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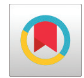
RESEARCH ARTICLE

Preparation of hyperimmune serum against multidrug-resistant *Escherichia coli* isolated from animal products in Bali and Nusa Tenggara, Indonesia: An epidemiological perspective



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ABSTRACT

Background and Aim: The global rise of antimicrobial resistance (AMR), particularly multidrug-resistant *Escherichia coli* (MDR *E. coli*), poses a significant threat to public health, animal health, and environmental safety. Traditional antibiotics are increasingly ineffective, necessitating alternative approaches such as immunotherapy. Hyperimmune serum represents a potential solution to combat MDR pathogens by providing immediate passive immunity. The present study aimed to produce and evaluate hyperimmune serum against MDR *E. coli* isolated from animal products in Bali and Nusa Tenggara, Indonesia, as a novel immunotherapeutic strategy against AMR.

Materials and Methods: Five MDR *E. coli* isolates resistant to eight antibiotics were obtained from the Quality Testing and Certification Center for Animal Products in Bogor, Indonesia. Isolates originated from Bali, West Nusa Tenggara, and East Nusa Tenggara. Bacterial isolates were cultured in Yeast Tris (YT) broth medium supplemented with antibiotics. Bacterial proteins were extracted, solubilized, and emulsified with Freund's complete and incomplete adjuvants. A 2-year-old horse was immunized with prepared antigens through intramuscular injections over 6 weeks, followed by intravenous administration. Serum was collected pre- and post-immunization. Confirmation of the hyperimmune serum's specificity and neutralizing capability was performed using an *in vitro* agar plate test.

Results: Successful growth of MDR *E. coli* isolates was confirmed in antibiotic-supplemented media, whereas no growth was observed in the antibiotic-sensitive native control. Hyperimmune serum obtained post-immunization exhibited strong *in vitro* specificity, recognized homologous MDR *E. coli* isolates, and formed significant neutralization zones (5–6 mm) around agar diffusion disks. Pre-immunization serum showed no reactivity, demonstrating effective production of specific antibodies. The neutralization zones were slightly smaller than those achieved with chloramphenicol control, indicating effective but slightly reduced bactericidal activity.

Conclusion: The study successfully demonstrated that hyperimmune serum generated from MDR *E. coli* isolates from animal products has specific neutralizing activity against homologous MDR bacterial strains. These findings support the potential application of hyperimmune sera as an immunotherapeutic tool to mitigate AMR-related infections. Further research is necessary to standardize Immunoglobulin G concentrations, determine optimal immunization protocols, and evaluate clinical efficacy *in vivo*. The hyperimmune serum preparation represents a promising advancement for tackling MDR bacterial infections and contributes substantially to One Health strategies aimed at reducing the public health threat posed by AMR.

Keywords: antimicrobial resistance, hyperimmune serum, immunotherapy, multidrug-resistant *Escherichia coli*, One Health.

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INTRODUCTION

The discovery of antibiotics in 1928 was soon followed by the emergence of antibiotic resistance plasmids in various microbes, a phenomenon known as antimicrobial resistance (AMR), first identified in the 1960s and progressively increasing to the present day [1, 2]. Antibiotic resistance is a natural evolutionary process by which microorganisms defend themselves to survive. Molecular mechanisms involved in AMR include genetic mutations, enzyme modifications, gene transfer among commensal or pathogenic bacteria [3, 4], and epigenetic modifications [5]. A significant example of enzyme modification is the production of β-lactamase enzymes, which neutralize penicillin and thereby render bacteria resistant [6, 7].

Due to AMR, the global community faces severe challenges related to emerging and re-emerging infectious diseases, which are projected to result in 10 million deaths annually by 2050 [8, 9]. The COVID-19 pandemic, an example of such emerging diseases, has significantly impacted global health and socio-economic stability. However, the threat posed by AMR is equally critical, given that its emergence was predictable yet inadequately addressed, with the situation projected to worsen [10, 11]. Rising resistance to currently available antimicrobials (including antibiotics, antivirals, antifungals, and antiparasitics), coupled with dwindling therapeutic options, contributes to increased morbidity and mortality [10]. This is primarily because the discovery and development of effective antibiotics typically lag behind the rate at which AMR, particularly multidrug resistant *Escherichia coli* (MDR *E. coli*) emerges [1]. According to the World Health Organization (WHO), the AMR crisis ranks among the top three global health threats, identified as the third leading cause of death after cardiovascular diseases [12]. In 2019, AMR was directly responsible for approximately 1.27 million deaths, and nearly 5 million deaths were associated with drug-resistant infections, according to a report published in January 2022. This number is expected to rise to 10 million per year by 2050, surpassing cancer-related fatalities [13].

Managing AMR is complex and requires coordinated efforts across various sectors. Multiple initiatives have been undertaken to control the rapid spread of AMR. International organizations, such as the World Organization for Animal Health (formerly Office International des Epizooties), the WHO, and the Food and Agriculture Organization, have adopted the One Health framework to address AMR comprehensively and have developed global action plans since 2015. The One Health concept integrates human, animal, and environmental health, recognizing their interconnectedness in addressing global challenges like AMR. This approach highlights the necessity of cross-sectoral cooperation among human health, animal health, and environmental sectors to mitigate AMR spread effectively. Within the AMR context, One Health emphasizes rational antibiotic usage, comprehensive resistance surveillance, and appropriate management of antimicrobial waste to prevent environmental contamination. Furthermore, it promotes strengthening regulatory frameworks, fostering innovative research, and educating the public to enhance awareness about the impacts of inappropriate antibiotic use. By harmonizing efforts from diverse sectors, One Health aims to offer a holistic and effective response to combat AMR, which poses a critical threat to global health.

Understanding the roles played by the food, agriculture, and environmental sectors in AMR is essential for preventing infection and curbing the development and dissemination of resistant microorganisms. Under the One Health principle, five key strategies have been outlined for nations to control AMR and minimize its adverse impacts: increasing public awareness, enhancing surveillance systems, implementing rigorous infection prevention measures, promoting antimicrobial stewardship, and advancing research and development [14]. Despite the implementation of a global action plan under the One Health framework since 2015, AMR incidence remains significantly high and is projected to increase over the next decade. A contributing factor to this continued rise could be the disproportionate emphasis placed on upstream rather than downstream interventions.

The limited progress in developing novel antibiotics, coupled with evolving insights into host-pathogen interactions, has stimulated interest in immunological strategies to counteract AMR. One promising approach involves utilizing commonly encountered MDR *E. coli* strains to develop specific anti-MDR *E. coli* immunoglobulins G (IgG). Antibody-based therapies have historically proven effective in preventing and treating various bacterial infections, as exemplified by anti-tetanus serum, anti-rabies serum, and anti-snake venom, traditionally produced using horses as the host species [15]. Essentially, human infections with MDR pathogens pose significant therapeutic challenges, limiting antibiotic treatment options and heightening the risk that AMR will indeed become a leading cause of mortality by 2050.

Despite numerous global initiatives and strategic interventions under the One Health framework to mitigate AMR, progress in controlling MDR *E. coli* remains limited. Previous research has primarily focused on the development of new antibiotics and antibiotic stewardship programs, but these approaches alone have been insufficient in addressing the accelerating spread of AMR effectively. Immunotherapeutic strategies, such as the use of hyperimmune serum, represent a promising yet underexplored alternative. At present, there is limited empirical evidence regarding the production, efficacy, and specificity of hyperimmune serum targeting MDR *E. coli*, especially isolates originating from animal

products in Indonesia. Addressing this gap is crucial for developing innovative solutions to combat AMR and ensuring integrated, sustainable health management across human, animal, and environmental sectors.

This study aimed to address the identified research gap by producing and evaluating hyperimmune serum against MDR *E. coli* isolates obtained from animal products in Bali and Nusa Tenggara, Indonesia. Specifically, the study sought to determine the specificity, effectiveness, and potential therapeutic application of hyperimmune serum as an innovative immunological intervention to mitigate the impacts of AMR within a One Health context.

MATERIALS AND METHODS

Ethical approval

This study was approved by the ethical commission of Udayana University (Letter No. B/217/UN14.2.9/PT.01.04/2022).

Study period and location

The study was conducted from May to October 2023 at the Laboratory of Immunology, Udayana University, in conjunction with the Disease Investigation Center of Denpasar Bali.

Sources of *E. coli* isolates

A total of five *E. coli* isolates were kindly provided by the Center for Quality Testing and Certification of Animal Products in Bogor, Indonesia, in response to request letter No. 6261/PK.300/F5/09/2019. These isolates originally came from Bali, West Nusa Tenggara, and East Nusa Tenggara and had been previously submitted to BPMSH for bacterial typing and resistance analysis. The isolates, labeled as 002/034, 002/038, 002/042, 002/043, and 002/048, all harbored plasmids conferring resistance to eight different antibiotics, namely, ampicillin, amoxicillin, ciprofloxacin, cephalosporin, gentamycin, streptomycin, sulfamethoxazole, and tetracycline, and were thus classified as MDR *E. coli*. In addition, an antibiotic-sensitive native *E. coli* isolate without resistance plasmids was cultured as a negative control (Table 1).

Culturing and isolation of targeted MDR *E. coli*

All of the coded *E. coli* isolates were cultured carefully, adhering to standard bacterial growth protocols and established biosafety measures described by Tenaya *et al*. [16] and Tenaya [17]. Initially, a bacterial stock stored at −80°C was thawed, and a single dose was immediately inoculated into 50 mL of Yeast Tris (YT) medium (pH 7.2), containing 10 g of bacto-yeast extract and 5 g of NaCl per liter of double-distilled water, supplemented with antibiotics (ampicillin and kanamycin). This culture was incubated overnight at 37°C with shaking. Subsequently, 10 mL of the resulting cloudy bacterial suspension was transferred into 1 L of soy broth medium and incubated again overnight. Bacterial cells at the mid-logarithmic growth phase were harvested by centrifugation at 1,000× *g* for 30 min, washed 3 times with phosphate-buffered saline (PBS, pH 7.2), and then resuspended in 50 mL PBS to approximately 10^9 cells/mL. Cells were then re-centrifuged under the same conditions to obtain the cell pellet. The harvested *E. coli* cells were chemically disrupted using trypsin, gently mixed by inversion for 18 h at 4°C, and centrifuged again at 20,000× *g* for 30 min at 4°C. The soluble proteins present in the supernatant were precipitated by adding eight volumes of absolute acetone for 18 h at −20°C, followed by centrifugation at 2000× *g* for 10 min at 4°C to collect the targeted protein pellet. The pellet was subsequently resuspended in PBS, and its protein concentration was quantified using a commercial protein assay kit (Bio-Rad Laboratories, CA, USA). Finally, aliquots were prepared to contain a protein concentration of 100 mg/mL.

Horse hyperimmune sera

Antisera targeting proteins from MDR *E. coli* were produced using a 2-year-old male horse. The bacterial isolates exhibiting the strongest growth, designated as 002/038 and 002/048, originated from Gianyar and Manggarai districts, respectively (Table 2). These isolates were combined and utilized as antigens to immunize the horse. Initially, a blood sample was obtained to serve as a baseline serum control. Subsequently, the horse received an injection containing 2.5 mL of MDR-*E. coli* proteins emulsified with 2.5 mL of Freund’s complete adjuvant (Sigma-Aldrich, Darmstadt, Germany), administered aseptically at two deep intramuscular

Table 1: Origin of the MDR-*E. coli* and control *E. coli* isolates used in this study.

No.	Codes	Provinces of origins	Districts
1	002/034	Bali	Denpasar
2	002/038	Bali	Gianyar
3	002/042	Western Nusa Tenggara	Mataram
4	002/043	Eastern Nusa Tenggara	Kupang
5	002/048	Eastern Nusa Tenggara	Manggarai
6	Control *E. coli*	Bali	Karangasem

E. coli=Escherichia coli

Table 2: The bacterial growth score obtained using similar broth culture media.

No.	Codes	Provinces of origins	Districts	Score cell growth
1	002/034	Bali	Denpasar	+++
2	002/038	Bali	Gianyar	++++
3	002/042	Western Nusa Tenggara	Mataram	+++
4	002/043	Eastern Nusa Tenggara	Kupang	+++
5	002/048	Eastern Nusa Tenggara	Manggarai	++++
6	Native *E. coli*	Bali	Karangasem	-

(No bacterial growth), +++ (Strong growth), ++++ (Very strong growth), as indicated by the thickness of the cell pellets measured in cm, *E. coli=Escherichia coli*

sites on the neck. This immunization procedure was repeated 2 weeks later. Thereafter, the animal received weekly vaccinations for 4 weeks using MDR-*E. coli* proteins emulsified in Freund's incomplete adjuvant. Finally, the horse was administered 2.5 mL of the antigenic protein without any adjuvant intravenously, on two separate occasions at 1-week intervals. Blood samples were collected through jugular vein puncture 2 weeks after the initial immunization and 1 week after the final immunization. Collected sera were aliquoted and stored at −20°C until further use.

Serological confirmation of hyperimmune sera

Confirmation of the homologous antigen-antibody reaction after immunization was conducted through an *in vitro* plate assay, following standard protocols with minor modifications [18]. One full loop of MDR *E. coli* isolates coded 002/038 and/or 002/045, cultured in soy broth media, was suspended in 1 mL of sterile physiological sodium chloride solution at pH 7.2. Using sterile cotton, the bacterial suspension was then evenly spread across the surface of agar media and allowed to dry briefly. Subsequently, several paper discs were prepared by placing drops of undiluted hyperimmune sera collected before and after immunization onto them. In addition, disks containing chloramphenicol as an antibiotic control were carefully placed onto the agar surface at specific intervals. Plates were incubated at 35°C for 16–18 h, after which they were examined for the presence of the expected reactions.

RESULTS

Culturing and MDR-resistant *E. coli*

All five MDR *E. coli* isolates utilized in this study were successfully cultured in the medium, despite the presence of antibiotics. The bacterial cells exhibited rapid growth following overnight incubation, as evidenced by the formation of a dense, cloudy suspension (data not shown). After centrifugation, the harvested cells displayed growth scores comparable to those observed in tick cell pellet cultures. In contrast, no bacterial growth was detected in the control culture containing native *E. coli* without antibiotic resistance plasmids (Table 2).

Vaccine preparation and the production of horse hyper-immune sera

Two crucial steps for preparing the vaccine as an immunogenic antigen included isolating the targeted protein from the bacterial culture and solubilizing the isolated protein using an appropriate Freund's adjuvant. The targeted protein isolation and the preparation of a water-in-oil phase vaccine mixture were successfully accomplished and subsequently used to immunize an experimental horse. A total volume of 100 mL of blood was collected 2 weeks after the initial immunization and again 1 week following the final injection of MDR *E. coli* proteins, using a plain tube to separate serum. The collected blood sample was centrifuged at 2,800× *g* for 5 min at 5°C, after which the serum was aliquoted and stored at −20°C until further analysis. Serum neutralization was confirmed through an *in vitro* agar gel test; however, IgG concentration was not quantified during this preliminary study.

Serological confirmation of hyperimmune sera from MDR *E. coli* isolates

The in vitro assay confirmed that the hyperimmune serum specifically recognized and reacted with the *E. coli* isolates, as demonstrated by clear zones around the hyperimmune serum disk on the agar when exposed to the homologous MDR *E. coli* isolate coded 002/038, originating from Bali (Figure 1). A similar reaction was also observed between the hyperimmune serum and isolate 002/048 from Eastern Nusa Tenggara. The reaction observed was notably stronger with hyperimmune serum collected 1 week after the final immunization (antibody) compared to serum collected 2 weeks post-immunization. In addition, no reaction occurred with the pre-immunization sera, indicating the successful production of specific antibodies through the immunization process. However, a strong and distinct reaction was observed between the isolates and chloramphenicol, confirming that the bacteria remained sensitive to this antibiotic. The extent of the reaction exhibited by the MDR *E. coli* isolates was quantified in millimeters (Table 3).

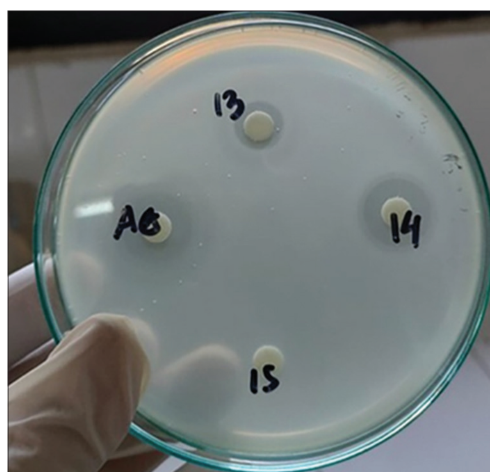

Figure 1: Neutralization test results between antibody (Immunoglobulin G [IgG]) and Multidrug-resistant *Escherichia coli* (MDR *E. coli*) isolate. AB=Antibody (IgG in horse serum) 1 week after the final vaccination; 15=Serum before vaccination; 14=Chloramphenicol antibiotic control; 13=Antibody (IgG in horse serum) at week 3 post-vaccination, indicating a neutralization reaction between the MDR *E. coli* isolate and the serum (IgG) at week 2 (13) and 1-week post-vaccination (AB). The MDR *E. coli* isolate also remains sensitive to chloramphenicol (antibiotic control), as evidenced by the appearance of a neutralization zone outside the white paper (dish). No homologous antibody was detected in the serum collected before vaccination (15).

Table 3: The score reaction of MDR *E. coli* isolates coded 002/038 and 002/048 to hyperimmune serum and chloramphenicol in an agar plate measured in mm.

No.	Isolates	Serum*	Serum**	Serum***	Chloramphenicol
1	002/038	-	3	6	7
2	002/048	-	2	5	6.5

*Serum collected before immunization; **Hyperimmune serum collected 2 weeks after immunization; ***Hyperimmune serum collected 1 week after the last immunization. Notes: Hyperimmune serum collected 1 week after the last immunization had the strongest score, suggesting that it contained the highest antibody titer compared with the other sera but relatively lower than chloramphenicol. *E. coli=Escherichia coli*

DISCUSSION

Various strategies have been employed to control AMR, which significantly threatens global health; however, the incidence of AMR continues to rise [2]. This suggests that previous approaches have not been sufficiently effective, highlighting the need for improved preventive and therapeutic solutions [19]. One of the strategies implemented is the limitation or prohibition of antibiotic use as feed additives. Nevertheless, antibiotics continue to be widely used for therapeutic purposes without proper supervision by authorized professionals. This study aimed to produce serum against antibiotic-resistant microorganisms as an immunotherapy model, an approach not previously reported. Five isolates of *E. coli* characterized as resistant to eight different antibiotics, known as MDR *E. coli*, were utilized to generate hyperimmune serum to assess whether the serum could effectively neutralize the homologous bacteria.

The propagation of MDR-*E. coli* isolates in YT medium supplemented with dual antibiotics (ampicillin and kanamycin) was successfully conducted, whereas no growth occurred in the antibiotic-sensitive negative control (*E. coli*). This outcome indicates that the bacterial isolates genuinely harbored plasmids conferring resistance to the eight antibiotics tested, including kanamycin. This characteristic resembles that of commercially available competent *E. coli* strains, such as DH5-α or BL-21, commonly employed to clone targeted plasmids for research purposes [17, 18]. Moreover, this study demonstrated that MDR-*E. coli* is widely distributed across various regions in Bali, West Nusa Tenggara, and East Nusa Tenggara, suggesting these bacteria could serve as reservoirs of MDR-*E. coli*, posing significant threats to human and animal health in these areas.

Moreover, the five MDR-*E. coli* isolates employed in this study exhibited resistance to eight distinct antibiotics, indicating extensive and uncontrolled antibiotic use across these regions. Tenaya *et al*. [20] reported a notably high prevalence (73.8%) of *E. coli* contamination exceeding the allowable microbial limits in animal-derived products from these provinces, although the specific prevalence of MDR-*E. coli* was not determined. Similar findings were recently reported in Japan, where 84.6% (11/13) of *E. coli* isolates demonstrated AMR [21]. This underscores the significant societal issue posed by AMR, especially MDR-*E. coli*, affecting both humans and animals. Consequently, developing neutralizing antibodies to combat AMR is critically important in the event of accidental infections.

The soluble proteins isolated and used in this study exhibited pathogenic and immunogenic characteristics that stimulated the production of specific hyperimmune sera, despite being precipitated using absolute methanol. Comparable protein extraction methods have previously been employed successfully in generating polyclonal and monoclonal antibodies against *Serpulina pilosicoli* [16], suggesting that absolute methanol precipitation does not denature the proteins. Undiluted hyperimmune serum produced in this study demonstrated clear biological activity, displaying a robust reaction with homologous MDR-*E. coli* isolates, evidenced by distinct neutralization zones. Such a reaction was absent when tested with pre-vaccination serum, confirming the presence of neutralizing antibodies within the hyperimmune serum, even though specific IgG antibodies were not isolated for a stronger confirmation. A pronounced immunological response between intact bacterial cells of isolates 002/038 and 002/048 and the hyperimmune serum indicated that the protein utilized for hyperimmune serum production was derived from the bacterial outer envelope.

In addition, the serum contained antibodies specifically recognizing the bacterial cell membrane proteins, confirming homologous antigen recognition. Tenaya *et al*. [16] demonstrated that proteins extracted in a similar manner were effective in generating polyclonal and monoclonal antibodies specifically targeting the outer envelopes of genus-specific bacteria from diverse geographic regions, confirmed by western blotting and electron microscopy analyses. However, using horses for antibody production carries potential risks associated with emerging infectious diseases. Alternative approaches have been considered safer and more efficient, such as producing antibodies (immunoglobulin Y [IgY]) in chickens [22, 23]. Therefore, appropriate biosecurity measures must be strictly implemented when utilizing horses experimentally.

CONCLUSION

The present study effectively demonstrated the production of hyperimmune serum targeting MDR *E. coli* isolates from animal products originating in Bali and Nusa Tenggara, Indonesia. Key results revealed significant specificity and neutralizing capacity of the serum against homologous MDR *E. coli* strains, clearly demonstrated by distinct neutralization zones during *in vitro* assays. These findings validate the potential of hyperimmune serum as an innovative immunotherapeutic strategy to combat the increasing threat posed by AMR.

A major strength of this research lies in its novel exploration of hyperimmune serum as an alternative solution to traditional antibiotic treatments. This approach particularly addresses a critical gap in existing AMR management strategies. By highlighting the viability and specificity of antibody-mediated responses against resistant bacterial strains, the study provides foundational evidence for immunological interventions.

Nevertheless, several limitations were noted. The current investigation did not quantify or standardize the specific IgG concentration within the hyperimmune serum, limiting the generalizability and direct applicability of the results. Furthermore, the absence of an *in vivo* evaluation restricts the assessment of clinical efficacy, safety, and potential side effects. The experimental reliance on horse-derived antibodies may also present biosecurity concerns related to emerging infectious diseases.

Future studies should focus on addressing these limitations by standardizing antibody titers and conducting comprehensive *in vivo* evaluations to confirm clinical effectiveness and safety profiles. In addition, research exploring alternative antibody-production hosts, such as chickens IgY antibodies, would enhance biosecurity and practical applicability. Further investigation is warranted to integrate this promising immunotherapy approach into broader One Health frameworks aimed at mitigating the global AMR crisis.

AUTHORS' CONTRIBUTIONS

IWMT, KKA, and TKS: Designed the research concept, analyzed data, and drafted the manuscript. IKS, IMS, and RMDM: Performed immunization and collected serum samples. IAPA, NMH, and HS: Conducted laboratory testing, interpreted and analyzed data, and revised the manuscript. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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