Locally bred rabbits (*Lepus nigricollis* Cuvier) for antibody production

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Abstract

*Locally bred rabbits (*Lepus nigricollis *Cuvier) have been used extensively as experimental models in Indonesia. Moreover, the rabbit is considered as a convenient host for antibody production. This study aimed to investigate whether the locally bred rabbit can be used for immunoserum production using available facilities. The animals were obtained from a local supplier and acclimatized in the animal facility. Live suspensions of common bacteria,* Escherichia coli *and* Staphylococcus aureus *were prepared as the antigens. Bacteria at various doses were given orally or injected intraperitoneally on day 1 and day 7. Rabbits without antigen induction served as controls. Blood samples were collected on days 0, 3 and 9 and processed into serum.*

Immunosera were incubated with the respective bacteria and allowed to agglutinate. Agglutination was observed directly and confirmed by microscopic observation. Agglutination scores and immunoserum titers were obtained. Immunosera from the control and baseline immunosera before antigen induction reacted positively to the bacteria. However, immunosera after antigen induction, especially from the intraperitoneal injection groups gave higher agglutination scores and higher titers compared with the control. Immunoserum and antibody production required bacteria-free laboratory facilities and animal hosts.

Keywords: Rabbit (*Lepus nigricollis* Cuvier), Immunoserum, Antibody, *Escherichia coli*, *Staphylococcus aureus*.

Introduction

Rats (*Rattus novergicus*) and mice (*Mus musculus*) are the most common animal models used in Indonesia for pharmacological and pharmacokinetic studies i.e. hepatoxicity of curcumin in Wistar rat[9] and distribution of radiopharmaceuticals in mouse cancer model[14]. Additionally, locally bred rabbits (*Lepus nigricollis* Cuvier) have been used extensively as experimental models in Indonesia. For example, locally bred rabbits have been used in toxicity tests[15] and in the field of pathological studies[18].

Rabbits are also preferable hosts for antibody or immunoserum production due to the large amount of blood that can be drawn frequently. Drawing 40 mL of a rabbit's blood in a one-month period does not affect their survival[2]. Mice and rats as a laboratory animal lack interest as hosts for immunoserum production because of their limited blood volume.

Meanwhile donkeys, sheep, goats and horses are used industrially but require advanced care and their maintenance is costly compared with that of rabbits[11]. Compared with sheep or goats, rabbits are more manageable in many developing countries with limited research facilities including Indonesia. New Zealand rabbits have been used for a long time in immunoserum production and are still used today[16]. Local rabbits are the result of cross-breeding for various type of rabbits that are not well documented and are widely found on local farms in Indonesia. These various breeds have disease resistance and heat tolerance[22].

The limited availability of immunoserum and antibodies in Indonesia has become an obstacle in research development. Most of the immunoserum products are still imported, so they have high costs and take quite a long time for shipping. Thus, to meet the demand for immunosera, an initial step toward the independent production of immunoserum is needed. Antibacterial immunosera can be produced easily by vaccinating a rabbit as the host animal[7,16,20].

This study was conducted to determine the utility of locally bred rabbits for immunoserum production in the available facilities. Rabbits were vaccinated with the bacteria orally or intraperitoneally and their blood was sampled and processed into serum. Oral antigen administration can induce the production of IgA and IgG immunoglobulins as responses to mucosal and systemic immunity through the activation of Peyer's patches on the intestinal lumen[13,26]. Intraperitoneal vaccination will cause antigens to spread rapidly into the major lymphoid nodules in the abdominal cavity, so that they can induce antibody production more efficiently[12].

The antigens used in this study were *Escherichia coli* and *Staphylococcus aureus* which are common bacteria. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains were chosen due to their non-pathogenic characteristics and ability to induce an immune response in the host animal[3,17,19]. The use of locally bred rabbits and common bacteria as antigens with low pathogenicity was within the capabilities of the facilities available for this study. The doses used in this study were 10^3, 10^5 and 10^7 CFU which are known to be able to induce an immune response in rabbits[8]. Antibodies may be raised two days after

vaccination and their serum levels increase rapidly in the blood on the second and third days[23,24].

Material and Methods

Bacterial strain: *Escherichia coli* strain ATCC 25922 and *Staphylococcus aureus* strain ATCC 25923 were used as antigens. *E. coli* strain ATCC 25922 is a non-pathogenic gram-negative bacterium possessing serotype O6 and is classified as biosafety level (BSL) 1[17]. *S. aureus* ATCC 29523 strain is a non-disease-associated gram-positive bacterium that is commonly found on the mammalian cutaneous layer and demands BSL 2 for handling[5].

Rabbit (*Lepus nigricollis* Cuvier) as the host animal: Male adult (8- to 12-week old) locally bred rabbits (*Lepus nigricollis* Cuvier) were obtained from a local farm in Yogyakarta (Imogiri, Bantul, Yogyakarta, Indonesia). The rabbits were then acclimatized for a week in the Experimental Animal Development Unit (UPHP), Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada (UGM) before study initiation. During acclimatization, the rabbits were placed in a steel cage (40 cm × 40 cm × 30 cm) inside the animal facility. The temperature and humidity were maintained at 27ºC and 80% respectively with a 12/12 hour light/dark cycle. Peanut leaves and mineral water were given *ad libitum*.

Preparation of bacteria as the antigen: Bacteria were grown in nutrient broth medium and incubated inside a shaking incubator at 37°C overnight with constant shaking (120 rpm). After incubation, the optical density was measured by using a spectrophotometer at a wavelength of 600 nm. The achieved density was then converted to a bacterial concentration by using the conversion factor OD_{600} 1 equivalent to 5×10^8 CFU/mL for *E. coli*[21] and to 1.5×10^8 CFU/mL for *S. aureus*[4]. The measured bacterial suspensions were then serially diluted in phosphate-buffered saline (PBS) until the desired concentrations of 10^3, 10^5 and 10^7 CFU/mL were reached. A final volume of 10 mL of each dilution was prepared.

Immunization and blood sampling: Immunization was performed on rabbits that were assigned to several groups depending on the dose variation and administration routes. The grouping of host animals (A–N) is shown in table 1. Each group consisted of 2 to 3 rabbits.

The rabbits were kept in the Experimental Animal Cage facility, Research Center for Biotechnology UGM. The cage was made from bamboo (80 cm × 50 cm × 50 cm in size) containing 2–3 rabbits per cage and was placed in a room (4 × 3 m) with a temperature and humidity of 25 ± 1ºC and 80% respectively with a 12/12 h light/dark cycle. BR1 *comfeed* rabbit and mineral water were given *ad libitum*. The condition of the rabbits was monitored daily every morning and evening by a general physical check. The biological waste was immediately disinfected with sodium hypochlorite before disposal. This study was approved by the Ethical Commission of LPPT UGM (certificate number: 00086/04/LPPT/XI/2018).

All rabbits in each group were treated according to the experimental design schedule shown in figure 1. Immunization was performed twice with a one-week interval and blood was drawn before the first immunization and two days after each subsequent immunization. Blood samples were collected in a blood tube without anticoagulant and kept overnight 4°C until fully coagulated.

Blood processing into immunoserum: Fully coagulated blood samples were then centrifuged for 15 min at 10,000 rpm to separate the serum from the remaining blood clot[25].

Table 1
Group design of host animals for immunoserum production

Tested Bacteria	Route	Dose per rabbit	Group
E. coli	Per oral	10^3 CFU	A
		10^5 CFU	B
		10^7 CFU	C
	Intraperitoneal	10^3 CFU	D
		10^5 CFU	E
		10^7 CFU	F
E. coli negative control			G
S. aureus	Per oral	10^3 CFU	H
		10^5 CFU	I
		10^7 CFU	J
	Intraperitoneal	10^3 CFU	K
		10^5 CFU	L
		10^7 CFU	M
S. aureus negative control			N

The obtained immunoserum samples were coded as follows depending on the blood sampling time: S0 = day 0 (before the first immunization); S1 = day 3 (after the first immunization); and S2 = day 9 (after the second immunization). The obtained immunoserum was then kept in a −20°C freezer until use.

Agglutination test for immunoserum titer: Immunoserum quality was assessed by reacting the serum with the bacterial suspension in PBS to detect the presence of antibody based on the agglutination reaction between the antibody in serum and its specific bacterial antigen[10]. A dilution series of serum samples (200 mL/well) was prepared in a 24-well plate. Each sample was diluted twofold in double rows horizontally until a 2048× dilution reached as shown in figure 2. One hundred microliters of 10^7 CFU/mL bacterial suspension was then added to each well containing the dilution series of serum samples. A mixture of PBS and the bacterial suspension served as the negative control while PBS without anything added served as the solvent control.

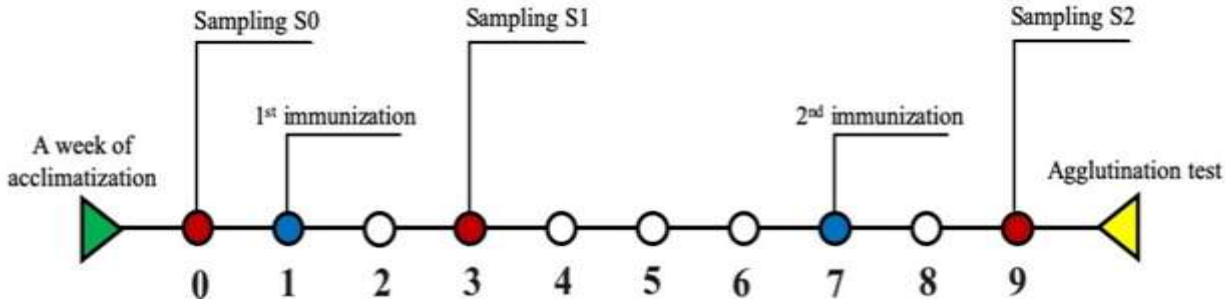

Figure 1: Experimental design for immunoserum production in rabbits. The figure shows treatment schedule of the experiment. Every circle represents a day, the red circle for blood sampling and the blue one represents immunization day. Rabbits were acclimatized for a week before the first blood sampling.

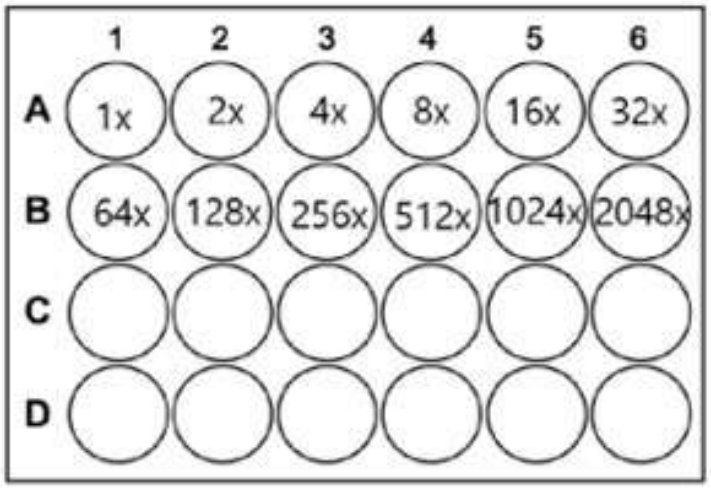

Figure 2: Immunoserum dilution chart in a 24-well plate

Table 2
Immunoserum titer and agglutination scoring chart[10]

Qualitative Data			**Semi-Quantitative Data**	
Immunoserum titer	Turbidity categories	Microscopic description	Agglutination score	Agglutination percentage
The highest dilution that still shows positively reacted serum forming agglutination	High turbidity	Large and many lumps appeared	4	100%
	Turbid	Slightly large lumps observed	3	75%
	Moderate turbidity	Small and many lumps observed	2	50%
	Slightly turbid	Few small lumps	1	25%
	Clear	No lumps detected	0	0%

Serum to which the bacterial suspension had been added was then incubated overnight at room temperature to enhance agglutination[1]. A positive reaction was characterized by the appearance of agglutination after the addition of bacterial culture. Agglutination was observed directly and confirmed microscopically at 10× magnification using an inverted microscope.

Data analysis: The obtained data were categorized as qualitative and semi-quantitative. The immunoserum titer was considered as qualitative data described as the highest dilution of the respective sample that still showed positive agglutination. Turbidity resulting from agglutination between the serum and bacteria was then scored according to the chart in table 2. Turbidity was observed directly and confirmed under a microscope. The score was converted to a semi-quantitative agglutination score adapted from Han et al[10]. The agglutination score was used to determine the limit of a serum dilution series that was considered as the titer value of the respective immunoserum.

Results and Discussion

Immunoserum production requires health maintenance of the host animal. Host animals needed to be acclimatized in a certain environment to minimize contamination that might induce unintended immune responses. Moreover, the caging environment should be controlled so as not to increase the stress level of the host animal. A stressful environment could lead to loss of appetite and cause premature death in the rabbit[6]. One problem that occured in this research was the early death of host animals, so additional batches were needed. The premature death of host animals was probably caused by stress due to environmental interference including light and sound. Therefore, caging conditions must be maintained in such a way as to prevent premature death.

The agglutination score is a semi-quantitative assessment of the results of agglutination that occurs between the antigen (bacteria) and antibodies in the serum (antibacteria). The greater is the agglutination, the higher is the agglutination score. Determination of the agglutination score was carried out by comparing the test group with the negative control and solvent control. The agglutination score was further confirmed by comparing the results of direct and microscopic observation. The representation of the agglutination score is shown in figure 3. Agglutination scores for each host animal group observed only on undiluted samples in the 24-well plate are presented in table 3.

The agglutination scores in the negative (untreated) control groups were fairly high. This could be caused by contamination or pre-exposure of the host animals to *E. coli* and *S. aureus* bacteria before they were used in the study. Moreover, cross- reaction between the rabbit immunoserum and antigen suspension could also produce false- positive agglutination. Immunoserum is indeed a polyclonal antibody preparation that allows other type of antibodies to react to the *E. coli* and *S. aureus* antigen epitopes at once by assuming that these two bacteria possibly share the same epitope on their surface. Therefore, contamination related to the bacterial preparation that was used for agglutination reactions must be minimized.

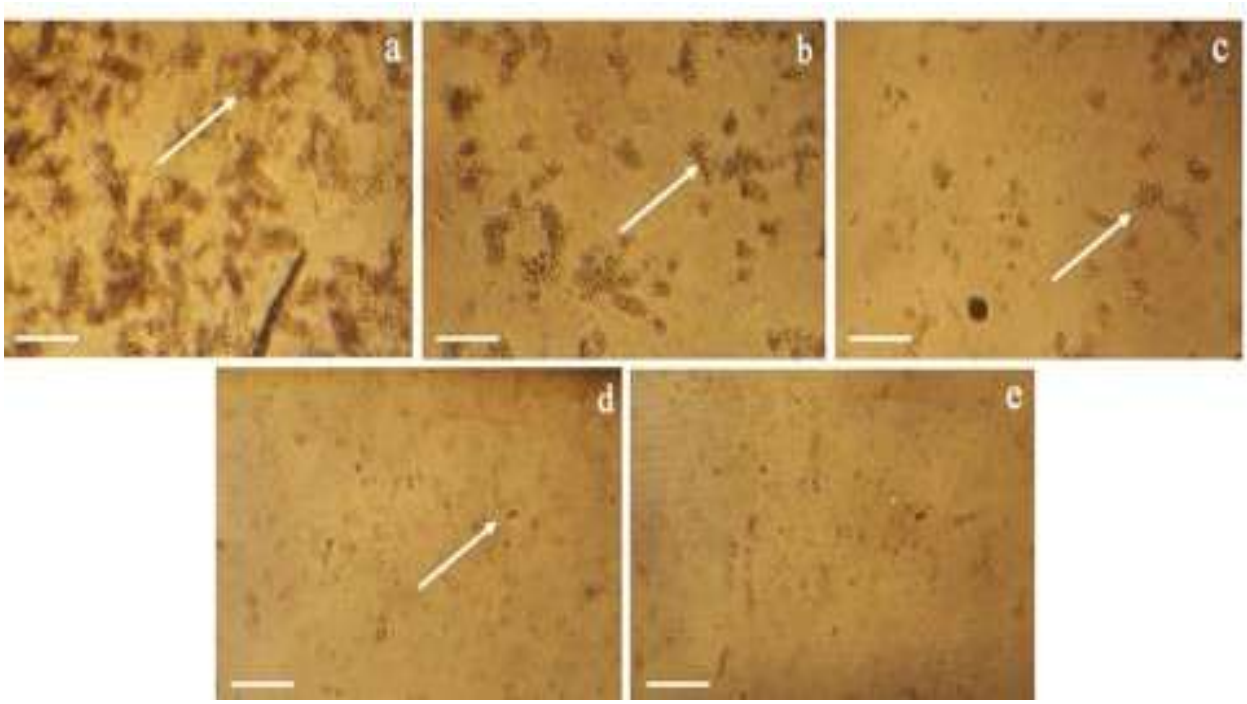

Figure 3: Visual representation of agglutination scores. The determination of agglutination score of reacted immunoserum and its respected antigen was confirmed by observation under an inverted microscope at 10x magnification. a, b, c, d and e represent score of 4, 3, 2, 1 and 0, respectively. The arrow points the agglutination. Scale bar = 100 µm

Table 3
Agglutination score of undiluted serum

Tested Bacteria	Administration Route	Dose per Rabbit	Group	Average of Undiluted Serum Agglutination Score*		
				S0	S1	S2
E. coli	Per oral	10^3 CFU	A	3	3	3.67
		10^5 CFU	B	3.33	3.33	3.33
		10^7 CFU	C	3.33	4	4
	Intraperitonial	10^3 CFU	D	3.5	4	4
		10^5 CFU	E	3	4	3.5
		10^7 CFU	F	3.5	4	4
E. coli negative control			G	2	2	3
S. aureus	Per oral	10^3 CFU	H	3	3	3
		10^5 CFU	I	3.5	4	3.5
		10^7 CFU	J	3.5	3.5	3.5
	Intraperitonial	10^3 CFU	K	3	3	3.5
		10^5 CFU	L	3.5	3	3.5
		10^7 CFU	M	4	3.5	3.5
S. aureus negative control			N	3	3	3

*S0, S1, and, S2 samples were obtained respectively on the day before first immunization, two days after first immunization and two days after second immunization.

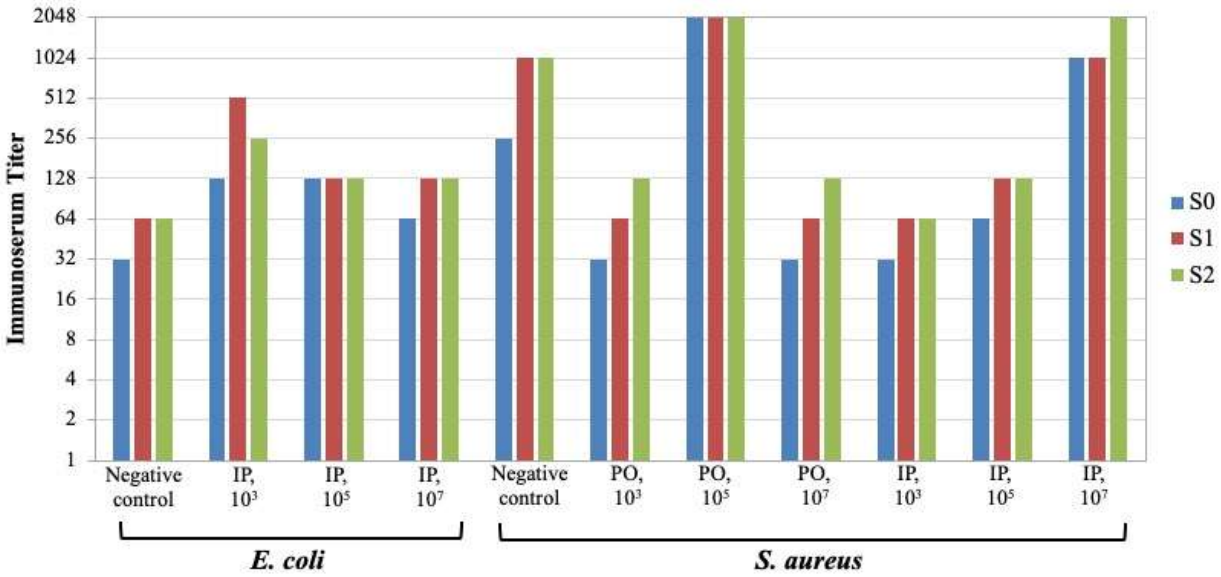

Figure 4: Immunoserum titer in various groups. Titer values were achieved from the observation on the agglutination reaction. Microscopic observation was conducted to confirm the obtained score data. S0, S1, and, S2 samples were obtained respectively on the day before first immunization, two days after first immunization and two days after second immunization. IP indicates intraperitoneal injection; PO indicates per oral administration. The dose for each group is listed under the respected group in CFU/mL

The bacterial suspension used in testing must be ensured to be free from other bacteria or any foreign substance, so that unintended cross- reactions between the undesired antigen and antibodies in the serum can be avoided. Further identification of the bacterial suspension to ascertain the presence of related antigens and possible contaminants could overcome the possibility of undesired antigen presence. This condition was a shortcoming of this study because the antigen was not rechecked before being tested. Agglutination scores from pre-treatment serum (S0) for each test animal in the treatment group were high (Table 3). Therefore, repeated antigen suppression of the test animal did not cause a significant difference in the S1 and S2 immunoserum compared with the S0 immunoserum.

The immunoserum titer was determined from the highest dilution which still produced a positive result for the agglutination reaction. The immunoserum titer value for each group of test animals is shown in figure 4. The immunoserum titer of the untreated negative control of the

host animal in S0, S1 and S2 blood sampling showed a high titer value. Based on this finding, it can be concluded that the negative control of the test animal had been contaminated by related antigens (*E. coli* or *S. aureus*). Contamination by these bacteria might unpredictably come from a variety of sources including food, drinks and animal feces, considering rabbits are coprophagous, meaning that they like to eat their own feces.

Immunoserum titer values of both the negative control and treated groups were relatively high. Treated groups before and after immunization with *E. coli* or *S. aureus* bacteria perorally or intraperitoneally at all doses showed similar results when compared with the negative control group. Therefore, this study has not been able to show differences in the effects of oral and intraperitoneal administration of bacteria in the production of immunoserum.

This study had several disadvantages including the use of the bacteria *E. coli* and *S. aureus* which are considered as common bacteria with a ubiquitous distribution in the environment. These bacteria live and grow in a supportive environment including food and beverages. Local rabbits that were used in this study had been exposed to related bacteria, so even the negative control group and pre-immunization blood sampling (S0) already produced anti-*E. coli* and anti-*S. aureus* immunoserum. To overcome this problem, it is necessary to use host animals and facilities maintained at the minimum, a semi-specific pathogen-free category.

An immunoserum production study also requires testing of bacteria before it is given to the host animals. This pretest is to ensure that the proposed antigen does not contain contaminants in the form of foreign matter or other undesired bacteria. This contamination could cause the unpredictable formation of antibodies to these contaminants. Tests on antigen suspensions before they are applied to host animals can be performed with bacterial detection test kits i.e. by PCR techniques.

Conclusion

Immunoserum production using locally bred rabbits (*Lepus nigricollis* Cuvier) could not be performed in the available facility in this study. Host animals were not standardized and using common bacteria (*E. coli* and *S. aureus*) might have interfered in the experimental system cause contamination.

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