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Overexpression and purification of *YidRv* gene from the hypervirulent *Klebsiella pneumoniae*, and the ability of the gene product in inducing a humoral response

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ABSTRACT The *yidRv* gene, isolated from the hypervirulent *Klebsiella pneumoniae* (hvKP), is a novel gene with an unknown function; however, it has exhibited high homology to the *yidR*, a gene recognized as potential vaccine candidate. The aim of this study was to clone the *yidRv* gene from the Indonesian hvKP and to investigate its overexpression in *Escherichia coli*. In the experiment, *yidRv* was cloned into pET21 to construct pYik23. Recombinant protein YidRv was produced by growing *E. coli* BL21 (DE3)/pYik23 in LB medium with ampicillin at 29 °C, inducing protein synthesis with 0.5 mM IPTG for 20 hours. Purification was performed using Ni-NTA resin, and the purified protein (50 µg) was administered to BALB/c mice to test for the production of IgG, IgM and IgA on 2 days before and day 19th and 37th after the first vaccination. The results show a significant induction of IgG and IgM, but not of IgA antibodies. In conclusion, the *yidRv* gene was overexpressed in *E. coli* BL21 (DE3) at high levels in soluble form, with the recombinant protein able to be purified to 90% homogeneity. The recombinant YidRv demonstrated the ability to stimulate the generation of both IgM and IgG antibodies.

KEYWORDS Hypervirulent; *Klebsiella pneumoniae*; Overexpression; Recombinant antigen YidRv

1. Introduction

Hypervirulent *Klebsiella pneumoniae* is considered as a more threatening pathogenic bacteria than conventional *Klebsiella pneumoniae* (c-KP) due to its excessive production of capsular polysaccharides that lead to resistance against several antibiotics (Russo and Marr 2019). A previous study found that the gene sequence of *yidRv*, obtained through successful amplification from hvKP genomes of pneumonia patients, exhibited a 99.75% similarity to the *yidR* gene in c-KP. The c-KP *yidR* gene encodes a potential outer membrane protein and is conserved across 308 *Klebsiella* strains found in both human and bovine hosts (Yang et al. 2019). The *yidR* gene is thought to mediate the hyper-adherence phenotype via the ATP/GTP binding protein. Furthermore, the recombinant YidR (YidR-rec) protein has demonstrated a strong protective capacity against *K. pneumoniae* infection in a mouse model (Rodrigues et al. 2020) and the antigen is a potential vaccine candidate. Therefore, this study aimed to determine whether YidRv exhibits a strong protective capacity against *K. pneumoniae* infection and can be considered a vaccine candidate.

According to bioinformatic analysis, *yidRv* contains epitopes that may stimulate T- and B-cells (Permadi et al. 2024). A critical element in eliminating pathogenic bacterial infections entails triggering targeted immune responses, including humoral (antibody-based) and cellular (T-cell-based) immunity. Macrophages also play a crucial role in eliminating cells hosting persistent pathogens, while activated T cells release cytokines and chemokines that disrupt the spread of microbes in infection-specific manners (Thakur et al. 2019). In contrast, B/-plasma-cells induce specific long-term protective memory, to produce antibodies, a hallmark of adaptive immunity (Kervevan and Chakrabarti 2021).

In the development of a prototype vaccine for hvKP, the production of antigens needs to be scaled up. *Escherichia coli* BL21(DE3) was selected as a host in this investigation due to the possession of an inducible T7

RNA polymerase-dependent expression system, facilitating easy adjustment of protein production levels (Du et al. 2021). To aid in purification, recombinant protein DNA sequences are often altered to incorporate specific residues at the N- or C-terminus. Some frequently used tags are polyhistidine (His-tag), glutathione S-transferase, maltose-binding protein, and thioredoxin (Booth et al. 2018). His-tag, being relatively small (2.5 kDa), is generally considered to have minimal impact on the function and structure of most proteins (Booth et al. 2018). On the other hand, another report showed that using His-tag antigen with specific adjuvants could improve antibody response (Chung et al. 2023).

This study reported the successful cloning and overexpression of *yidRv* from hvKP in *E. coli* BL21(DE3). The gene product was purified by employing the immobilized metal affinity chromatography technique. The purified recombinant protein was then tested to provoke an immune response in mice particularly in synthesizing IgG and IgM antibodies.

2. Materials and Methods

2.1. Construction of a pYidRv recombinant plasmid

The entire *yidRv* sequence (1,227 bp) from hvKP (Permadi et al. 2024) was subjected to codon optimization and was chemically synthesized by GenScript (USA). The yidRv DNA sequence was then inserted into pET21(a) (5,443 bp) at the *Nde*I (Jena Bioscience, Germany) and *Xho*I (Jena Bioscience, Germany) cleavage sites to create pYik23 which was then introduces into *E. coli* BL21(DE3) through the heat shock technique. *E. coli* BL21(DE3)/pYik23 was cultured and screened on Luria broth (LB) supplemented with 100 µg/mL ampicillin (Macklin Biochemical Co, China). PCR was carried out to confirm the existence of *yidRv* gene.

2.2. Growth condition of E. coli BL21(DE3)/pYik23

E. coli BL21 (DE3)/pYik23 was cultured in LB medium with ampicillin (100 µg/mL) until an optical density was reached at 600 nm (OD_{600}) of 0.6. The culture was aerobically incubated in a shaker incubator at 37 °C with 200 rpm shaking. The addition of 0.5 mM isopropyl-B-D-thiogalactopyranoside (IPTG) (Promega, UK) induced the expression of *yidRv* after lowering the temperature to 29 °C. After 20 h, the cells were harvested through centrifugation at 6000 × g for a duration of 15 min at a temperature of 4 °C. The pellet was cleansed with phosphate-buffered saline (PBS), frozen in liquid nitrogen, and kept at -20 °C.

2.3. Overexpression of the gene product (YidRv protein)

The cells were defrosted and suspended in 2.5 volumes of 50 mM lysis-equilibration-wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, at pH 8.0) with 1 mM phenyl methyl sulphonyl fluoride (Sigma Aldrich, USA), and lysozyme followed by disruption using an ultrasonicator at 40% for 10 sec 10 times on ice. The resulting cell extract was centrifuged for 15 min at 8,000 × g, and the supernatant was subjected to protein purification with IMAC resin. The expression of YidRv-rec protein was analysed using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie Brilliant Blue R-250 dye (Thermo Fisher, UK). Furthermore, target proteins were identified by comparison with pre-stained protein standard markers (Transgene Biotech, China). The presence of YidRv-rec was confirmed using the Western blot method.

2.4. Protein purification using the Ni-NTA purification system

YidRv-rec protein purification was conducted using Ni-NTA resin (Probond, Invitrogen, UK). As much as 300 µL of supernatant extract was mixed with 2 mL Ni-NTA resin, and the mixture was loaded onto cartridges and allowed to flow with gravitation. The resin was washed twice with a buffer composed of 50 mL 1× native purification buffer (pH 8.0) containing 10 mM imidazole. Elution of the nickel-bound protein was achieved with elution buffer (1× native purification buffer with 250 mM imidazole, pH 8.0) applied twice. All collected fractions were analyzed by 10% SDS-PAGE.

2.5. Mouse immunization and polyclonal antibody collection

Approval for all experimental animal procedures was granted by the Ethics Committee of the University of Brawijaya under approval no. 147-KEP-UB-2023. A total of 12 female Balb/c mice, aged between six and eight weeks, were acquired from Indo Animal Lab (Bogor, Indonesia) and acclimatized for seven days before vaccination treatment. The mice were assigned into three groups, namely without vaccination, vaccinated with adjuvant and vaccinated with YidRv-rec protein. The third group received intraperitoneal injections of 100 µL of PBS containing 50 µg YidRv-rec protein emulsified using Freund's complete adjuvant at 1:1 (v/v) three times at 2-week intervals (at weeks 0, 2, and 4). Blood samples were collected through cardiac puncture on 2 days before the first immunization, and 19 and 37 days after the first immunization to determine the antibody response. Subsequently, serum was isolated by centrifugation and the supernatant was incubated overnight at 4 °C with 25% (v/v) ammonium sulfate with gentle agitation. The mixture was then centrifuged at 5,000 × g for 10 min at 4 °C. The pellet was resuspended in PBS overnight and after another centrifugation at 10,000 × g for 10 min, the final supernatant was stored at -20 °C (Babu et al. 2017).

2.6. Measurement of antibody level using iELISA method

The frozen serum were thawed at room temperature. iELISA plate wells were coated with 100 µL of coating buffer solution containing 8 µg of YidRv-rec protein and incubated overnight at 4 °C. After washing with PBS on

the following day, the plate was blocked using 1% bovine serum albumin for 3 h at room temperature followed by incubation with the mouse sera diluted in PBS at 1:1,000 ratio. Another PBS wash was carried out, and 50 µL of peroxidase-conjugated secondary antibodies targeting mouse IgG (1:5,000), IgM (1:1,000) and IgA (1:3,000) (Invitrogen, USA) were added. The mixture was incubated overnight at 4 °C. The following day, 50 µL of substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) was added. The reaction was stopped using a stop solution and the absorbance was measured at a wavelength of 450 nm on an ELISA reader.

2.7. Western Blot analysis

Western blotting method is referred to a technical protocol by Biorad (Mishra et al. 2017). In this process, a filter paper was moistened with a transfer buffer containing 25 mM Tris base, 191 mM glycine, and 20% methanol, soaked with SDS-PAGE gel. The transfer process to the nitrocellulose (NC) membrane lasted 90 min, at 20 V and 300 mA. Subsequently, the membrane was mixed with primary antibody 6×-His tag monoclonal antibody in 0.05% tris-buffered saline (TBS)-tween (with a ratio of 1:1,000) and incubated overnight. Anti-mouse secondary antibodies were dissolved in TBS-tween with a ratio of 1:2,000, incubated for 60 min, and washed three times using TBS-tween. Secondary antibodies were further diluted in 1 mL of TBS-tween solvent at a ratio of 1:1,000 and incubated for 40 min followed by washing with TBS-Tween. One millilitre of TMB substrate was added into NC membrane and incubated for 30 min until a band appeared. The reaction was then stopped with water and the membrane was the air-dried and scanned.

3. Results and Discussion

3.1. Construction of pYik23

The DNA encoding the entire YidRv protein was chemically synthesized and subjected to codon optimization. Multiple alterations were made in the sequence. Initially, codon usage was adjusted to match the preferred codons of *E. coli*, resulting in an improved codon adaptation index (CAI) from 0.55 to 0.95 (Figure 1). As a reference, a CAI of 1.0 indicates optimal adaptation for high expression in the target organism, while values exceeding 0.8 are typically favorable.

The GC content and secondary structure of the mRNA were optimized to enhance stability. This was achieved by eliminating stem-loop structures, that could hinder ribosome binding and mRNA folding as well as avoiding potential slippage events during translation. Regulatory elements within the DNA sequence known to negatively impact gene expression were also modified. The *E. coli* ribosome binding sites and the putative Shine-Dalgarno sequence were omitted (Table 1).

The optimized segment was then inserted into pET21(a) to produce pYik23. The transformation of pYik23 to *E. coli* BL21(DE3) competence cell yielded 52 clones. A total of 34 clones were tested, and all were positive for carrying the insert (Figure 2).

3.2. Overexpression of yidRv in Escherichia coli

This study achieved successful transformation of the *yidRv* gene into *E. coli* BL21(DE3), resulting in its overexpression at 29 °C. To induce transcription, 0.5 mM IPTG was added. The YidRv-rec protein was primarily synthesized in soluble form, as it was chiefly found in supernatant (S).

TABLE 1 Comparison of optimized and original codon related to the target restriction enzymes sites, polymerase slippage site, ribosome binding site, and Shine Dalgarno like sequence in the DNA sequence of *yidRv*.

	optimized	original
NdeI(CATATG)	1(1)	1(1)
XhoI(CTCGAG)	1(1228)	1(1228)
Polymerase slippage site 1	0	1
Polymerase slippage site 2	0	1
Frameshift element	0	0
*E. coli*_RBS(AGGAGG)	0	2(623,1181)
PolyT(TTTTTT)	0	0
Chi_sites(GCTGGTGG)	0	0
T7Cis(ATCTGTT)	0	0
PolyA(AAAAAAA)	0	0
SD_like(GGRGGT)	0	2

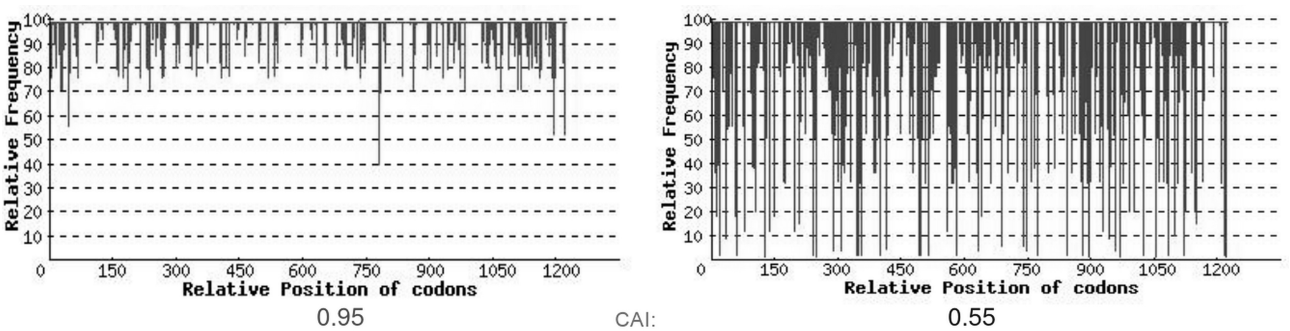

FIGURE 1 Variation in codon preference throughout the coding region of the gene sequence. The increase of relative codon frequency usage in each position into more than a frequency value of 40% after codon optimization (left) and before codon optimization (right).

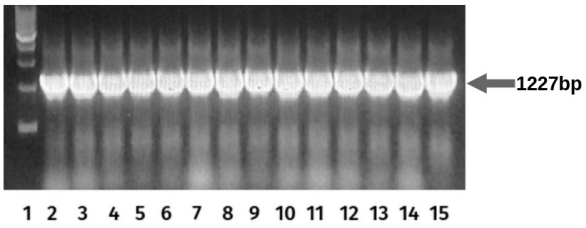

FIGURE 2 PCR amplification of *yidRv* in *E. coli* BL21(DE3)/pYik23 clones. All lanes (2–14) show a band in the position of 1.227 kb. Protein *YidR* served as a positive control and is shown on lane 15. Lane 1 serves as a pre-stained DNA marker.

As shown in Figure 4 (lanes 6 and 8) the band correlating with 43 kDa in the supernatant of the protein extract of *E. coli* BL21(DE3)/pYik23 appeared thicker than in the pellet (Figure 3, lanes 7 and 9).

This result has great potential for increased protein production using a fermenter where the conditions for bacterial growth, such as aeration and pH are ideally fulfilled and automatically controlled. Based on the results, *E. coli* BL21/pYik23 successfully expressed YidRv as much as 10% of the total protein. The amount was slightly less than the protein yield produced by YidR-rec from c-KP which can achieve 15% of the total protein (Rodrigues et al. 2020). Moreover, the protein was primarily produced in soluble form.

Western blot analysis was used to confirm the identity of the protein band observed at approximately 43 kDa as YidRv-rec. This analysis used an antibody specifically recognizing proteins containing a His-Tag epitope. The YidRv-rec protein is novel and has not yet been produced elsewhere. Protein Ag38-rec, which contain His-tag, was a positive control cross-reacting with the His-Tag antibody (Figure 4, lanes 9 and 10). The anti-His-Tag antibody solely recognized a band at approximately 43 kDa, corresponding with the expected molecular weight of YidRv-rec protein.

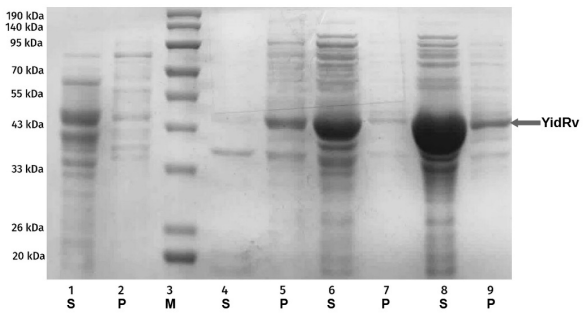

FIGURE 3 SDS-PAGE stained with Coomassie Blue to show recombinant YidRv overexpressed at 29 °C. The supernatant of *E. coli* BL21 crude extract without a plasmid before (lane 1) and after 3 h of IPTG induction (lane 2). The supernatant (lane 4) and pellet (lane 5) of *E. coli* BL21/*pYik23 before IPTG induction. The supernatant (lane 6) and pellet (lane 7) of* E. coli BL21 (DE-3)/pYik23 after 3 h, and supernatant (lane 8) and pellet (lane 9) 20 h post IPTG induction. P: pellet; S: supernatant. Lane 3: pre-stained protein marker.

3.3. Purification of the recombinant YidRv protein

Purification of the YidRv-rec protein was achieved using Ni-NTA resin, showing high efficacy, as evidenced by a distinct band at approximately 43 kDa in the eluted fraction (Figure 5, lane 7). This band was absent in both the flowthrough and washing fractions (Figure 5, lanes 2-6), indicating minimal non-specific interactions during the purification process.

3.4. Detection of polyclonal antibodies

Induction of the humoral immune response in mice using the YidRv-rec antigen with incomplete Freund adjuvant was determined based on the optical density ratio (OD) values of IgG, IgM and IgA using iELISA method. Antibody measurement demonstrated that the group vaccinated with YidR-rec and adjuvant had substantially higher levels of both IgG and IgM antibodies, compared with the group induced with adjuvant only (p = 0.00). IgM antibody production exhibited a peak at day 19, followed by a decrease by day 37. While the IgG titer remained elevated until day 37. IgA titers appeared flat on day 19 and remained very low until day 37. In general, the YidRv-rec antigen appeared to possess medium immunogenicity (Figure 6).

3.5. Discussion

This study investigated the overexpression of the *yidRv* gene originating from an hvKP bacteria strain using a heterologous expression system *E. coli* BL21(DE3). A previous study reported a high degree of DNA sequence similarity (99.75% homology) between the *yidRv* gene isolated from Malang, Indonesia, and its counterpart from c-KP. Based on this DNA sequence homology, YidRv-rec protein produced was expected to share similar immunological properties with that of c-KP. The *yidRv* gene was chemically synthesized and underwent codon optimization. In the field of protein expression, modifying the codon usage pattern (codon optimization) is a widely employed technique to improve the production of soluble recombinant proteins. A study has reported the substantial impact of codon optimization on protein expression levels, with some examples showing increases exceeding 1000-fold (Mauro 2018). Moreover, using this strategy, it can accelerate the rate of translation elongation by addressing limitations arising from differences in codon usage preferences and transfer RNA (tRNA) availability between various host organisms (Mauro 2018). The optimization enabled expression of the YidRv protein in *E. coli* BL21(DE3) reaching up to 10% of total cellular protein. However, we found that the yield was lower compared to the expression levels achieved with traditional YidR-rec production methods, which can reach up to 15% of total protein (Rodrigues et al. 2020). The observed discrepancy in protein yield might be attributed to variations in culturing conditions. Rodrigues et al. (2020) employed a fermenter system that provided a controlled environment for bacterial growth, including optimized parameters like aer-

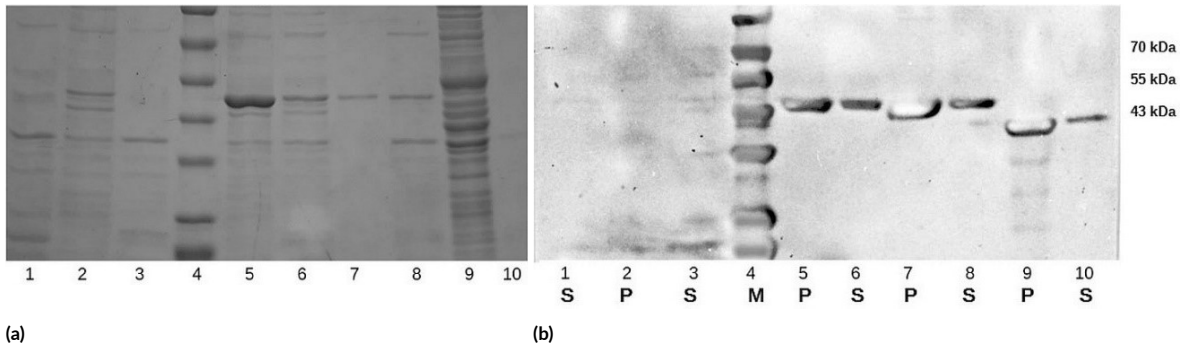

FIGURE 4 Analysis of the YidRv protein after induction with 0.1 and 1 mM IPTG for 3 hours using SDS-PAGE (a) and the western blot method (b). The supernatant (lane 1) and pellet (lane 2) of *E. coli* BL21(DE-3) without plasmid. The pellet (lane 2) and supernatant (lane 3) of *E. coli* BL21(DE-3)/pYik23. Pre-stained protein marker (lane 4). The pellet (lane 5) and supernatant (lane 6) of *E. coli* BL21(DE-3)/pYik23 induced with 0.1 mM IPTG. The pellet (lane 7) and supernatant (lane 8) of *E. coli* BL21(DE-3)/pYik23 induced with 1 mM IPTG. Ag38 protein serves as positive control (lane 9 and 10). S: supernatant; P: pellet.

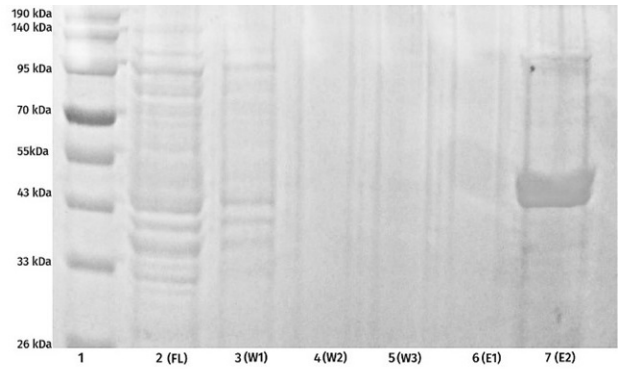

FIGURE 5 Purification of YidRv-rec protein fused with His-tag using Ni-NTA resin. Lane 1: protein marker; 2: flow through (FL); 3: washing 1 (W1); 4: washing 2 (W2); 5: washing 3 (W3); 6: washing 4 (W4); 7: elution.

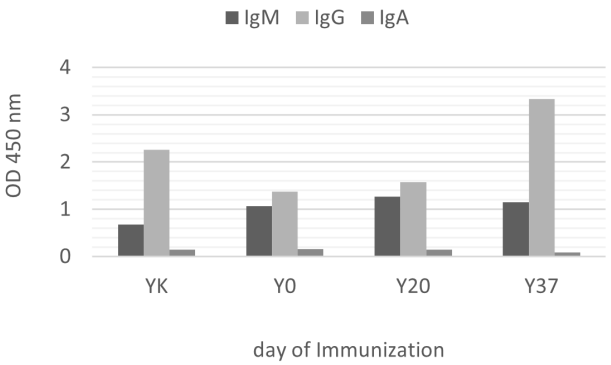

FIGURE 6 The antibody response in mice model against YidRv-rec protein determined using iELISA. Mice were induced with YidRv-rec antigen at day 0, 20 and 30. YK is uninduced mice serves as negative control. The estimation of IgG, IgM and IgA antibodies in serum was measured at day 2 (Y-2) before the first vaccination and day 19 (Y19) and 37 (Y37) after first vaccination by iELISA using YidRv-rec as coating antigen. The developed HRP reaction was measured as absorbance at 450 nm.

ation and pH. In the present study we used conventional methods for bacterial growth, including LB media and an incubator shaker.

The *yidRv* gene from hvKP Malang, Indonesia, was successfully overexpressed in *E. coli* BL21 (DE-3). However, under conditions of 37 °C, YidRv-rec was dominated by inclusion bodies, where approximately 75 of the protein was found in the pellet, and 25% was in the supernatant in a soluble form. Several strategies were performed to increase the protein solubilization i.e lowering induction temperature into 29 °C and prolonged IPTG induction. At 29 °C, the translation process is slower, and the chaperone folded the nascent peptide more slowly, allowing more protein to be translated in soluble form (Soleymani and Mostafaie 2019). A combination of low temperature and proper timing of IPTG induction (20 h) produced more soluble recombinant protein. However, a very low temperature could slow down protein folding by chaperones as in the case of overexpressing recombinant proteins at 25 °C (Ravitchandirane et al. 2022).

In the process of purification, numerous recombinant proteins experience alterations to DNA sequences by incorporating particular residues at either end of the protein. These additional sequence fragments (or tags) bring distinctive characteristics to the protein. As an illustration, a purification tag might include a unique epitope that can be identified by an antibody fragment fixed on a chromatography column (Zhao et al. 2018). Common tags include His-tag, glutathione S-transferase, maltose-binding protein, and thioredoxin. Furthermore, protein tags can be designed to incorporate a recognition motif for a specific protease. For instance, a tobacco etch virus (TEV) protease cleavage site can be introduced. Cleavage by the cognate protease typically leaves the purified protein with a short residual amino acid sequence.

One crucial step in producing recombinant proteins is purifying protein target. It is important to conduct a short, efficient purification process to maximize the yield of purified protein while maintaining a high immune response. To avoid loss during protein purification steps, YidRv was designed to be purified using IMAC method in which the 6× histidine-tag was inserted on the C terminal of the plasmid vector. The choice of this position is due to several recombinant protein being fused with the histidine-tag in-

serted on the N-terminus and failing to be overexpressed (Lopez et al. 2019). Moreover, this position is considered capable of increasing protein solubility reaching 80% solubility (Krupka et al. 2016). Adding His-tag to antigens with specific adjuvants could improve antibody response (Chung et al. 2023). In this study, *yidRv* can be successfully overexpressed in *E. coli* Bl21-(DE3), with 80% of the protein being soluble.

The YidRv-rec protein demonstrated the ability to provoke a humoral immune response to produce IgG and IgM but not IgA, antibodies. The basal level of the humoral immune response on the first day post vaccination showed levels of IgM, IgG, and IgA antibodies, an indication of natural immunity. In the first week, Naïve B-cells proliferate into short-lived plasma cells that rapidly produce pathogen-specific antibodies such as IgM. Then, over time, these plasma cells undergo switching to produce IgG. As a result, IgM-producing plasma cells decrease on third week while IgG level still elevates (Akkaya et al. 2020). The elevated IgG level after 37 days suggested that YidRv might possess high immunogenicity. A previous study reported elevated levels of poly-specific IgG antibodies in mouse livers post-infection with carbapenem-resistant *Klebsiella pneumoniae* (Banerjee et al. 2021). Furthermore, IgG monoclonal antibodies facilitate external mechanisms that eliminate hvKP by strengthening biofilm inhibition as well as promoting the deposition of complement and neutrophil extracellular traps, leading to a reduction in the spread of bacteria to organs (Diago-Navarro et al. 2018). This study represents the first demonstration that the YidRv-rec protein can provoke humoral immune response, particularly IgG antibodies. Concerning the fact that IgA was not induced efficiently after vaccination with YidRv-rec protein, it could be because we used uninfected mouse. A study demonstrated that more effective IgA induction was achieved in pre-exposure patients with the related pathogen (Sano et al. 2022).

4. Conclusions

In conclusion, the novel *yidRv* gene from hvKP, could be overexpressed in soluble form in the heterologous system *E. coli* BL21(DE3). The best expression conditions were at 29 °C, with an induction of 0.5 mM IPTG for 20 h. The purified recombinant YidRv as an antigen significantly induced the synthesis of IgG and IgM but not IgA antibodies. Although *yidRv* showed high homology to *yidR* from c-KP, further study was needed to characterize the biological function and the feasibility of vaccine development.

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Authors' contributions

TYMR, IH designed the study. MJA, EFG, NSP carried out the laboratory work. TYMR, SRP, IH analyzed the data. TYMR, SRP, AKW, IH wrote and reviewed the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare have no pecuniary or other personal interest.

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