The 3rd ISTAP, Faculty of Animal Science, Gadjah Mada University, 2002 *ISBN : 979 – 97243 – 2 - 5*

DEVELOPMENT OF ROUGH MUTANT *BRUCELLA ABORTUS* VACCINE FROM LOCAL FIELD STRAIN TO ERADICATE BRUCELLOSIS IN INDONESIA

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Abstract

Brucellosis in cattle causes abortion and infertility. *B. abortus* strain 19 (S19) vaccine has been used to prevent bovine brucellosis. However, the S19 vaccine induces abortion in pregnant cattle and persistent infection after vaccination, which is indistinguishable by brucellosis surveillance testing from those productions by natural infection with field strains. The study reported here was designed to develop a rough mutant *B. abortus*, named strain RB27, which was derived from virulent local field strain. The RB27 was derived by repeated passage of smooth strain 158 on Trypticase soy supplemented with 1.5% bacto agar (TSBA) and varying concentrations rifampin. In order to stabilise the putative mutation, the RB 27 was *in vitro* passaged in guinea pigs as well as *in vitro* passaged on unsupplemented TSBA plates. The result showed that the RB 27 strain did not revert to smooth form and did not change in its virulence.

Key words: Brucella, Abortus, Mutant, Local strain, Vaccine, Rifampin

Introduction

Bovine brucellosis caused by *Brucella abortus* is a disease that causes abortion and reduces infertility in cattle (Enright, 1990) and causes severe illness in humans (Young, 1983). Prevention of this disease on cattle can be achieved by vaccination with *B. abortus* strain 19 (S19) (Nicoletti, 1990). However, since the initiation of the National Brucellosis Eradication Program in Indonesia using *B. abortus* vaccine S19 has not been intensively applied, the prevalence of brucellosis in cattle has not been drastically reduced.

S19 vaccine has two major limitation, those are, has ability to induce abortion when given to pregnant cattle (Nicolletti, 1990) and induces production of antibodies to the lipopolysaccharide O-side chain that are detected in serological tests (Sutherland and Searson, 1990). Consequently, it is difficult to interpret the results of the serological tests because the result of positive test may indicate the

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In order to stabilize the putative mutation, the RB27 organisms were *in vivo* passaged in Guinea pigs as well as *in vitro* passaged on unsupplemented TSBA plates. After several passages the strain did not revert to the smooth form. This indicating that strain RB 27 is stabled both *in vitro* and *in vivo*. The strain did not require CO_2 or blood in the medium for growth; this is similar to the other mutant strains such as strain 45/20 and RB51 (Schurig *et al.*, 1984 and Roop *et al.*, 1987).

Table 1. Ratio of spleen weight per body weight of Guinea pigs injected i.m. with $1x10^8$ CFU of RB27 at 1 through 5 weeks post inoculation

Weeks after inoculation	Mean spleen (gram)	Mean body weight (gram)	Ratio of spleen/ body weight (gram)
1	0,615	429	0,143
2	0,753	483	0,156
3	0,513	389,5	0,132
4	0,753	250	0,301
5	0,493	378	0,130
Mean	0,625	385,9	0,162

Table 2. Reactions of Guinea pigs to inoculation of RB27 strain and virulent *Brucella* strains

	Strain RB27	Strain virulence[a]
Enlarged spleen/ nodules	none	detected
Spleen weight	0,625 gram	>2 gram
Percentage spleens yielding *Brucella*	40%	>50%
Means colonies/gram spleen	84,5	>100
Mean spleen/body weight ratio	0,162	>0,2

[a] Source: Alton *et al.* (1967)

The virulence test was carried out to check that there has been no change in the virulence of the strain *B. abortus* RB27. The result showed that, after inoculation of this strain in the guinea pigs there were no enlarged spleen and/or nodules, the spleen weigh less than 1 gram and also the mean colonies per gram spleen about 100. According to the rough guide for interpreting that reaction, the strain RB27 did not virulent (Alton *et al.*, 1967). Therefore, RB27 would appear to be an excellent vaccine candidate.

Conclusion

B. abortus mutan strain RB27 is stable both *in vitro* and *in vivo* and did not change in virulence. Therefore, the result of this present study suggests that RB27

strain could be used as a vaccine candidate of brucellosis. Further study is needed to determine the efficacy.

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occurrence of natural infections of cattle with field strains of *B. abortus* or vaccination of cattle with *B. abortus* S 19 (Morgan, 1977 and MacMillan, 1990).

Recently, a rough mutant strain of B. *abortus* RB51 (SRB51) has been proposed for vaccination cattle of all ages (Steven *et al.*, 1995; Schurig *et al.*, 1991; Olsen 2000). Vaccination of calves with SRB51 did not induce abortion and infection (Cheville *et al.*, 1996). Moreover, this mutant vaccine did not induce positive antibody responses to current brucellosis serological surveillance tests (Steven *et al.*, 1994). However, this SRB 51 has not been available in Indonesia. The purpose of this study is to develop a rough mutant of *B. abortus* strain from local field strain and test its stability and virulence as a vaccine candidate for Bovine Brucellosis.

Material and Method

Derivation of Rifampin-Resistant Mutants of *B. abortus* 158

A rough mutant, strain RB27 was derived from virulent smooth *B. abortus* strain 158 with refer to the method reported by Schurig *et al.* (1991). The S158 was grown on Trypticase soy agar (Difco) + 1.5% bacto agar (TSBA) plates supplemented with rifampin in the concentrations of 25μg to 300μg at 37^{0}C with 5% CO_2 for 48 hour. Single colonies of RB27 showing the desired characteristics (rifampin resistance and rough colony morphology) were maintained on slant agar as a master seed stock.

Stability Test of Strain RB27 *In Vitro* and *In Vivo*

The strain RB27 was passaged *in vitro* 30 times on unsupplemented TSBA plates at 37^{0}C, 5% CO_2 approximately every second day of growth. Following each passage, colonies were inspected for roughness by auto agglutination in acriflavine. The *in vivo* passage was done by infected two guinea pigs with SRB27 intramuscularly (1x10^8 CFU) and killed a month later; colonies isolated from the spleen were pooled and reinoculated into 2 guinea pigs. This procedure was repeated until 10 passages were completed.

Virulence Test of Strain RB27 in Guinea Pigs

Test for the virulence of strain RB27 was done by injecting Guinea pigs intramuscularly in the thigh with 1x10^8 CFU of RB27 strain. The guinea pigs were then weighed and killed at 8 weeks post inoculation. Spleens were removed aseptically and weighed, and observed whether there are any lesions. Each spleen was ground in 10 volumes of diluents and 0.4 ml of the suspension inoculated onto each of 2 plates of TSBA plates. The plates were incubated for 48 hours at 37^{0}C. The number *Bruccella* colonies per gram of spleen were counted. The ratio of the spleen per body weight of each guinea pig was then calculated.

Results and Discussion

The utilization of rifampin on TSBA media to obtain a rough colony in this study based on the previous study that organisms resistant to rifampicin are less virulent than rifampicin susceptible strains (Moormant and Mandell, 1981). The *B. abortus* strain 158 was passaged 5 times on varying concentrations of rifampin resulted in the appearance of colonies which demonstrated rough Brucella (RB) colonies. Colonies of 48 h old culture of RB27 were stained with acriflavine and bacterial cells from these cultures autoagglutinated. Single colony that designated RB was passed further on various concentrations of rifampin, resulting in a clone, designated RB27 (Figure 1).

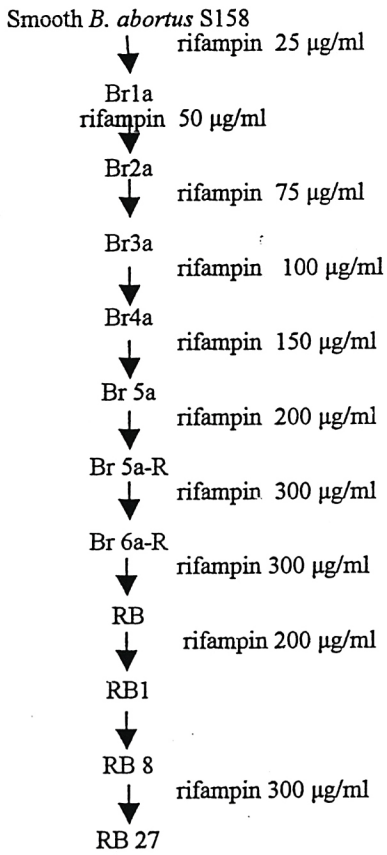

Figure 1. Derivation of B. *abortus* strain RB27 on TSBA agar plates supplemented with rifampin at the concentration shown.

extensively studied. Variation in the growth hormone gene has been reported in many cattle breeds (Hoj *et al* 1993; Lucy *et al* 1993; Sutarno, 1998), and recently Schlee *et al.* (1994) reported that different concentrations of plasma growth hormone related to different growth hormone genotypes of cattle. Mitochondrial DNA has also become another area of interest for studying the maternal inheritance of many traits in livestock, as well as in population genetic studies. Mitochondrial DNA polymorphism has been reported within and between breeds of mostly European cattle (Sutarno & Lymbery 1997), and recently, Sutarno *et al.* (2002) reported a significant correlation between mitochondrial DNA polymorphism and fertility in beef cattle. The aims of the study were therefore to: Investigate polymorphisms in the growth hormone gene, and analyse the association between growth hormone gene polymorphism and growth trait of Indonesian local cattle.

Materials and Methods

Experimental Cattle

The cattle used in this study were of Indonesian local cattle comprising of three different breeds, Bali cattle, Madura cattle and Ongole derived (PO) cattle. The samples were consisting of 50 Madura cattle, 50 of Bali cattle and 50 Ongole derived cattle.

Blood Collection

Blood was collected by venepuncture into a 50ml Venoject tube containing heparin as anticoagulant. About 10ml of this blood was aspirated and stored at -70°C for future reference. White blood cells were then isolated from the remaining 40ml of blood.

Isolation of White Blood Cells

Whole blood was dispensed into centrifuge tubes, and then spun at about 1500g for 15-20 minutes. The buffy coat was removed with a pipette, transferred to 10ml or 20 ml centrifuge tubes, topped up with TE-1 buffer and centrifuged at 2000g for 10-15 minutes. The pellet was resuspended in 1ml of TE-2 buffer, transferred to 1ml Nunc storage tube, and frozen at -84°C.

Extraction of Genomic DNA From White Blood Cells

The genomic DNA was extracted using Wizard genomic DNA purification system from Promega, as instructed by the manufacturer.

Genotyping

Polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP) (Hoj *et al.*, 1993; Schlee *et al.*, 1994) were used to detect polymorphisms in both of the growth hormone gene.

PCR Amplification of Growth Hormone Gene

Growth hormone locus 1 (GHL1), a 223 bp region spanning intron IV and exon V, and growth hormone locus 2 (GH-L2), a 329 bp region spanning exon III and exon IV of the growth hormone gene were amplified by PCR using primers GH1 / GH2 and GH5 / GH6 (Mitra *et al.*, 1995) respectively.

The primers used to amplify these fragments were:

GH1: 5'-GCTGCTCCTGAGGGCCCTTCG-3'
GH2: 5'-GCGGCGGCACTTCATGACCCT-3'
GH5: 5'-CCCACGGGCAAGAATGAGGC-3'
GH6: 5'-TGAGGAACTGCAGGGGCCCA-3'

All amplification reactions were performed in a 25µl reaction mix consisting of 200 ng of template DNA, 0.15 µM each of the oligonucleotide primers, 200 µM each dNTPs, 2 mM $MgCl_2$, 10x buffer and 1.5 units Taq DNA polymerase (Promega) in 0.2 ml PCR reaction tube.

RFLP Analysis

The PCR products were used directly in restriction enzyme digestion reactions. Amplified DNA of growth hormone locus 1 was digested with restriction endonuclease *Alu*I (Promega) to identify the *Alu*I site polymorphism, and amplified DNA of growth hormone locus 2 was digested with restriction endonuclease *Msp*I (Promega) to identify the *Msp*I site polymorphism. Electrophoresis was performed using horizontal gels, in electrophoretic cells (Bio-Rad, Richmond, U.S.A) for 90 minutes at 55 volts. Ethidium bromide was included in the gel at a final concentration of 0.12□g/ml. After electrophoresis, DNA was visualized under UV-illumination and photographed using Polaroid type 57 films with a red filter.

Association Analysis

Associations between molecular genotypes and quantitative traits were estimated from ANOVA model:

Model: $Y_{ijklmn} = \square + B_i + S_j + L_k + D_l + Y_m + G_n + e_{ijklmn}$

Where □ is the least square mean value, B_i is the effect of breed, S_j is the effect of sex, L_k is the effect of line (selected or control), D_l is the effect of the age of the individuals dam, Y_m is the effect of year of birth, G_n is the effect of haplotype and e_{ijklmn} is the residual error.

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