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Potency of Cell Wall Protein of Pasteurella multocida as Hemorrhagic Septicemia Vaccine on Swamp Buffaloes

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ABSTRACT

Vaccine testing of cell wall protein (Pasteurella multocida) aims to control hemorrhagic septicemia in (swamp buffalo) carried out at the Microbiology Laboratory of the Faculty of Agriculture, Lambung Mangkurat University, and Laboratory of Molecular Genetics, Faculty of Saint, Airlangga University. For field testing, it was carried out in Tampakang Village, Hulu Sungai Utara Regency and Banua Raya Village, Bati-Bati, Tanah Laut Regency. Safety tests using experimental animals (mice and swamp buffalo). The serum obtained was tested serologically using Enzyme-Linked Immunosorbent Assay (ELISA). 100% vaccine safety test for mice and buffalo live without clinical symptoms as the characteristics of animals affected by hemorrhagic septicemia. With the ELISA method, the result of vaccinated swamp buffalo is positive antibody formation by seeing the increasing Optical Density (OD) value ƛ 450 ƛm, before the vaccine 0.292 after vaccination 0.748 and 1.576 and 1.821 after the booster. Based on observations in the laboratory and in the field, both safety tests and potential test cell wall protein vaccines Pasteurella multocida of local isolates can be used to prevent hemorrhagic septicemia that attacks swamp buffalo, both laboratory and field scale.Keywords: Vaccine candidate, Pasteurella multocida, safety test, ELISA test.

***Key words**: Vaccine candidate, Pasteurella multocida, safety test, ELISA test*

INTRODUCTION

Constraints faced by buffaloes in South Kalimantan vary, such as reduced grazing land which results in a reduced availability of natural food which causes buffalo body defenses to be reduced so that it is susceptible to disease, one of these diseases is Pasteurellosis caused by the bacterium Pasteurella multocida (P. multocida) and its variants. This bacterium can cause serious illness; this organism can infect many wild and domestic animal species, and can cause acute or chronic diseases (Alwis, 1992) in Herliani and Sulaiman (2011).

Pasteurella multocida is the main causative agent of SE disease in buffalo, pigs, goats, deer and camels caused by serogroups Pasteurella multocida A, B and E; Fowl Cholera (FC) in poultry caused by Pasteurella multocida sero-group A (Eriksen et al. 1999; Wilkie et al., 2012), in addition Pasteurella multocida is also a major contributor to several respiratory disorders of Bovine Respiratory Disease Complex (BRDC) in cattle, and pneumonia in pigs and sheep (Gyles et al., 2011).

One effort to control bacterial infection *Pasteurella multocida* is through prevention efforts using vaccines. Various vaccines have been tried with varying degrees of protection (Carter *et al*., 1991 and Mosier, 1993 in Herliani and Sulaiman, 2011). In Indonesia, vaccine killed broth Bactria put into use from 1910-1970 (Syamsudin, 1993). There are currently several types of anti-vaccines *Pasteurella multocida* on the market, but the ability to protect is limited to only a few weeks (Supar and Tati Ariyanti, 2007). *P. multocida*

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is a pathogenthat still needs a lot of study. This is caused by the variety of diseases caused, as well as the number of host species affected, and the way the infection is not yet understood (Wilkie *et al*., 2012).

Recently, research on *P. multocida* has been widely reported, for example regarding virulence genes, and the nature of *P. multocida* as commensal, opportunistic, primary pathogens and metabolites as determinants of pathogenicity to the host (Gyles *et al*., 2011). *P. multocida* as a commensal bacterium can survive for a long time and reproduce without causing infection in the host (Diallo and Frost 2000). However, *Pasteurella multocida* commensal can also infect a host when the host is under stress, changes in the expression patterns of virulence factors in *Pasteurella multocida*, and a decrease in host immunity due to other microbial infections (bacteria, viruses or parasites). As a result *Pasteurella multocida* is able to reproduce rapidly in the upper respiratory tract and cause *Bovine Respiratory Disease Complex* (BRDC), despite the strong suspicion that Mannheim hemolytic as a predominant pathogen to BRDC (Griffin I. 2010; Kubatzky 2012).

Research into vaccine development beginning in the 1990s showed that the vaccine *P. multocida* local isolate provided better protection against homologous and heterologous strain testing in cattle and buffaloes (Ramdani, 1997). Similar results can be seen that the inactivated vaccine *P. multocida* local isolate from ducks can provide better protection compared to imported strains against homologous and heterologous strains in experimental ducks (Supar *et al*., 2001a, b; Herliani and Sulaiman, 2010).

Vaccination using live vaccines from the bacterium *Pasteurella multocida* apparently can increase the immune response that is high enough to somatic antigens and can increase resistance to infection given challenge. Vaccines are administered by aerosol, subcutaneous and intradermal (Mosier, 1993). Infected animals or infected animals are the main sources of infection for nearby animals. In addition, pasteurellosis can be contracted from latent infections in stressed animals. Stress factors other than due to weather changes and mismanagement are also often closely related to stress due to long-distance travel from the area of origin of livestock to abattoirs. (Crater and Alwis 1989; Alwis, 1981; Alwis and Vipulasiri, 1980).

Research on vaccines has been carried out, but no vaccine has been used for a long time. this might be due to the vaccine being used that is not protective, due to differences in antigenic properties between the isolates used with isolates in the field (which infect livestock) in accordance with the statement (Syamsuddin 1977 and Sommerset, *et.al*., 2005) the ideal vaccine must meet several requirements that are determined including immunogenic and protective, safe, stable and effective and efficient.

In South Kalimantan the vaccination was carried out with an inactivated *P. multocida* Katha strain from Myanmar. This vaccine is widely available in the market, but there are many variations in quality, efficacy or efficacy, and the composition of the vaccine used. Inactivated vaccines with oil adjuvants are very concentrated, making it difficult to apply, other than that the storage time is relatively short because they are easily damaged at room temperature, and often cause adverse local reactions.

METHODOLOGY

The isolate bacteria that founded in prior experiment in 2015 was used in this experiment. The bacteria was stored in the microbial laboratory of the Agriculture Faculty ULM, before it was cultured in BHI agar media. The bacteria isolate was identified based on phenotype character ie morphology, biochemistry, and physiology. Morphological observation refer to Cappucino and Sherman (2001).

ISSN: 2354-5844(Print)
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Preparation of Cell Wall Protein

Preparation of *Pasteurella multocida* cell-wall protein was based De Boer dan Schaad method (1990).

Safety Test of HS Vaccine

Eight mices were vaccinated by *intraperitoneal* with 0.5 ml vaccine per mouse. Other 4 mices were not vaccinated as control, and then all mouses were observed for 7 days. Two buffaloes about 4-5 months of age with body weight around 30 kgs were inoculated by intramuscular (IM) vaccine with minimal dose 4 ml and the observed for 21 days. Another buffalo was not vaccinated as the control also observed for 21 days. The vaccine is accomplished the standar if the vaccinated mices and the vaccinated buffaloes, also the controls are not shown clininical dissease symptoms.

Potency Test of Vaccine

Two buffaloes about 4-5 months of age with body weight around 30 kgs were inoculated by intramuscular (IM) with vaccine that made in 1st year experiemet with minimal dose 4 ml (BW up to 40 kg = 4 ml) and the observed for 14 days. After 14 days, the buffaloes were given boostering vaccination

Measuring of Antibody Titres

Measuring of antibody of blood serum samples was conducted by *Enzyme –Linked immune Sorbent Assay (*ELISA) method that develoved by Biology Department of UNAIR.

RESULTS AND DISCUSSION

Result of staining Pasteurella multocida bacteria from SE infected buffaloes seen on Figure 1. Identification of biochemistry refers to Cappucino dan Sherman (2001). Results of bacteria test were seen on Table 1. Based on the test, it can be concluded that sample bacteria was stored in the microbial laboratory of the Agriculture Faculty ULM and tested its pathogenesis on Balb C mice was Pasteurella multocida bacteria, this finding confirms to statement of De Alwis (1992) end Woolcock (1992), that Pasteurella multocida is bacteria with shorth stick form, size 1 – 2 µm X 0.3 – 1.0 µm, and gram-negative.

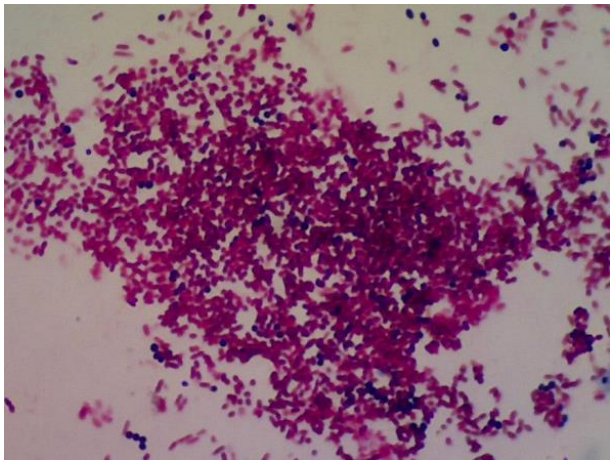

Figure 1. Gram staining of P. multocida bacteria in those isolated from lung organs 100x enlargement.

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Table 1. The results of testing the biochemical and physical properties of *Pasteurella multocida* bacteria

Characterization	Results			Information
	Plate 1	Plate 2	Plate 3	
Gram	Negative	Negative	Negative	The bacteria looks red
Form	Coccobacillus	Coccobacillus	Coccobacillus	Bacteria are small rods and both ends are rounded
Spores	Negative	Negative	Negative	Under a microscope, bacteria appear to be entirely blue
Media Mac Conkey	Grow	Grow	Grow	The bacteria form small, round, and serrated colonies
Hemolysis	Negative	Negative	Negative	In blood agar media, faded red does not occur
Motility	Negative	Negative	Negative	Bacteria only grow on puncture marks
O/F	Positive	Positive	Positive	Change media color from green to yellow
Glucose	Positive	Positive	Positive	Change media color from red to yellow
Lactose	Positive	Positive	Positive	Change media color from red to yellow
Monnitol	Positive	Positive	Positive	Change media color from red to yellow (longer time)
Indole	Positive	Positive	Positive	Red ring is formed
Urease	Negtive	Negative	Negative	Not change media color
Oxidase	Positive	Positive	Positive	the color on the oxidation stick turns blue
Catalase	Positive	Positive	Positive	Seen bubbles on the media after adding H_2O_2 solution

By giemsa staining of blood couting glass, the bacteria was seen bipolar, capsuled, and not motile. The bacteria have characteristic anaerobic facultative, easy grow on simple (basal) media, but it will grow rapidly with riched media by serum or blood. Growing colony in media agar inhibited hemolysis of red blood, the colony have characteristic round with plate periphery, concave elevancy, greyish color, diameter 1-3 mm and with specific odor.

Result of Safety Test

For safety testing, the results show that during experiment all mices and buffaloes still life without any clinical symptoms such as rainy noses, red eyes, swollen neck area, apathetic body, or increasing temperature, it is simmilar to report by Priadi dan Natalia (2002). The body temperature observed during experiment was around 38^0 – 39^0C. This indicate that the vaccine is safe to use in treated animals, with dose 10 ul for mices and 4 ml for buffalo, the results can be seen on Table 2 and Table 3.

ISSN: 2354-5844(Print)
ISSN: 2477-5223 (Online)

Table 2. The data of safety test on balb C mice.

Treatments	No	Clinical Symptoms	Dead Mice						
			1	2	3	4	5	6	7
Control Mice	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
Vaccinated Mice	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
Results		All (100%) treated animals (Balb mice c) live and have no symptoms of hemorrhagic septicemia							
Result Value		Vaccines are safe to use							

Table 3. The Data Security Test on Swamp Buffaloes.

Buffalo	No	CS	Length of Observation (21) days																				
			1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Vaccinated	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Results			All (100%) treated animals (buffaloes) live and have no symptoms of hemorrhagic septicemia.																				
Result Value			Vaccines are safe to use																				

CS = Clinical Symptoms

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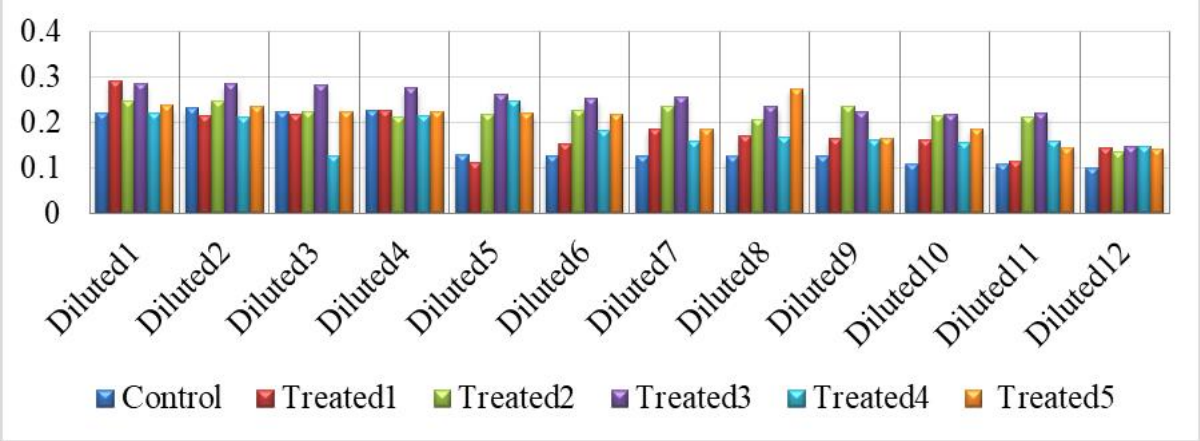

Figure 2. Swamp buffalo antibody titers before being immunized in various dilutions. C = negative control, P= Immunization treatments with cell-wall protein 100 ul, 1 - 5 = Replication

The Potency Test

By the potency test show that all treated buffaloes live and does not show any clinical symptoms. The potency test was conducted by ELISA serological testing, this because ELISA serological testing can detect sensitively even for view protein antibody in serum and needed only small serum (Gazela, 1980). ELISA testing can detect until less than 0.0005 ug protein per-ml antibody (Tizard, 1988). Serum was taken from *vena jugularis*, and then it was tested its antibody production.

Antibody titres were determined by ELISA technic. Antibody was diluted from dari 2^1, 2^2, 2^3 until 2^{12}. Testing of antibody titres by ELISA is more sensitive and specific because the ELISA technic based on reaction of antigens (Ag) dan antibodies (Ab). The antibody titres was determined at value of OD ƛ 595 ηm, in 3 time measurement (before immunization, 1st, 2nd, and 3rd of immunization).

The antibody titres of treated buffaloes before vaccinated (Ag of cellwall sel *P. multocida)* can be seen on Figure 2. The highest OD values are 0.292; 0.248; 0.286; 0.248; and 0.274. Otherwise, OD values before immunization was less than 2 time OD of negative control. This finding indicated that undeveloped yet of antibody against cell-wall of protein.

The antibody titre after 1st immunization was taken in after treated buffaloes immunated by Ag (cellwall protein of *Pasteurella multocida*), as seen on Figure 3. The high OD values are 0.719; 0.748; 0.734; 0.728 and 0.713. Otherwise, OD valuesafter 1st immunization was larger than 3 time OD negative control. This fainding indicate that 1st immunization can stimulate antibody formation against the cellwall protein. The binding between Ag and Ab still can be detected obviously at diluted 2^{-1} until 2^{-6}.

The antibody titre after 2nd immunization was determined after treated buffaloes immunated with Ag (cell wall protein of *P. multocida),* as seen on Figure 4. The high OD values are 1.313; 1.453; 1.576; 1,467 and 1.361. Otherwise, OD value safter 2nd immunization was larger than 6 time OD negative control. This fainding indicate that 2nd immunization can stimulate antibody formation against the cellwall protein. This finding also confirmed the finding Tizard (1988) mentioned that vaccinated animal can produce more antibodies.

ISSN: 2354-5844(Print)
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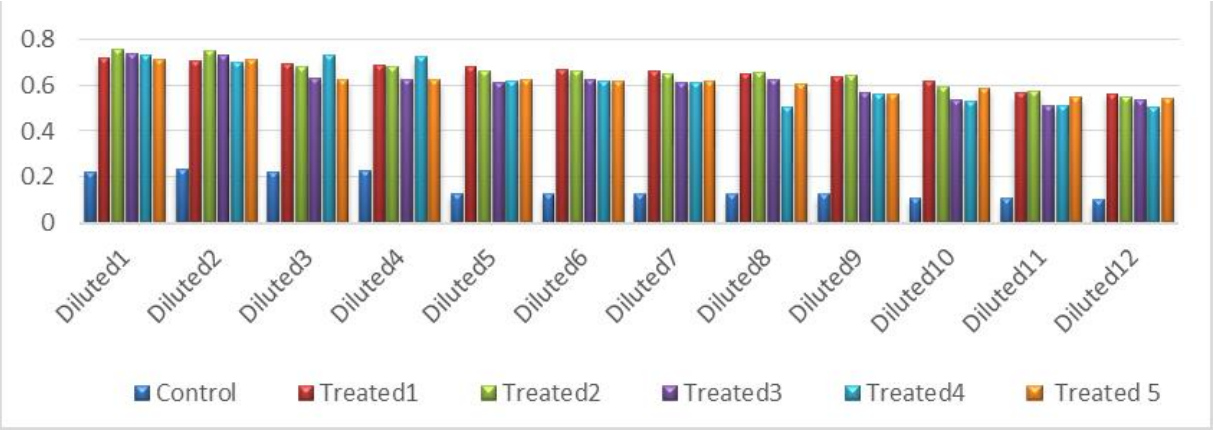

Figure 3. Swamp buffalo antibody titers after 1st immunization in various dilutions.
Note: C = negative control, P= Immunization treatments with cell-wall protein 100 ưl, 1 - 5 = Replication

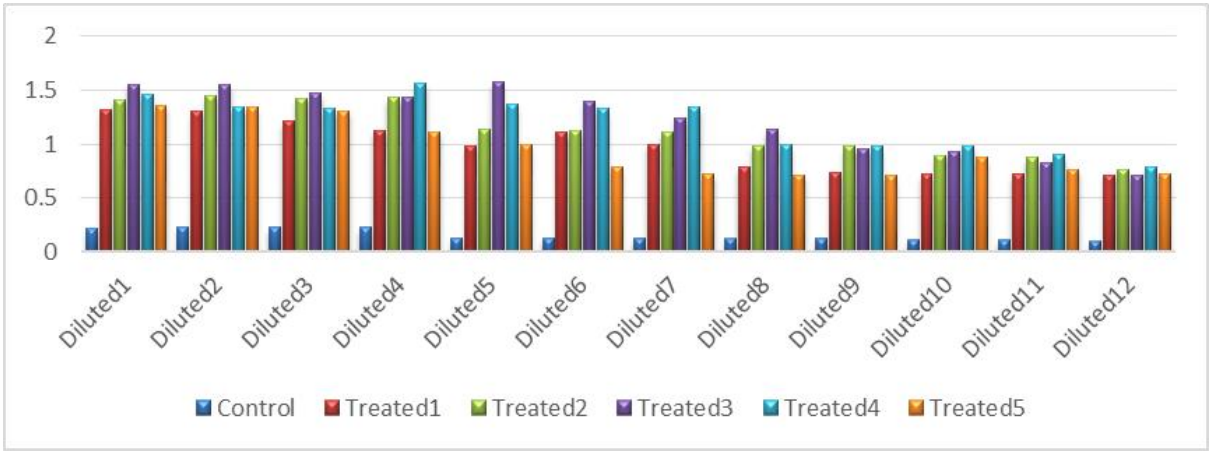

Figure 4. Swamp buffalo antibody titers after 2nd immunization in various dilutions.

Note: C = negative control, P= Immunization treatments with cell-wall protein 100 ưl, 1 - 5 = Replication

The antibody titre after 3rd immunization was determined after treated buffalo's immunated with Ag (cellwall protein of *P. multocida)*, as seen on Figure 5. The high OD values are 1.821; 1.752; 1.775; 1.673 and 1.741. Otherwise, OD value safter 3nd immunization was 0.220, larger than 7 time OD negative control. This fainding indicate that 3nd immunization can stimulate antibody sharply formation against the cellwall protein.

Anova test of antibody titres show that treatments affect significantly on antibody titres ($p < 0.05$), then further test was done by LSD. There are significant different among results of antibody titres, antibody

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ISSN: 2477-5223 (Online)

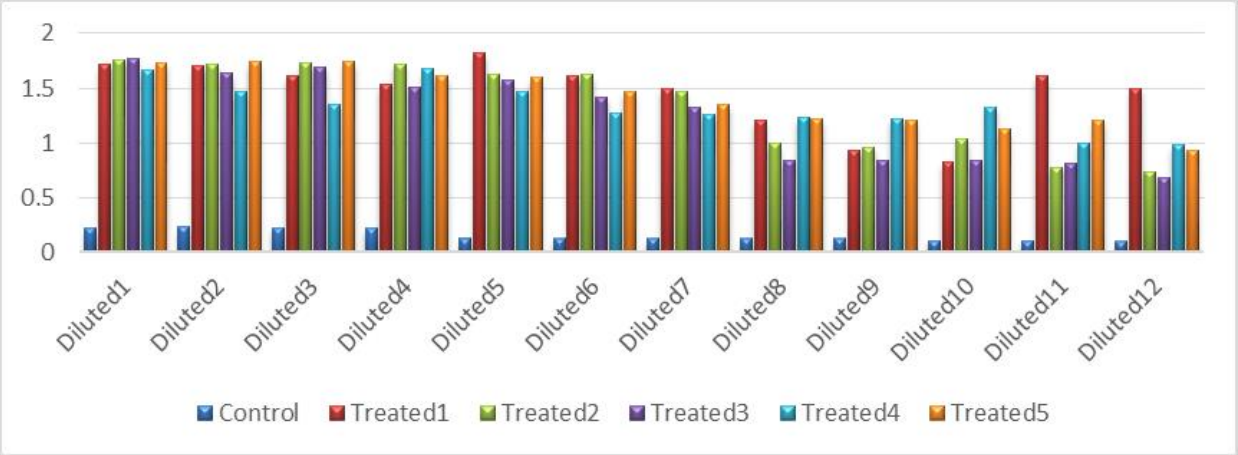

Figure 5. Swamp buffalo antibody titers after 3rd immunization in various dilutions. C = negative control, P= Immunization treatments with cell-wall protein 100 ul, 1 - 5 = Replication

Table 4. The Antibody titers in buffalo after immunization

Immunazation Time	Peak OD Values at					Mean ± SD	Repeated Dilluted at				
	1	2	3	4	5		1	2	3	4	5
Before Immunization	0.292	0.248	0.286	0.24	0.724	$0.268^{d} \pm 0.0230$	2^{-1}	2^{-2}	2^{-1}	2^{-5}	2^{-8}
1st Immunization	0.719	0.748	0.734	0.728	0.712	$0.6846^{c} \pm 0.0681$	2^{-1}	2^{-2}	2^{-1}	2^{-1}	2^{-1}
2nd Immunization	1.313	1.453	0.576	1.467	1.361	$1.434^{b} \pm 0.1020$	2^{-1}	2^{-2}	2^{-5}	2^{-1}	2^{-1}
3rd Immunization	1.821	1.752	1.775	1.675	1.741	$1.7528^{a} \pm 0.05$	2^{-5}	2^{-1}	2^{-1}	2^{-4}	2^{-2}

Hints : Numbers followed by different letters indicate there is significance at 5% tested with Anova and LSD

titre before immunization (0.2680) is significant different with antibody titres at immunization I (0.6846), II (1.4340) and immunization III (1.7528), as seen on Table 4.

The immunity system can differ between part of body system self and foreign thing entering the body. In general, the immunity system can divide into humoral immunity and seluler immnunity.

However, two mechanism of immunity can not separate one and another. The two systems can increase effectiveness each other, and interact to produce harmonic biological activities (Fenner dan Fransk, 1995).

Non-spesific immunity system is natural immunity system in the body but it has less protective system. All disease agents that entering into body will be destroyed this kind immunity because of non specific immunity of definite disease. In otherhand, specific immunity system consist of cell mediated immunity and antibody mediated immunity or well known as the humoral immunity system (Butcher dan Miles, 2003).

The humoral immunity sytem consist of antibody and fluid that are secreted from body organ. In otherhand, seluler immunity system is in form of macrophage, lymphosit, and neutrophil in cells. Antigens of cellwall protein *P. multocida* produce immun respons in the form of phagocytosis antigen by polimorphonuclear phagocyt cell or macrophage. If in the process of phagocytosis there are still antigens that are not yet phagocytic, then the antigen will

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stimulate a specific immune response to form antibodies.

Antibodies are produced by central lymphoid organs consisting of the bone marrow and thymus gland, especially by lymphocyte cells. There are two types of lymphocyte cells, namely B lymphocyte cells and T lymphocyte cells. These two cells work together to produce antibodies in the body. The antibodies and antigens have very specific relationships. This condition is seen when antigens enter the body. At that time, T lymphocyte cells immediately detect the characteristics and types of antigens. Then T lymphocytes react quickly by binding to the antigen through the surface of the receptor. After that, T lymphocyte cells divide and form clones. While the membrane surface produces monomeric immunoglobulin. Antigen molecules and antibody molecules bind to one another and the bonds of these molecules are placed on the macrophage. Sequentially, macrophages present antigens to B lymphocyte cells. Then, B-lymphocyte cells proliferate and become mature form, so that it can form antibodies againts to cellwall protein of *P. multocida* by cell B (Austyn dan Wood, 1994; Bellanti, 1993).

Increased antibody titers after immunization I, II, and III showed an immune response to antigens of the cell wall protein of P. multocida. Immunization with the same antigen increases higher antibody titer. This is supported by results of Goldsby *et al.* (2000) and Herscowitz (1993), after the onset of antibodies, biosynthesis of active antibodies begin, so that there is an increase in antibody concentration logarithmically, reached the highest antibody titer after 812 days. According to Austyn and Wood (1994), Goldsby *et al.* (2000), and Herscowitz (1993), the second exposure to the same immunogen will cause the addition of immune response in the form of imonocompetent cells and accelerated antibodies. In the secondary response, the latent period is shorter, the antibody synthesis rate is faster, the antibody titer peak lasts longer, the antibody power is higher, there are more memory cells and more IgG.

CONCLUSION

Based on the results of observations in the laboratory and the field, safety test or potential test for the vaccines of Pasteurella multocida cell-wall protein can be used to prevent hemorrhagic septicemia that attacks swamp buffaloes, both laboratory scale and field scale.

RECOMENDATION

Further research is needed to test potential in other areas, so that the vaccine obtained can be used for field testing (*in vivo*).

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