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Immune response of rabbits after vaccination against Septicemia epizootica based on protein A matrix of *Staphylococcus aureus*

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ABSTRACT

Septicemia epizootica (SE), caused by *Pasteurella multocida*, is an acute infectious disease affecting livestock. The formulation of a *P. multocida* vaccine derived from Indonesian strains, and hyperimmune serum was developed using *Staphylococcus aureus* containing protein A as a matrix to enhance the binding between components. This study aimed to evaluate the humoral immune response in Hycole rabbits following vaccination with a vaccine formulated using a *Staphylococcus aureus* protein A-based matrix. Three types of inactivated *P. multocida* vaccines adjuvanted with Montanide ISA 70 M VG (Sepic®) were prepared in different formulations. (1) Vaccine with 5 parts *S. aureus* protein A suspension to 1 part hyperimmune serum, (2) Vaccine formulated with a ratio of 1 part *S. aureus* protein A suspension to 2 parts hyperimmune serum, and (3) Whole-cell *P. multocida* vaccine without matrix. A commercial SE vaccine was used as a control. A total of 16 rabbits were divided into four groups, including treatment and control. The vaccines were administered intramuscularly twice, with a two-week interval between doses. Antibody titers were measured using the ELISA method in the second week after the primary immunization and in the second week following the booster dose. The study results showed that all groups experienced an increase in antibody titers following vaccination, with varying levels of effectiveness. The commercial vaccine (vaccine 4) induced the highest immune response, followed by vaccine 1, which emerged as the most promising experimental candidate. Vaccine 2 demonstrated the lowest response, indicating that its effectiveness needs to be re-evaluated.

Introduction

Epizootic septicemia (SE) is known as a snoring disease caused by the bacteria *Pasteurella multocida* serotype B:2 (Lestari *et al*., 2025). SE is a major problem in tropical countries because it causes death in a short time (Chanda *et al*., 2024). In Indonesia, 1,156 cases of SE were recorded in Aceh between 2006 and 2010 (Nurliana *et al*., 2019). Meanwhile, SE disease occurs almost every year in Kupang Regency, East Nusa Tenggara (NTT), which causes a decline in the livestock population (Berek *et al*., 2015). Therefore, SE vaccination should be done routinely. *P. multocida* is a Gram-negative, bipolar, short coccobacillus-shaped bacterium (0.5–1.5; 0.25–0.5 mm) with rounded ends classified in the *Pasteurella*ceae family (Orynbayev *et al*., 2019). *P. multocida* originating from the 2016 NTT outbreak has been successfully isolated and has high immunogenicity potential as a SE vaccine agent (Noor *et al*., 2021). One approach in vaccine development is the use of *Staphylococcus aureus* Protein A as a carrier matrix to increase vaccine effectiveness.

S. aureus is a Gram-positive, coccus-shaped bacterium, shows positive results in catalase and coagulase tests, does not form spores, and is non-motile (Hornik *et al*., 2021). These bacteria are widely distributed in nature and are known as commensal bacteria (Krismer *et al*., 2017). *S. aureus* has protein A on its cell surface. Protein A in *S. aureus* has a high ability to bind the Fragment constant (Fc) part of Immunoglobulin G (IgG) in various animal species, so it has the potential to be used as a matrix for both antibody detection systems and in vaccine development (O'Seaghdha *et al*., 2006; Kurnia *et al*., 2024). Protein A-based vaccines from *S. aureus* assist the immune system by increasing the efficiency of antigen phagocytosis by immune cells such as macrophages or dendritic cells, facilitating the processing and presentation of antigen epitopes to the surface of phagocytic cells and activating the adaptive immune system, especially T cells, to develop immunity against *P. multocida* (Shi *et al*., 2021).

Protein A matrix-based vaccine consists of *S. aureus* Protein A (SpA), anti-*P. multocida* serum (AbPm) and *P. multocida* antigen (AgPm) (Mandelli *et al*., 2024). Protein A has a role in binding to Fc of rabbit serum anti-*P. multocida* and the serum will bind to *P. multocida* antigens, so it is expected that the *S. aureus* protein A-based matrix vaccine contains more *P. multocida* agents to induce an immune response against the cause of SE disease (Sulica *et al*., 1979). To support the stability and immunogenicity of the *S. aureus* protein A-based matrix vaccine, oil or aluminum hydroxide adjuvants are added (Meeusen *et al*., 2007). Evaluation of vaccination results is carried out by measuring the antibody titer formed using the Enzyme Linked Immunosorbent Assay (ELISA) technique (Aydin *et al*., 2025). This study aimed to determine the humoral immune response of rabbits to the SE vaccine based on *S. aureus* protein A matrix and to evaluate the effectiveness of its use by measuring the Optical Density (OD) value of the serum produced using the ELISA technique.

Materials and methods

Time and place of research

Research design included exploratory research, namely developing an SE vaccine using *S. aureus* protein A as a carrier matrix, as well as experimental research, namely injecting the protein A-based matrix vaccine into rabbits and observing the immune response using the ELISA method.

This research was conducted from March 2024 to March 2025 at the Genomics Laboratory of the National Research and Innovation Agency

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and the Animalium Science Center of PT. Mitra Global Animalia Cibinong, Bogor, West Java. The ethical permit for the research was obtained from the Ethics Commission of the National Research and Innovation Agency number 059/KE.02/SK/03/2024.

Preparation of vaccine materials and adjuvants

The vaccine materials used are *P. multocida* NTT bacterial isolate, anti-*P. multocida* NTT serum, *S. aureus* 2996C isolate, and Montanide ISA 70 M VG vaccine adjuvant (Sepic®).

Pasteurella multocida NTT

Reconstitution was carried out on the *P. multocida* NTT isolate from the Balitvet Culture Collection (BCC) archive and then re-identified following previous research procedures (Desem *et al*., 2023). Conventional identification includes morphological observation with Gram staining, subculture on Mac Conkey agar media, Triple Sugar Iron Agar (TSIA) Test and biochemical tests using the API 20 NE kit (BioMérieux, France). Molecular identification was carried out to target the *kmt* gene specific to *P. multocida* (Townsend *et al*., 1998). Whole-cell antigen production was carried out using the heat-killed method. The isolate was cultured in liquid Brain Heart Infusion (BHI) media which was incubated for 24 hours in an incubator shaker. The culture was then washed three times with phosphate-buffered saline (PBS) solution by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the resulting precipitate (pellet) was collected and inactivated by heating at 56°C for one hour in a water bath.

Serum hiperimun anti-P. multocida

The anti-*P. multocida* hyperimmune serum used was the result of production in previous studies. Detection of the homology of hyperimmune serum antibodies against *P. multocida* isolates was carried out using antigen-antibody reactions with the Agar Gel Precipitation Test (AGPT) (Amanu *et al*., 2017).

Staphylococcus aureus Protein A

In this study, *S. aureus* 2996C isolate from previous research archives was used as a vaccine matrix. *S. aureus* 2996C was cultured on blood agar media at 37°C for 24 hours. Bacterial cells were collected by centrifugation (9,000 rpm for 10 minutes), then the sediment obtained was washed three times using phosphate buffer saline (PBS, pH 7.4). Furthermore, the cells were suspended in PBS solution at a McFarland concentration of 4 and incubated for 1 hour at 60°C in a water bath and collected to facilitate the binding of previously produced IgG anti-P. mutlocida serum.

Adjuvant

Montanide ISA 70 M VG (Sepic®) is used to stabilize and increase the immunogenicity of the vaccine to be produced. The ratio used is 60 volumes of vaccine agent and 40 volumes of Montanide ISA 70 M VG.

Staphylococcus aureus protein A based matrix vaccine formulation

The vaccine preparations used in this study were 4 types. The SE vaccine based on the *S. aureus* protein A matrix was made with three different compositions, namely (1) SE vaccine with a protein A matrix code 5+1, (2) SE vaccine with a protein A matrix code 1:2. Meanwhile, 2 SE vaccines were also prepared for control and comparison, (3) inactivated SE vaccine *P. multocida* (code WC), and (4) commercial SE vaccine. To equalize it with the commercial vaccine, the adjuvant Montanide ISA 70 M VG was added to the four types of vaccines made.

Subculture of *S. aureus* and *P. multocida* bacteria was carried out in parallel for 18-20 hours then the pellets were collected after two washes with PBS. The bacterial culture was prepared with a concentration of 109 or equivalent to Mc Farland 4 then inactivated by heating. The SE vaccine matrix protein A code 5+1 was made by mixing 5 ml of inactivated *S. aureus* suspension with 1 ml of *P. multocida* hyperimmune serum (IgG) suspension. After incubation and obtaining the pellets, resuspension was continued using sterile Phosphate Buffer Saline (PBS) and the addition of inactivated *P. multocida* suspension [109 CFU] until 5 ml of adjuvanted SE vaccine volume was obtained. Meanwhile, the SE vaccine matrix protein A code 1:2 was constructed by making a 1:2 ratio between the volume of *S. aureus* and *P. multocida* hyperimmune serum. After incubation and centrifugation, the pellets were resuspended using PBS and re-incubated with *P. multocida* antigen. The addition of adjuvants to each vaccine was made using a ratio of 40:60, where 40% was adjuvant and 60% was SE vaccine material based on protein A matrix. While the SE vaccine without matrix only contained *P. multocida* suspension with Montanide ISA 70 M VG adjuvant.

Immune response

Each type of the four vaccines was injected into male Hycole rabbits aged 4-6 months with a body weight of 1.8-2.2 kg. Vaccine injection was carried out into the femoral muscle intramuscularly with a dose of 3x108 CFU per head or equivalent to 0.3 ml. Booster vaccination was given 2 weeks after primary vaccination.

Anti-*P. multocida* serum in rabbits was collected before primary vaccination, 2 weeks after primary vaccination, and 2 weeks after booster vaccination to be tested serologically. The ELISA test was conducted using an in-house ELISA method.

Data analysis

The data generated from the re-identification of the bacterial isolates used in this study were analyzed descriptively and presented in the form of figures and tables. The results of the *P. multocida* genome sequencing were analyzed using the Pathosystems Resource Integration Center (PATRIC), a web-based bioinformatics platform and the ELISA serology test results were analyzed using the EXCELL Workbook, ANNOVA or Kruskal Wallis programs.

Results

Culture and biochemical characteristics of P. multocida isolates

The results of the culture of *P. multocida* isolates on 5% sheep blood agar media showed that the NTT *P. multocida* isolate stored in freeze-dried form could still grow well and was confirmed as *P. multocida* using the API 20 NE Biomereux kit (Figure 1).

Molecular identification and Whole Genome Sequencing results

Detection of the presence of the species-specific gene *P. multocida* (*kmt*) using a pair of OIE standard primers showed a PCR product of 460 bp when electrophoresed on agarose gel. Amplification of the *kmt* gene in *P. multocida* isolates is presented in Figure 2. While the PATRIC analysis dendogram illustrates the comparison between *P. multocida* NTT and the reference isolate from GenBank in Figure 3.

Hyperimmune Serum anti-Pasteurella multocida NTT

Anti-*P. multocida* serum resulting from antibody production showed a precipitation reaction on agar gel media (Figure 4).

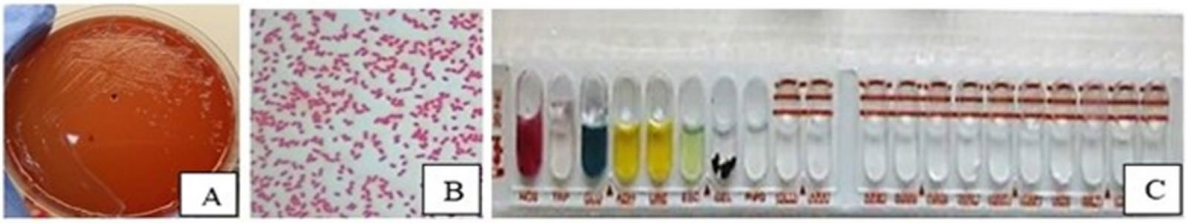

Figure 1. Reconstitution Results of *P. multocida* NTT isolates. (A) on 5% sheep blood agar media; (B) *P. multocida* Gram negative at 100X Magnification; and (C) Confirmation of *P. multocida* biochemical test with API 20NE kit (Biomereux)

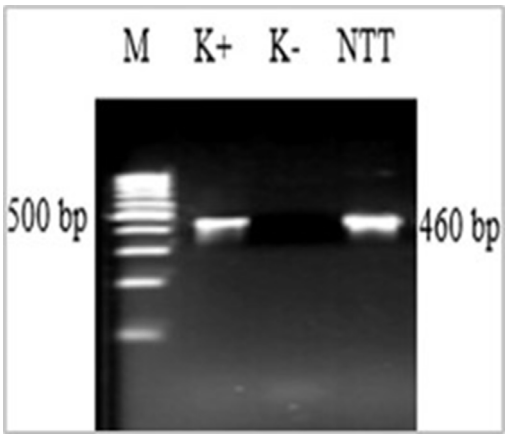

Figure 2. Specific gene amplification (*kmt*) of *P. multocida* NTT.

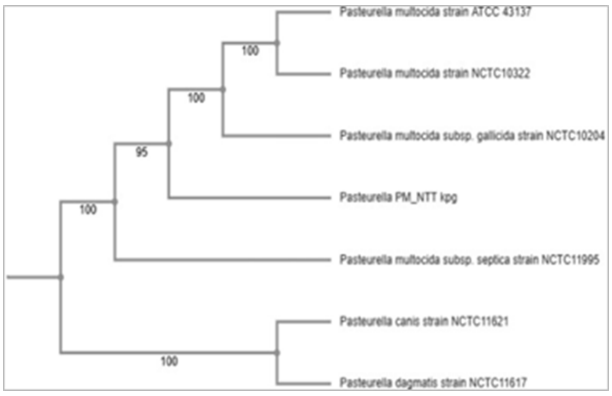

Figure 3. Phylogenetic tree from comparison of the *P. multocida* NTT genome with 6 *P. multocida* species from Genbank using PATRIC.

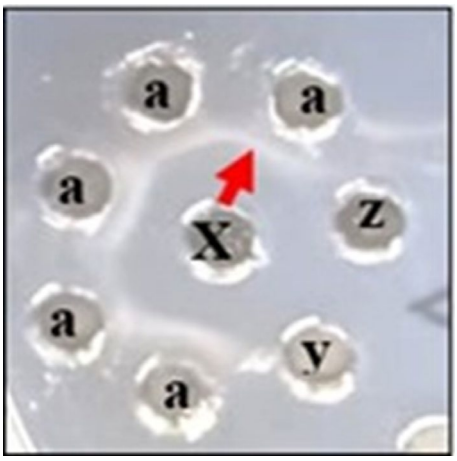

Figure 4. Precipitation line between *P. multocida* antigen (X) and antibody (a) is visualized and not formed between antigen and negative control (y & z) in Agar Gel Precipitation Test.

Re-identification of Staphylococcus aureus Protein A

Colonies grown on Baired Parker Agar (BPA) culture showed typical *S. aureus* morphology, namely round, black colonies with a clear zone around them. The Gram test showed Gram-positive coccus-shaped bacteria arranged like grapes.

The protein A detection test on *S. aureus* 2996C isolate was carried out using the Serum Soft Agar (SSA) method which gave compact colony growth results in SSA media when compared to protein A-negative isolates with diffuse colony growth (Figure 5).

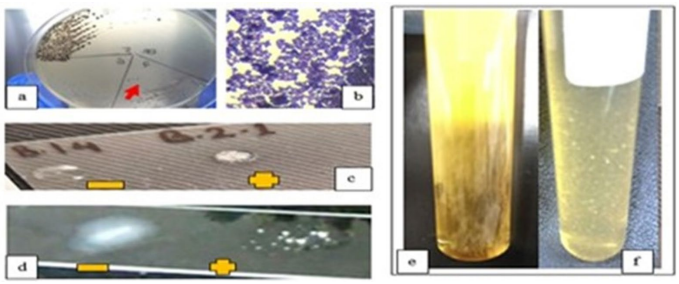

Figure 5. Identification of *S. aureus* is seen in: (a) BPA media; (b) positive Gram stain; (c) positive catalase test; and (d) positive coagulase test.

S. aureus was molecularly confirmed using the Polymerase Chain Reaction (PCR) technique by targeting the 23S rRNA, nuc, coa and spa genes (encoding protein A). The PCR products were then electrophoresed on a 1.5% agarose gel and visualized on a UV transilluminator. The resulting gel displayed amplicons measuring 1250 bp for the 23S rRNA gene, 279 bp for the nuc gene, 600 bp for the coa gene and 110 bp for the spa gene (Figures 6 and 7).

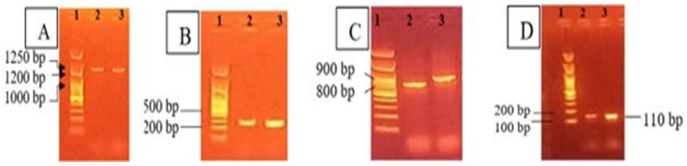

Figure 6. Visualization of *S. aureus* amplicons (column 3) on 1.5% agar gel with ATCC 25923 control (column 2) and 100 bp DNA marker (column 1): (A) 23S rRNA gene (1250 bp); (B) nuc gene (279 bp); (C) coa gene (800-900 bp); and (D) spa gene (110 bp).

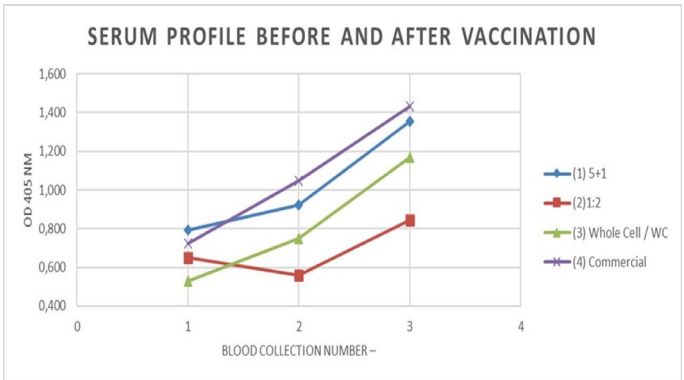

Figure 7. Optical Density (OD) ELISA values of anti-*P. multocida* serum rabbits treated with vaccines 1, 2, 3, and 4 before vaccination, 2 weeks after primary vaccination and 2 weeks after booster vaccination.

Discussion

P. multocida culture on blood agar media appeared as grayish mucoid colonies with a diameter of 1–2 mm, glistering like dew and does not hemolyze blood. On Mac Conkey agar media, *P. multocida* cannot grow because it is inhibited by the bile content in the media (Hart and

Champlin, 1988). Carbohydrate fermentation tests were carried out together with H2S formation tests on TSIA (triple sugar iron agar) media. The results obtained showed that the TSIA media that was pierced with *P. multocida* culture changed color from red to yellow at the base (butt) and slant, no gas was formed, no H2S was formed, oxidase and indole tests were positive and testing using the API 20NE kit (Biomereux) presented a numerical profile of 3000004 (% ID = 96%).

The taxonomic results of *P. multocida* NTT have a closeness of 95% with *P. multocida* subsp. *septica* strain 11995 and 100% with *P. multocida* subsp. *gallicida* strain NCTC 10204 and the *P. multocida* group strain NCTC 10322 and *P. multocida* ATCC 43137 in phylogenetic alignment. PATRIC is a web-based bioinformatics platform designed for the analysis of pathogenic microbial genomes, including bacteria and viruses (Wattam *et al*., 2014).

In Figure 4, anti-*P. multocida* serum in wells 1-5 contains antibodies targeting the antigen. A precipitin loop will form in the equivalence zone when the antibody and antigen diffuse through the gel. The precipitin line formed remains stable because the complex is too large to continue to diffuse through the gel (Deshmukh *et al*., 2020). These results indicate that there is a strong and specific bond between the antigen and antibody (Høiby, 2025).

S. aureus is a Gram-positive bacterium that is shaped like grapes when observed under a microscope. It has protein A on the surface of its cells which can be detected with Serum Soft Agar (SSA) media. The SSA method utilizes the properties of protein A which is able to interact with the Fc part of immunoglobulin G (IgG) in various mammalian species. When *S. aureus* containing protein A is cultured on soft agar media enriched with rabbit serum, it will show the presence of compact colonies (crystals). This indicates that bacterial growth is inhibited due to the bond between protein A and Fc IgG. Protein A has the ability to bind to crystallizable fragments (Fc) of immunoglobulin G (IgG) of various mammalian species (Choe *et al*., 2016). Conversely, if the *S. aureus* colony that grows in SSA medium appears diffuse, it indicates that the bacteria do not produce protein A, or protein A is covered by a bacterial capsule (Wolf *et al*., 2022). SSA is a method that has been proven to be able to detect the presence of protein A in *S. aureus* strains simply and economically.

The spa gene encodes *S. aureus* protein A. This protein is attached to the cell wall and has the ability to interact with several host components (Palmqvist *et al*., 2002). Protein A from *S. aureus* will bind to the Fc domain of immunoglobulin (Ig) and the Fab domain part will bind to the antigen (O'Seaghdha *et al*., 2006).

The results of rabbit vaccination using *S. aureus* protein A matrix-based vaccine in this study are presented in Figure 7. All four SE vaccines can induce antibodies in experimental rabbits. The SE vaccine based on *S. aureus* protein A matrix in this study showed that it can induce antibody responses and the vaccine formulation appeared better. The Kruskall Walis statistical test was used to analyze the Elisa results based on the sampling time (before, after primary vaccination, after booster vaccination) and showed a significant difference between the groups before vaccination and after the 2nd vaccination. However, the analysis results showed no difference in the results of the serum Elisa OD test against *P. multocida* based on the type of vaccine.

In this study, the prepared vaccine contains a bond between *S. aureus* protein A which functions as a matrix for the Fc part of *P. multocida* hyperimmune serum IgG. The Fab part of IgG will bind to the *P. multocida* antigen. The existence of a complex bond between the three components has been demonstrated by Kurnia *et al*. (2024) in a different study. The complex is the result of a binding reaction between the *Clostridium perfringens* toxin detection kit and the *C. perfringens* toxin produced on a laboratory scale (Neumann *et al*., 2021). After entering the body, the SE vaccine based on *S. aureus* protein A will stimulate the body's immune system by helping the phagocytic activity of dendritic cells (macrophages) in displaying *P. multocida* antigen epitopes to the surface of their cells (as Antigent Presenting Cells) (Karauzum and Datta, 2017). After the *P. multocida* antigen enters the body, it will be phagocytosed by dendritic cells. Next, the dendritic cells will process the antigen and display specific parts of the antigen called epitopes on the surface of the cell on MHC II (ten Broeke *et al*., 2013). In this case, dendritic cells act as Antigen Presenting Cells (APC) or antigen presenting cells (Guilliams *et al*., 2014). Epitopes displayed on the surface will be recognized by T cells in the adaptive immune system, which then triggers a specific immune response to *P. multocida*, namely the formation of antibodies and activation of other immune cells aimed at killing or neutralizing bacteria that cause SE disease.

Antibodies in this study are proteins formed from the immune response to vaccine antigens that enter the rabbit's body. Antigens recognized by the immune system will stimulate the formation of antibodies as a defense mechanism of the body. In general, the rabbit's immune system is not much different from the immune system in mammals. The organization of the rabbit's lymphoid system (eg, spleen, lymph nodes, appendix, and GALT) is very similar to that of other mammals, so rabbits are often used as models in immunology and polyclonal antibody production (Esteves *et al*., 2018). Rabbits have innate and adaptive immune systems that include B and T cells, as well as immunoglobulin production, like other mammals (Neave *et al*., 2018). This suggests that rabbits and other mammals have homologous basic immune mechanisms.

Conclusion

In this study, a prototype of the SE matrix vaccine consisting of *S. aureus* protein A, rabbit anti-*P. multocida* serum and *P. multocida* antigen was successfully created. The SE matrix vaccine prototype formulation of 5 parts by volume of *S. aureus* with 1 part by volume of anti-*P. multocida* serum with Montanide ISA 70 M VG adjuvant was able to induce antibodies in experimental rabbits after vaccination. The results showed that all groups experienced an increase in antibody titers after vaccination, with varying effectiveness. The commercial vaccine (vaccine 4) produced the highest immune response, followed by vaccine 1 as the most promising experimental candidate. Meanwhile, vaccine 2 showed the lowest response, so its effectiveness needs to be reviewed. The need for a longer observation period for the formation of antibody titers from the matrix vaccine results, as well as a challenge test on laboratory animals.

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Conflict of interest

The authors have no conflict of interest to declare.

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