3rd International Conference on Tropical and Coastal Region Eco Development 2017

IOP Conf. Series: Earth and Environmental Science **116** (2018) 011001 doi:10.1088/1755-1315/116/1/011001

FOREWORD FROM THE CHAIR OF THE 3RD ICTCRED 2017

On behalf of the Organizing Committee, I would like to extend our warmest welcome to you at the International Conference on Tropical and Coastal Region Eco Development (ICTCRED) 2017. This annual conference is the third event after the second has been successfully conducted in 2016 at Bali. This conference is organized by Research and Community Services Institute (LPPM), Diponegoro University. The conference aims to provide a forum for researchers, academicians, professionals, and industries to expose and exchange innovative ideas, methods, and experience in the areas related to tropical life sciences and coastal development.This conference also provides forum for researchers and scientists to exchange ideas and their current achievements.

In this year, 215 abstracts from various universities and research centers from many countries have been received. However, after in-depth review, only 147 high quality papers are accepted for oral and poster presentation in this conference. In addition, we cordially invite seven highly respected researchers in various fields as keynote speakers in this conference, to share their knowledge and expertise. I am grateful of each one of them for setting aside their valuable time to participate in this conference.

Moreover, I would like to announce that the ICTCRED 2017 Committee has signed an agreement with the Institute of Physics (IOP) to publish the conference proceeding in their Scopus-indexed *IOP Conference Series: Earth and Environmental Sciences (EES)* after a series of review. We do hope that the collaboration with IOP will increase the visibility of this conference papers to international levels which also give benefits to authors and also their institutions.

Finally the success of this conference lies not only in the quality of papers but also on the dedicated team efforts of the organizing committee. We thanks to the keynote speakers for the participation in this conference. I would like to acknowledge Institute of Physics (IOP) for the collaboration in publishing the conference proceedings. Indeed, I would like to thank the Scientific Committee members for their effort in reviewing and evaluating the papers for maintaining the quality of the conference. Last but not least, all staffs of The Research and Community Services Institute, Diponegoro University, deserves our great appreciation for their unlimited supports.

To all delegates, I hope that the 3rd ICTCRED 2017 event will be memorable not only from the scientific perspective but in the joy of meeting with other scientists for mutual collaboration. I wish you enjoy the conference as well as the beautiful nature and great traditions of Yogyakarta.

Chair,
Organizing Committee of ICTCRED 2017

Dr. Agus Trianto

FOREWORD OF THE DIRECTOR

Dear distinguished speakers, delegates, ladies, and gentlemen

I am very pleased to welcome you all to this international conference, Tropical and Coastal Region Eco-development, which acts as a forum for those interested in tropical and coastal development issues. Diponegoro University commits to provide an opportunity for scientific society to always play important role in disseminating ideas and research results especially in the area of coastal and tropical development, which is the main research field of our university. Hence, this conference offers a platform for extensive sharing and exchange of knowledge for the development of coastal and tropical areas.

The topics presented in this conference cover marine biodiversity, sustainable marine utilization and development, climate change on coastal and marine ecosystems and coral reef ecosystems and coastal management. In the tropical field, this conference deals with relevant ideas and knowledge addressing vital life sciences, tropical health and nutrition, tropical diseases and tropical food and energy. In addition, this conference also covers socio-economic aspects such exploration of tropical rainforests, deforestation, rising immigration, etc. Thus, it is clear that the International Conference on Tropical and Coastal region eco-development is a unique blend of coastal and tropical that nicely fits the current interest among the community concerned with sustainable coastal and tropical ecosystems.

Finally, we would like to express our gratitude to our distinguished keynote speakers, Prof. Susilo Wibowo, Dr. Hadiyanto, Prof. Gerard Pals, Prof. Junichi Tanaka, Dr. Roel H. Bosma, Prof. Tao Liu, and Prof. Soottawat Benjakul, who had been traveling all the way to Yogyakarta. Certainly we will have an important benefit of preparing the next generation of Indonesian scientists with international exposure. We thank our participants to present their research papers, to share extensively and exchange of ideas thoughts and discussions so that this conference facilitates the formation of networks among participants. We thank all invited guests who have shown their interests in coastal and tropical region development field. Many thanks to the organizing and scientific committee of ICTCRED 2017 who have work very hard to run the conference.

I wish you all a productive and successful conference.

Yours sincerely
Director of Research and Community Services Institute
Diponegoro University

Prof. Heru Susanto

WELCOME ADDRESS OF THE RECTOR OF DIPONEGORO UNIVERSITY

It's a great pleasure and honour for our University to be the host of the 3rd International Conference on Tropical and Coastal Region Eco Development 2017 organized by Research and Community Service Institute, Diponegoro University. The special acknowledgement, I address to the distinguished speakers Prof. Susilo Wibowo and Dr. Hadiyanto from Diponegoro University-Indonesia, Prof. Gerard Pals from VU University Medical Center – Netherlands, Prof. Junichi Tanaka from University of the Ryukyus – Japan, Dr. Roel H. Bosma from Wageningen University – Netherlands, Prof. Tao Liu from Ocean University of China, and Prof. Soottawat Benjakul from Prince of Songkla University. Thank you for the valuable time to deliver knowledge and share scientific information at this conference. I believe that this opportunity will provide the valuable information for us and deliberate some new research ideas for participants of this conference. For all the participants, I would also like to welcome you at this conference.

The origin of the conference theme is reflected from the idea of our Center of Excellence (CoE) which was established in 2012 representing our priority as a research university. Since the declaration of Diponegoro Universityas a research university, the main theme of every research result will be enhanced to the level of international benchmarking.

Diponegoro University, consists of 13 faculties, has strong human resources and research background related to the coastal development and tropical life sciences. It is also supported by integrated laboratory of marine and fisheries, which are located at Teluk Awur Jepara.

Coastal development and tropical life sciences are two important issues in Indonesia and need to be actualized by the government. The enormous potencies of Indonesia, large resources of marine area and their potential value of natural marine products extract, provide an opportunity to contribute for health energy and food. Recent issue has been arisen that the food and energy also can be exploited from the sea. Indeed, the exploration and exploitation of marine products must be considered on the impact of the environmental devastation. These issues are interesting topics which are reflected by large number of abstracts submitted to this conference. These interesting issues need to be discussed in this conference by sharing research finding and ideas. I am gratefull to see that this conference has enormous responds from the participants either from domestic or from other countries such Japan, China, US, and France, as reported by organizing committee.

Number of publication indexed by reputable database has been set as an indicator for world university rank including Indonesia. Therefore, Diponegoro University also encourages all scientists and academic staffs to increase their publication records in these international reputation journals. Currently, Diponegoro Universityis in the 6th position among universities in Indonesia for the number of

publications in reputable International journals. I sincerely express appreciation to the organizing committee for their effort to realize this conference.

By the end of my short welcome address, I hope our foreign guests take advantage of their stay here to enjoy Yogyakarta and its wonderful places. It is a beautiful and historical city to visit with a wonderful and unique traditional art dance, stunning sunset, great sceneries and interesting shopping.

Once again, it is my great pleasure to welcome you all to the 3rd International Conference on Tropical and Coastal Region Eco Development 2017. I wish you a pleasant two fully scientific days of conferences and I hope you can get a fruitfull share with other scientists on current developed knowledge and perhaps seeking for potential collaboration of your interested field.

Thank you for your kind attention.

Prof. Yos Johan Utama
Rector

KEYNOTE SPEAKERS

Prof. Susilo Wibowo
Universitas Diponegoro – Indonesia
"Indonesia got obese; do we care? Genetics, epigenetic, and enviromental point of view"

Dr. Hadiyanto
Universitas Diponegoro – Indonesia
"Effects of sugar addition on the thermal degradation of phycocyanin from Spirulina sp."

Prof. Gerard Pals
VU University Medical Center – Netherlands
"Cancer and the environment"

Prof. Junichi Tanaka
University of the Ryukyus – Japan
"Exploration of coral reef organisms for bioactive molecules and related issues"

Dr. Roel H. Bosma
Wageningen University& Research – Netherlands
"Investing in climate change mitigation and adaptation on mangrove and aquaculture doubles benefits."

Prof. Tao Liu
Ocean University of China - China
"Research on complete mitochondrial genome of marine algae"

Prof. Soottawat Benjakul
Prince of Songkla University
"Valorization of fish processing by product"

Note from Editors

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Note from Editors

The 3rd International Conference on Tropical and Coastal Region Eco Development (ICTCRED) 2017 was held in Yogyakarta, Indonesia from 2nd to 4th October 2017. The conference was organized and fully supported by the Institute of Research and Community Services, Diponegoro University, Indonesia. Authors and participants from many countries made the conference truly international in scope. The scope of this conference varies from marine environment, marine products and their processing as well as oceanography technology, coastal environment management and policies. In addition, social science related to the coastal area development were also discussed.

This volume of *IOP Conference Series: Earth and Environmental Sciences* contains selected articles from those presented in the conference. After presentation, the revised papers were peer reviewed by fellow reviewers to ensure the quality of published materials. Finally, Editors decided to select and publish as many as 105 papers. It is hoped that the presented papers can offer more insight towards broad audience.

On behalf of Editors, we appreciate enormous work of all staffs and reviewers in the preparation of this volume. We would like to express our sincere thanks to all authors and presenters for their valuable contributions. We hope that the conference has been beneficial to all participants.

Guest Editors

Munawar A Riyadi, PhD
Diponegoro University, Indonesia
Chair of Scientific Committee - ICTCRED 2017

Scientific Committee/Editor

Organizing Committee

This conference has been conducted by Institute of Research and Community Services, Diponegoro University, Indonesia 2-4 October 2017

Peer review statement

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Peer review statement

All papers published in this volume of *IOP Conference Series: Earth and Environmental Science* have been peer reviewed through processes administered by the proceedings Editors. Reviews were conducted by expert referees to the professional and scientific standards expected of a proceedings journal published by IOP Publishing.

Effectivity Test Of Crude Protein Spore of *Myxobolus koi* as Materials Development For Sub Unit Vaccine To Prevent the Gold Fish (*Cyprinus carpio*, Linn) Dead by Myxobolusis

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Effectivity Test Of Crude Protein Spore of *Myxobolus koi* as Materials Development For Sub Unit Vaccine To Prevent the Gold Fish (C*yprinus carpio,* Linn) Dead by Myxobolusis

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Absract. Production of fisheries culture according to totality estimated is increasing about 4.9% per year. Gold fish is one of species that have economically value in Indonesia, but there are problem because of myxobolusis. The aims of this research are : Isolation of crude spore protein of *Myxobolus koi* by using SDS-PAGE and to analyze profile of *Myxobolus koi* protein that will be developed for sub unit vacsine that can protect koi fish infected by *Mxobolus sp.* The method of this research used experimental method and belonged to stage : Culture of *Myxobolus* by using spore scattered, characterization of Myxobolus proteine spore by using SDS-PAGE, Antibody polyclonal production by using ELISA methode to find out optical density of this antibody, and vaccination was done by dipping, with dose 600 µl per liter. The result showed that there were 5 bands of crude protein spore of *Myxobolus koi* with molecule weight (MW) 41.1 kDa, 51 kDa, 89.8 kDa, 121.7 kDa and 230,1 kDa. The highest Optical Density (0.699) from polychlonal antibody production by using crude spore protein of *Myxobolus koi* happened on the 42nd day. The result of the chellence test of the protein showed that the highest of leucocyte count happened on the fish immunized by crude spore protein and not infected by *Myxobolus koi*, but can increase the survival rate (SR) from 20% to 86,37%, so the crude spore protein of Myxobolus can be used for developtment of material for sub unit vaccine to prevent the myxobolusis.

Keywords : *Crude* protein spora, Leukocyte, *Myxobolus*, Myxobolusis, Optical Density.

1. Introduction

One of the species of freshwater fish that have a bright prospect is the goldfish (*Cyprinus carpio* Linn). Based on document from the Ministry of Fisheries and marine in 2010, it is stated that Indonesia recently mastered 7.5% freshwater fish trading, still lower if compared to Singapore which has reached 22.5%. This led to the development of the carp increased mainly through farming good intensively or taditional. However, many obstacles can cause a failure in the culture, in which the main constraint of very noteworthy is the emergence of an attack of the disease.

One of the diseases that often become parasiter is a Protozoan disease caused by *Myxobollus koi* called myxobolusis. Myxobolusis is a disease parasiter on fish caused by the sporozoa among other *Myxobolus* sp. Generally disease-causing organisms is known as its spore morphology, number, and location of the polar filament. The disease can cause serious problems on the goldfish of koi and can lead to the death of up to 60-90% with prevalence reaching 100%. Furthermore it is said that in 1974 and 1978 there was a case of *Myxobolus* attact in Indonesia that led to the deaths of up to 100% of the koi fish mainly on stadia seeds.

Fish Stricken by the disease of myxobolusis show difficulty to breathe because there was a nodule or cyst or nodule on the Gill filaments. In Blitar it was reported by farmers that in 2010 there was myxobolusis desease occuring on carp koi that was 3-5 cm with mortality reaching the 90%. The spread of this parasite occurs due to the transfer of parasites from one fish to another, either directly or through the host between certain phases of the life cycle of the parasite [8]. In 2002 mass death of goldfish occurred in Kulon Progo and Sleman, caused by a parasite *Myxobolus* sp. and *Henneguya* so that the loss experienced by the fish farmers was quite large. *Myxobolus* sp. is also found in the Ngrajek area of Magelang Regency in 2006 with prevalence reaching 91%, and then in goldfish ponds koi in Blitar with prevalence reaching 86% in 2010.

Prevention efforts and countermeasures against myxobolusis have been done using a disinfectant as well as other chemicals, but not yet able to meet the target and can even cause resistance and residues in the body of the fish. It is necessary to look for alternative prevention efforts that do not cause a negative impact. One of the efforts that have already begun to be developed at this time is by vaccination that can be done by means of immersion or injection [3]. [2] managed to find a antigenic carbohydrate isolated from glycoprotein.

Characterization of protein can be done by using the method of *Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophorosis* (SDS-PAGE) where the results can be known whether protein molecular weight is suitable for use as a vaccine. A protein core and anti-*Zoothamnium arbuscula* with cytoplasmic protein spasmin-1 on spasmonema. Immunoblotting analysis of results showed that the protein has a molecular weight of antigen of 68 kDa, 55 kDa and 71 kDa. This protein is thought to play a role in parasitic infestations on the host where the major protein is as an important ligand bonding in Ichthyiophthirius multifiliis and is the parasite's entry bridge into the cell.

Protein can be a good antigen when it has a molecular weight greater than 1,000 Dalton and the complexity of the structure. The antigen that is also referred to as imunogen is a material that can stimulate the immune response or material that may react with antibodies that are already there without regard to its ability to stimulate the production of antibodies.

The protein of spasmin on *Zoothamnium arbuscula* spasmonema can already be created Polyclonal antibody in hela cells. Immunoblotting analysis of the results shows that the location of the protein of antigens at molecule weight is 68/69 kDa, 55 kDa and 70 kDa. Proteins of the membrane *Tetrahymena* sp. which is the one with the *Ichthyophthyrius* familia *multifiliis* had most likely developed as a sub unit vaccine against the disease white spot on the fish. The antigenic membrane protein in Paramecium by SDS-PAGE and Western Blotting, with 61 kDa molecular weight, 63 kDa and 65 kDa.

Based on the background above, searching sub-unit of myxobolus material needs to be done to be developed as a sub unit vaccine that an provide protection against attacks on the carp myxobolusis, until the death of fish in the pond can be suppressed.

2. Research Methodology

This research was carried out from March to October by 2015, with the location of the research in the laboratory of Dry (*Kering)*, Fisheries and Marine Faculty, Airlangga University and the laboratory of molecular biology, the Faculty of Sciences Brawijaya University of Brawijaya, Malang.

2.1 Materials and Equipments

The main material for the isolation and characterization of a protein used are physiological NaCl, ethanol, solvents percoll gradient, pepsin, HCl, Ethylenediamenetetraacetic (EDTA), Kl, Na2HPO4, KH2PO4, trypsin, sodium citrat, NaHPO4, H2), NaHCO3, glucosa, phenols red 0.5%, NaOH, filter 0.22 um, bovineserum albumin, dextrosa, Ethyl Alohol (ETOH), proteinase, ForwardERIB1 primer pairs 5 '-ACCTGGTTGATCCTGCCAG-3 ' (2-20) and Reverse ERIB10 5 '-CCTCCGCAGGTTCACCTACGG-3 ' (2069-2070), 400 UM DNTP, 3 um Mgso4 , yellow and blue dye, agarose, Tribase Acetic and EDTA (TAE) buffer, sybrsafe, 100 DNAladder bp and bp 1, loading dey, tris-HCL, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), bromophenol blue, glycerol, SDS loading buffer, polyacrylamide, stacking gels, ammonium peroxidaisulphate (APS), glycine and Tetramethylenediamine (TEMED).

Materials for the production of Polyclonal Antibodies are rabbit from the Center Pusvetma/Veterinaria Farma, Surabaya, with an average weight of 2.385 kg 5 – 6 months and the male gender, Freund's complete adjuvants (Sigma), and Incomplete Adjuvants Freund's (Sigma).

The main equipment used are Haemocytometer, rubber pollicemen, microscope tube micrometer, autoclaf, centrifuse, swinging rotors, water bath sonicator and 1 set of tools of SDS-PAGE electrophoresis.

2.2 Research Methode

Research methods used in this research is descriptive method, where the blood picture and examination stages of counting of survival rate of carps are performed using 4 pieces of aquariums with a volume of 10 litres, for not exposed by a spore protein spores (given PBS) and not infected (K1), 1 for treatment of exposure to infection with spores and protein *Myxobolus koi* (K2), 1 piece of the Aquarium for treatment not exposed with protein and infected *Myxobolus koi* (K3) and 1 piece of akurium for the treatment of infection not exposed with protein and *Myxobolus koi* (K4). Dose used in this research is 600 micro litres per fish through injection (IM). Each of the aquariums is filled with 10 goldfish.

2.3 Sample preparation of *Myxobolus* sp. Spore.

The main ingredients of this research are nodules containing spores of *Myxobolus* sp. stadia adults are obtained from goldfish stricken by myxobolusis. The infected fish or there are nodules on gills first are cleaned using aquadest so that dirt stuck in the body of the fish is lost. Retrieval of nodules containing spores of *Myxobolus* is done manually by first taking the gills of carp and then using tweezers nodules on the gills slowly so that the nodules are not destroyed and turned so that the spores can be drawn wholly from the stricken organs (gills). Nodules containing spores of *Myxobolus* sp. are collected and washed with aqudest in order to be free from dirt and slime, and then broken down and crushed and then centrifugeded with speed 1,500 rpm. Spores will precipitate out. Deposits of spores are washed again with centrifugedion again with speed 1,500 rpm for 5 minutes. The deposits are taken and spores are counted in numbers and saved and given PBS.

2.4 Isolation of Spore Protein Crude of *Myxobolus* sp.

Spores that have been counted then arew ashed with PBS and centrifuged at speed 5000 rpm for 10 minutes. Leaching process is repeated twice. Pellet is resuspenced with buffer Lysis (0.32 M scrose (sucrosa) 54.8 gram, 1% v/v Triton X-100 0.5 ml, 10 mM Tris HCL pH 7.4 10 ml) so as to obtain a concentration ± 11ml. then on pellet suspension added 0.5 ml of EDTA 1 µ M and PMSF (phenylmethylsulfonyl fluoride) 5 mM as much as 0.5 ml. then sonication is done with ice in a sonicator waterbath (1 minute sonication, ½ minute break) repetitive 10 times. The results of the sonication is vortexed (½ minute vortex 1 minute rest) in ice, carried out repeatedly 15 times. Solution of sonication results is crude protein.

Analysis of protein concentration on research uses the method of Biuret and read with a UV-Visible spectrophotometer. Reaction with biuret method is the formation of the complex which is purple. This complex is formed when one or more peptides reacts with NaOH and CuSO4. Colour arising is due to Cu^{2+} ions complex with four nitrogen atoms that come from four of the ring peptide. Protein levels are calculated by converting on Bovine Serum Albumin curve (BSA) whose concentration is known.

2.5 The production of Polyclonal Antibody against Crude of Spores Protein Of Immunization of Spore Protein in rabbits

Adult healthy male Rabbit is injected intramuscularly with whole protein with a dose of 50-100 µg which was previously added adjuvants complete (Sigma) with the same comparison so that the final volume by as much as 500 µl. Injection is done under the skin at the four locations of the body that have loose skin. Injection was repeated with the same protein by the addition of adjuvants incomplete (Sigma) at 2 weeks after the first injection. Reinjection the next reset used the same protein with adjuvat the same way incomplete onwards until a high antibody titer was obtained. Prior to injecting, the first capture serum negative control in test as ELISA. Taking the serum before further carried out booster is to see any antibody response after injection with the same test.

2.6 Determination of Antibody Titer by ELISA Method

Titer of antibodies derived from rabbit serum that has been on immunization is determined by the method of ELISA. Negative control is taken from rabbits that are not in immunization by gender and age. The Cup of microtiter used is mikrotiter cup sumuran 96. Every sumuran is in the content with 100 µl of the antigen solution with the concentration 10 µg/ml in buffer coating and in incubation at a temperature of 20^0 C for 24 hours. The cup of mikrotiter is washed one time with wash buffer and then in each sumuran is added 100 µl of serum of rabbits immunizated and diluted with PBS. Rabbit serum is diluted in series, namely 2^0, 2^{-1}, 2^{-2}, 2^{-3}, …, 2^{-10}. The cup of mikrotiter is incubated at temperature 37^0 C for one hour and continued to be washed three times with wash buffer. Every sumuran is in the content with 100 µl of the conjugated solution of IgG goat antimouse peroxidase. Conjugate is diluted with PBS 10,000 X and BSA is added 1%. The cup of mikrotiter is incubated in temperature 37^0 C for one hour and then is washed three times with wash buffer. Each sumuran is added as many as 150 µl ABTS substrate µ l (one mg/ml in buffer substrate ABTS and 0.3 µl of hydrogen preokscide). The cup of mikrotiter is incubated in room temperature for 30-45 minutes. antibody titer is read with a spectrophotometer for ELISA (ELISA reader) at a wavelength of 405 nm. Antibody Titer indicates negative if the results of the reading of ELISA between control rabbit and the immunized rabbit gets almost the same optical density (OD) values immunization. Antibody Titer indicates positive when value OD immune rabbit higher or at least twice the value of the OD of control rabbit control.

2.7 Examination of Erithrosit Number

Hemosit is taken on the ventral part of the second abdominal segments using needle 25 G one syringe one ml entered 0.2 ml of solution modified with cold Alsever (AS 19.3 mM; Na citrate 239.8 mM NaCl 182.5 and glucose 6.2 mM EDTA; pH 7.2) as an anticoagulant. Counting the number of haemocyte is done with methods of May Grunwald-Giemza, namely using light microscopy (LM) with an enlarged 1000 times, then calculated with the Coulter counter ZM SUNDAY model (Counter Electronic Ltd), hemosit particle size range 0.4-800 µm, as supporting data can also be observed with the electron microscope (EM) by first centrifugede 700 X gravity at a temperature of 4^0 C for five minutes.

2.8 Examination Of The Differential White Blood Cells

The blood is shed on glass objects and made blood review with giemsa staining, later the type of cell is identified . Differential hemosit aims to find out the amount, type and percentage of cell hemosit. The number of hemosit is calculated up to 100 cells and look for the percentage of each type is found, done from the beginning to the end of the study.

2.9 Determination of Survival Rate (SR)

Determination of level of survival rate was conducted to analyze the ability of crude spore protein protection on goldfish. It is expressed in the form of a percentage of the number of koi fish that lives up to the experimental treatment against 30pasca the total number of fish kept. Fish survival is calculated by using the formula :

$$SR = \frac{Nt}{No} x100\%$$

Description:
SR = survival rate
NT = the number of fish that live at the end of the observation
No = number of fish that live in the beginning of the test

2.10 The parameters of the research and Data analysis

The main parameter of this research is the protein profile of *Myxobolus koi*, carp blood Picture (the number of Leukocytes and Erythrosit) and Survival Rate of the koi fish exposed with protein of *Myxobolus koi*. Data from the study results will be presented in the form of quantitative data and analyzed with Analyzed of Variance (ANOVA) [12].

3. Results And Discussion

The results of the examination and identification of spores of *Myxobolus koi* of koi fish can be seen in Figure 1 and 2. While the results of the determination of the characterization protein conducted using SDS-PAGE method can be seen in Figure 3.

Figure 1. Nodules on the gills of fish infected by *Myxobolus koi* (A)
and Nodules that contains the spores of *Myxobolus koi*

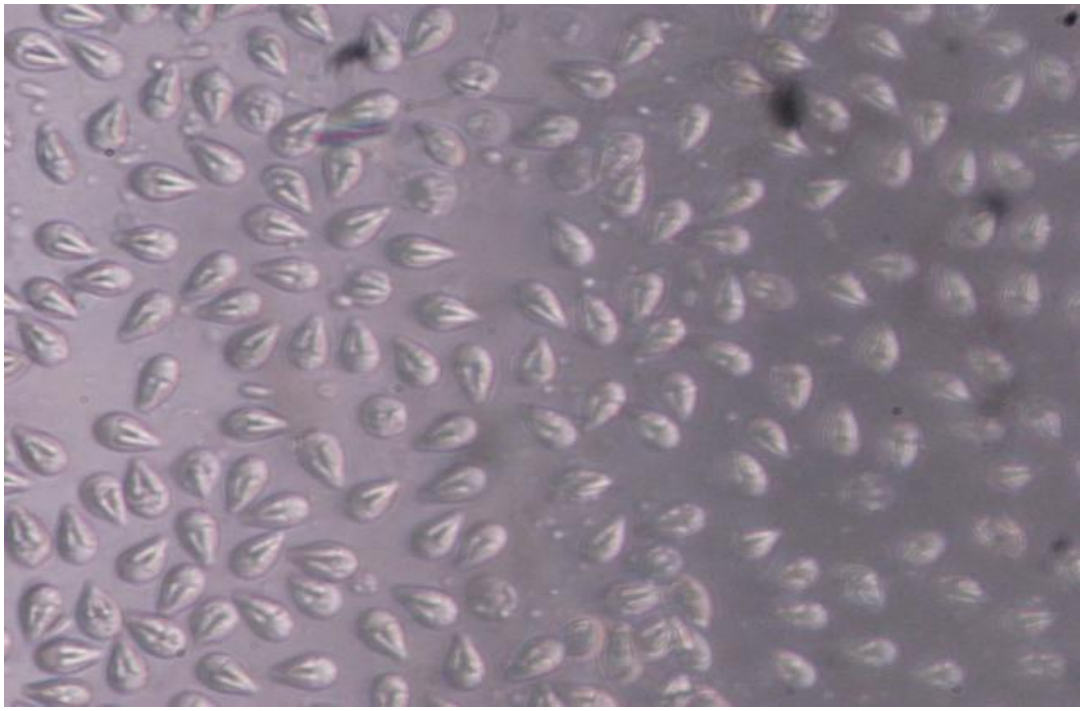

Figure 2. spore of *Myxobolus koi* (400x Zoom)

3.1 Isolation of Crude Protein

Samples of nodules containing spores of *Myxobolus* sp was obtained from Blitar, Tlogo village, Kanigoro district as many as 154. Isolation of proteins was carried out by taking 1 gram of tissue samples of nodules that were then made into crude protein and stored in a refrigerator with a temperature of 4 ° C in a solution of Tris-HCl 20 mM.

Crude protein concentration analysis is performed using the method of solution concentration using Biuret BSA 0 – 10000 ppm. Determination of the wavelength of the BSA was done in concentration of BSA at 4000 ppm so the obtained maximum wavelength was 541 nm.

3.2 Analysis of Crude Protein of Spores By The Method Of SDS-PAGE

Protein analysis is carried out using the method of SDS-PAGE which is a method to solve the protein based on molecular weight. SDS-PAGE uses the stacking gel used 3% and 12% separating gel and was run on 200 Volt voltages and 2 mA. The results of the SDS-PAGE in the form of band of proteins, where the band can be calculated from the weight of the molecule so that the character of the protein from the spores of *Myxobolus* sp. can be known. (Figure 3)

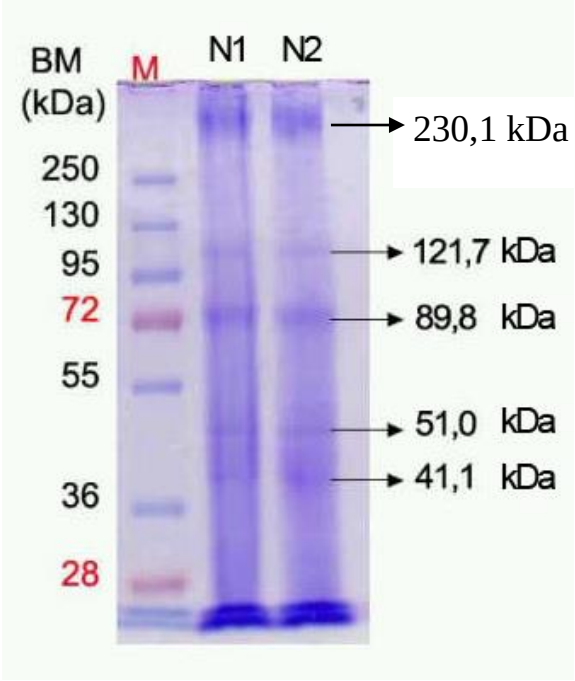

Figure 3. Showed that results of spore protein characterization of of *Myxobolus* sp. Using SDS-PAGE is invalidated 5 bands of protein with molecular weight (BM) 230.1 kDa, 121.7 kDa, 89.8 kDa, 51.0 kDa, 41.1 kDa.

3.3 Production of Polyclonal Antibodies

The result of the production of Polyclonal Antibodies from spore protein *Myxobolus koi* obtained Value OD that on day 1 is 0.233 and has increased up to day to 42. Every time the blood taking is done. boster OD Values on day 56 though done booster was done, it declined to be a 0547.

Table 1. The value of the Optical Density (OD) of Spore Proteins Polyclonal Antibody in rabbits by Indirect ELISA

Day to	Value of OD		
	The Rabbit serum infected by *Myxobolus*	Control Serum (-)	PBS control (-)
1	0.233	0.154	0.103
14	0.489	0.157	0.101
28	0.531	0.158	0.102
42	0.699	0.151	0.103
56	0.547	0.132	0.102

Value OD on day 42 reached 0.699 that shows more than 2 times the value of OD control. This is in accordance with the opinion of the Tizzard (1988) who says that animals given repeated vaccine will stimulate the formation of antibodies.

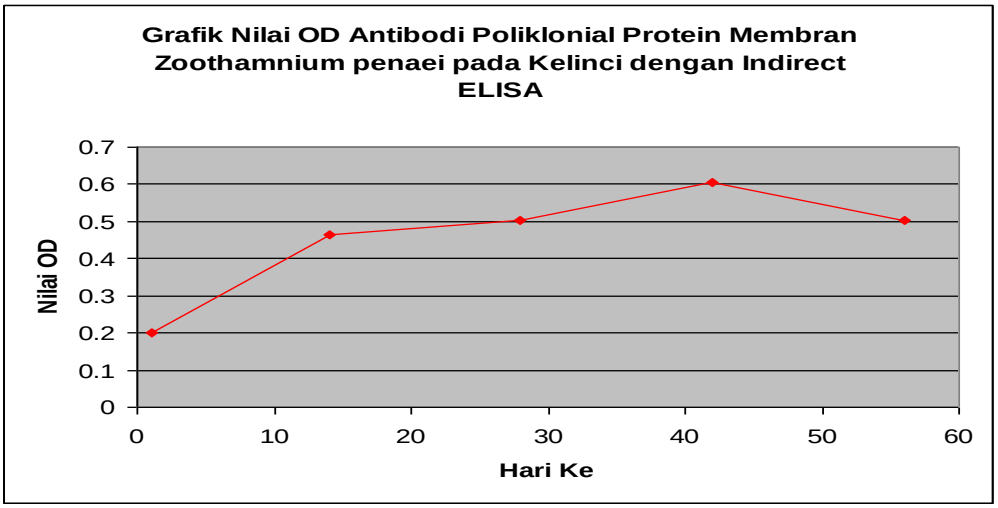

Figure 4. Graph of OD Value of Spore Protein Polyclonal Antibody of *Myxobolus koi* in rabbits by Indirect ELISA

ELISA test results showed that the crude spore proteins of *Myxobolus koi* can induce the formation of antibodies on goldfish. Table 1 shows the highest optical density values occusr on day 42. The increase in the value of the OD on the fish exposed later infected with crude spore protein *M. koi* shows the ability of the protein protection against myxobolusis infection. The mechanisms of the immune response in fish, when antigen (vaccine) enters into the body of the fish then the antigens is presented by MHC, antigen will be captured by receptors on T helper cells (2), and helper T cells (2) going to secrete cytokines, namely IL-2, IL-4, IL-6 aimed at the differentiation and proliferation of B cells, differentiation of B cells and plasma cells will produce memory cells. Next the cell plasma will synthesize specific antibody and bind antigens so as to prevent the movement of antigen and to ease the process of phagocytosis.

3.4 Examination results of Blood picture of Goldfish Exposed with Spore Protein

The results of the determination of the blood picture of Goldfish exposed with the spore proteins after 14 days of observance, with a dose of 600 µl is presented in table 2. Table 2 shows that the number of Erythrosit occurs on a control (K1) and lowest on K3 (infected by *Myxobolus koi*, not exposed by spore protein) namely 30.4% and the highest occurred in control (K1) by 91,13%, while on treatment of K3 (exposed by protein and infected by *Myxobolus koi*) amounted to 30.4%. For the number of leukocytes occurs on K3 that is 69.36% and lowest in 8.87% of K1, whereas in the treatments of K3 66,58%.

Table 2. The results of the determination of the Blood picture of a Goldfish exposed by spores protein after 14 days of maintenance.

Treatments	Image of Carp Blood after 14 days of maintenance (%)	
	Erythrosit	Leukosit
Control, no infection and no exposed Protein (K1)	91.13[a]	8.87[e]
Infected by *Myxobolus* and not exposed by Spores Protein a dose of 600 µ l/fish (K2)	32.42[b]	66.58[f]
Infected by *Myxobolus* and not exposed by Spores Protein with a dose of 600 µ l/fish (K3)	30.64[c]	69.36[g]
Not Infected by *Myxobolus* and not exposed by Spores Protein with a dose of 600 µ l/fish (K3)	69.33[d]	30.67[h]
Normal	96.5 – 98,0	14 - 36

Description: different Superscript on the same column indicates the existence of the real difference ($p < 0.05$)

3.5 Results of determination of Differensial Goldfish Leukocytes Exposed by Crude Spore Proteins

The results of the differensial determination of white blood cells in the goldfish exposed with crude spore protein after 14 days of observance, with a dose of 600 µl are presented in table 3. Table 3 shows that the results of the counting of the lymphocytes in the blood of carp at the highest is treatment K3 (fish without being vaccinated but infected with 80 spores/fish *M. koi*), namely in the amount of 86.37% and the lowest percentage is in the treatment of K namely 70.46%. The results of the counting of monocytes in the blood of carp at each treatment obtained the highest percentage in the treatment of K3 namely 23.33% and the lowest is K4 treatments at 9.24%. Percentage of observations of heterofil in blood of koi fish showed the highest percentage in the treatment of K4 namel 13.4% and lowest is in treatment of K2 (fish were vaccinated and infected by 80 spores/fish *M. koi*) of 6.7%. Observation of the number of eosinophils demonstrated the highest percentage on K3 treatment of 6.71% and lowest in the treatment of the K1 (control) of 6.8%. Observation of the number of basophils indicates the highest percentage in the treatment of 5.93% K3 and K1 is at the lowest treatment 2.23%.

Table 3. Results of determination of Differensial Leukocytes Carp exposed by Crude spore Protein after 14 days of maintenance.

Treatment	Differensial of Leukocytes Carp exposed by Crude spore Protein after 14 days of maintenance (%)				
	Limfosit	Basofil	Heterofl	Eosinofil	Monosit
Control, not infected and not exposed by protein (K1)	73.12	2.23	13.4	3.65	10.25
Infected by *Myxobolus* and exposed by spore Protein Spora dose 600 µl/fish (K2)	82.23	3.95	6.7	5.19	1.53
Infected by *Myxobolus* and not exposed by spore protein dose 600 µl/fish (K3)	86.37	5.93	12.6	6.71	23.33
Not infected by *Myxobolus* and exposed by spore Protein Spora dose 600 µl/fish (K4)	70.46	2.53	8	3.27	9.24
NORMAL	**54**	**2**	**14**	**7**	**23**

3.6 Results of determination of survival rate of Carps exposed by spore Protein

Survival rate of carp is expressed in percentage and calculated on day 14 presented in table 4. Table 4 shows that in spore protein protection test of *M. koi*, there is a noticeable difference ($p < 0.05$) between the treatment of survival rate between exposed and not exposed with spores of *Myxobolus koi* keeping after 14 days.

The results of calculation show that the highest at the treatment of survival rate is in K4 (fish are not infected and fish are exposed with spores of *M. koi*) namely 96.00% and treatment (control) of K1 94,00%, while the lowest treatment is K3 (fish are infected with 80 spores/fish of *M. koi* and not exposed) with the value of SR for 20%.

Table 4. The Results Of The Determination Of survival rate of Koi exposed by The Spores Protein after 14 days of maintenance

Tratment Group	Survival rate of koi fish after vaccination
Control, not infected and not exposed by protein (K1)	$94.00^{a} \pm 2.13$
Infected by *Myxobolus* and exposed by spore Protein Spora dose 600 µl/fish (K2)	$83.00^{b} \pm 6.43$
Infected by *Myxobolus* and not exposed by spore protein dose 600 µl/fish (K3)	$20.00^{c} \pm 3.54$
Not infected by *Myxobolus* and exposed by spore Protein Spora dose 600 µl/fish (K4)	$96.00^{a} \pm 2.46$

Description: different Superscript on the same column indicates the existence of the real difference ($p < 0.05$)

3.7 Water quality of maintenance medium

Water quality is the most important factor in fish farming. Water quality is affected by a variety of chemical substances that are dissolved in the water, that are temperature, pH, dissolved oxygen and ammonia.

4. Discussion

The results of the identification of spores shows that *Myxobolus* sp. obtained on goldfish gills in this research is *Myxobolus* sp. nodule samples obtained subsequently are made into protein samples by way of physical breakdown by crushing and disonicating to break down the cell. Sonication can be used to speed up the separation of particles in the sample by breaking Intermolecular interaction and then centrifuged with high speed and enough time to separate between the spores, nodules chains, and spore protein, and nodule protein. Sentrifus is a tool that is used to rotate the sample at high speeds and forced heavy particles accumulated into the base tube of sentrifus.

Crude protein concentration of nodules containing spores of *Myxobolus* sp. which is measurable by using UV-Visible Spectrophotometry is 5116.25 µg/ml. States that the lowest concentration of protein is needed for protein analysis of 1.2 µg/ml, so it can be inferred that the crude proteins of *Myxobolus* sp. protein analysis can be conducted using SDS-PAGE electrophoresis.

Stacking gel SDS-PAGE technique used was 3% and separating gel 12 % and being run on with 200 Volt voltages and 2 mA. This study used separating gel 12% protein because the spores of *Myxobolus* sp. ranged between 7 – 130 kDa. The protein which has a molecular weight of 20 – 200 kDa packed used separating gel 12%.

The results of the SDS-PAGE in this study showed 5 bands of protein with molecular weight (BM) 41.1 kDa, 51 kDa, 89.8 kDa, 121.7 kDa and 230.1 kDa. Protein band results obtained is not yet

known which the spore proteins and band proteins are and which the protein from tissue nodules is. The protein form spore of *Myxobolus cerebralis* has BM 7 kDa, 45 kda, 60 kDa and 130 kDa. Therefore, on this research protein bands with BM 41.1 kDa, 51 kDa, 89.8 kDa and 121.7 kDa and 230.1 kDa are crude of proteinof *Myxobolus* sp. which most likely is a highly immunogenic protein

Changes in the total number of leukocytes and erythrosit can be used as an indicator of the existence of the attack of infectious diseases in fish. Leukocytes in blood components is one that serves as a specific body defenses that will neutralize and destroy pathogens through phagocytosis. Lymphocytes is one form of leukocytes. figure 3 shows the percentage of lymphocytes in each treatment. The observations of lymphocytes showed the highest percentage of 86.37% in the treatment of K3 (not being exposed and infected with 80 spores/fish of *M. koi*) when compared with the treatment of K1 (control) of 73.12%. The increased number of lymphocytes in the fish infected with spores of *M. koi* is the body's defense system response of fish over the entry of pathogens. The antibody-producing lymphocytes function as immune to interference from disease.

Lymphocyte cell consists of two populations i.e. B cells and T cells. B cells have the ability to transform into plasma cells i.e. cells that produce antibodies. T cell immunity is as instrumental in cells interaction (cytotoxic T cells) and control the immune response (suppressor T cells). After the binding of an antigen with antigen receptor cell lymphocytes, lymphocyte cells will then divide and differentiate into effector cells and memory cells.

Monocyte percentage of each of the treatments is shown in Table 3. There was a decline in the number of monocytes in ttreatment K4 i.e. 9.24% compared with K1 (control) i.e. 10.25%. The decline was caused by the monocytes which fagositosised spores of *M. koi* that went in the body of the fish. This is in accordance with the opinion that the monocytes with macrophage will fagosit agents causing deseases that enter the body. The monocytes function as phagocytes against foreign objects which act as the agent of the disease.

The amount of heterofil in treatment K2, K3 and K4 has decreased when compared with the treatment of the K1 (control) as shown in Table 1. This is due to the increase in lymphocytes and monocytes, so heterofil is declining. Cell heterofil not do not play a role mainly in responding to an infection caused by a parasite and is more instrumental in infections caused by bacteria.

The results of the calculation against the number of eosinophils demonstrated the highest percentage on K3 treatment of 6.71% compared with treatment K (control) of 3.65%. Spore infection of M. koi increases the number of eosinophils in the blood of fish. The eosinophils is one of the body's defense cells that are dominant in the blood and will increase sharply in number in case of infection disease parasiter.

The results of the calculation showed that the lowest percentage basophils is 2.23% in K1 (control), then treatment K4 also shlows low value namely 2.53%. Tthe percentage of basophils in the blood of fish ranged from 0.17-0.194% and measuring 8-12 μm. The existence of basophils in blood circulation has been observed only in a small number of species of fish. Even more rarely basophils are found on examination of blood compared to eosinophils. The basophil granules contain eosinophils chemotaxis factor and type 1 hypersensitivity mediator when there is stimulation from allergens that cause the occurrence of snapping allergens on basophils, the content of basophils will release.

The results showed that spore proteins of *Myxobolus koi* were able to protect (protective nature) koi fish against myxobolusis. This is evident by the presence of increased survival of carp from 20.00% up to 83.00% to harvest takes place, so that it can be interpreted as a decline of the death of goldfish.

The results of measurements of water quality: temperature, pH and dissolved oxygen on maintenance as indicated in Table 3 show the average temperature of the medium maintenance of each treatment was about 27-28 ° C, pH of water 8 and dissolved oxygen of 5 ppm. It indicates that the condition of water quality of maintenance medium is still in normal conditions for the survival of the fish. The goldfish can live on temperature range 8-30 ° c. The levels of ammonia (NH3) before and after the treatment showed a difference in which ammonia before treatment of was 0 ppm while the after treatment was 0.5 ppm. The content of ammonia in the maintenance media tends to high caused by the presence of remnants of feed and fish metabolism excretion results.

5. Conclusion

5 profiles of Crude spora protein of *Myxobolus koi* are retrieved with molecular weight of 41.1 kDa, 51 kDa, 89.8 kDa, 121.7 kDa and 230.1 kDa. Crude spore Protein of *Myxobolus koi* can be developed as sub unit vaccine ingredients to prevent the death of goldfish, because it can improve the immune response of carp. Crude spore Protein *Myxobolus koi* can give protection to carps, so it can improve the survival rate of 20% up to 85%.

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