon Caulleryi</i> infection on broiler in endemic area of Indonesia	2020
4.	Potential Extract Ethanol Citrus Amblycarpa as a Bioinsecticide Against Aedes Aegypti Larvae	2021
5.	Protein Profile of Sporozoite of Leucocytozoon sp. from Culicoides sp.	2010
6.	Deteksi Cryptosporidium canis pada Anjing di Kota Surabaya	2020
7.	Eksplorasi Protein Antigenik <i>Leucocytozoon caulleryi</i> sebagai Kit Diagnostik Leucocytozoonosis pada Ayam Broiler	2013



















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8.	Uji reaktivitas protein 30 kDA bakteri <i>Aeromonas hydrophila</i> yang diisolasi dari ikan air tawar dengan teknik indirect ELISA.	2016
9.	Penambahan Sari Air Laut (Nigarin) Dalam Pengencer Skim Kuning	
).	Telur Terhadap Viabilitas Dan Motilitas Spermatozoa Sapi Limousin	2018
	Post Thawing	2010
10.	The Effectiveness of Ethanol Extract of Red Betel Leaf (Piper	
10.	· •	2020
	crocatum) Againts Mortality of Boophilus microplus Larvae In Vitro	
11.	Prevalence of Ectoparasites in Bean Goats on the Sub-District of	2020
	Prambon, District of Nganjuk	2020
12.	Repellent Effectiveness of Permot Leaf Ethanol Extract (Passiflora	2021
	Foetida Linn.) against Aedes Aegypti Adult Mosquitoes	2021
13.	Detection of Goat Digestive Tract Protozoa Through Feces	2021
	Examination in Kwanyar Sub-District, Bangkalan District	2021
14.	Identification and Prevalence of Digestive Tract Endoparasites of	
	Goats in Ujungpangkah, Gresik District	2021
15.	Morphology of surface ultrastructure of Duthiersia expansa(Cestoda	
	Diphyllobothriidea) from water lizards (Varamus salvator) from	2014
	Sidoarjo, Indonesia	2011
1.0		
16.	Antigenic Protein of Leucocytozoon caulleryi schizont Inducing	2017
	Cellular Immune Resonse: TLR-2 and CD4 as Marker	_ 5 _ 7

Adapun penelitian tersebut tidak perlu dilakukan Uji Etical Clearence karena tidak menggunakan hewan coba.

Demikian surat kerangan ini kami buat untuk dapat dipergunakan sebagai persyaratan pengusulan Jabatan Fungsional **Guru Besar**

Surabaya, 8 Agustus 2022



Dr. Mirni Lamid, drh., MP

