High yield production of pigeon circovirus capsid protein in the *E. coli* by evaluating the key parameters needed for protein expression

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**Abstract**

**Background:** Pigeon circovirus (PiCV) is considered to be a viral agent central to the development of young pigeon disease syndrome (YPDS). The Cap protein, a structural protein encoded by the *cap* (or C1) gene of PiCV, has been shown to be responsible for not only capsid assembly, but also has been used as antigen for detecting antibody when the host is infected with PiCV. The antigenic characteristics of the Cap protein potentially may allow the development of a detection kit that could be applied to control PiCV infection. However, poor expression and poor protein solubility have hampered the production of recombinant Cap protein in the bacteria. This study was undertaken to develop the optimal expression of recombinant full-length Cap protein of PiCV using an *E. coli* expression system.

**Results:** The PiCV *cap* gene was cloned and fused with different fusion partners including a His-tag, a GST-tag (glutathione-S-transferase tag) and a Trx-His-tag (thioredoxin-His tag). The resulting constructs were then expressed after transformation into a number of different *E. coli* strains; these then had their protein expression evaluated. The expression of the recombinant Cap protein in *E. coli* was significantly increased when Cap protein was fused with either a GST-tag or a Trx-His tag rather than a His-tag. After various rare amino acid codons presented in the Cap protein were optimized to give the sequence rCap<sub>opt</sub>, the expression level of the GST-rCap<sub>opt</sub> in *E. coli* BL21(DE3) was further increased to a significant degree. The highest protein expression level of GST-rCap<sub>opt</sub> obtained was 394.27 ± 26.1 mg/L per liter using the *E. coli* strain BL21(DE3)-pLysS. Moreover, approximately 74.5% of the expressed GST-rCap<sub>opt</sub> was in soluble form, which is higher than the soluble Trx-His-rCap<sub>opt</sub> expressed using the BL21(DE3)-pLysS strain. After purification using a GST affinity column combined with ion-exchange chromatography, the purified recombinant GST-rCap<sub>opt</sub> protein was found to have good antigenic activity when tested against PiCV-infected pigeon sera.

**Conclusions:** These findings shows that the *E. coli*-expressed full-length PiCV Cap protein has great potential in terms of large-scaled production and this should allow in the future the development of a serodiagnostic kit that is able to clinically detect PiCV infection in pigeons.

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Background
Pigeon circovirus (PiCV), is a non-enveloped virus and is considered to be the viral agent central to the development of young pigeon disease syndrome (YPDS). YPDS syndrome is a multifactorial disease that includes various unspecific clinical signs such as poor racing performance, weight loss, lethargy, anorexia, respiratory distress and diarrhea [1]. At present, PiCV is classified as a tentative member of circovirus family based on its particle size, its associated histopathology and the fact that it shares low-level DNA homology with psittacine beak and feather disease virus (BFDV) [2]. According to the previous reports on the genomic characterization of PiCV, PiCV has been characterized as having an ambisense single-stranded DNA genome of about 2.0 kb [3,4]. There are five open reading frames (ORFs) present on the ss-DNA; V1, C2, C3 and C4; these partially overlap within the PiCV genome. ORF C1 encodes a 30 kDa protein, which is the putative major component responsible for assembly of the viral capsid protein (Cap) [3]. ORF V1 encodes a non-structural protein with putative replication-associated protein (Rep) activity [4]. The ORFs C2, C3 and C4 encodes hypothetical proteins, the biological functions of which remain unclear. To date, some conventional methods have been used to detect the PiCV infection. These include electron microscopy, histological observation and molecular characterization of PiCV, PiCV disease virus (BFDV) [2]. According to the previous reports on the genomic characterization of PiCV, PiCV has been characterized as having an ambisense single-stranded DNA genome of about 2.0 kb [3,4]. There are five open reading frames (ORFs) present on the ss-DNA; V1, C2, C3 and C4; these partially overlap within the PiCV genome. ORF C1 encodes a 30 kDa protein, which is the putative major component responsible for assembly of the viral capsid protein (Cap) [3]. ORF V1 encodes a non-structural protein with putative replication-associated protein (Rep) activity [4]. The ORFs C2, C3 and C4 encodes hypothetical proteins, the biological functions of which remain unclear. To date, some conventional methods have been used to detect the PiCV infection. These include electron microscopy, histological observation and molecular characterization of PiCV, PiCV disease virus (BFDV) [2]. According to the previous reports on the genomic characterization of PiCV, PiCV has been characterized as having an ambisense single-stranded DNA genome of about 2.0 kb [3,4]. There are five open reading frames (ORFs) present on the ss-DNA; V1, C2, C3 and C4; these partially overlap within the PiCV genome. ORF C1 encodes a 30 kDa protein, which is the putative major component responsible for assembly of the viral capsid protein (Cap) [3]. ORF V1 encodes a non-structural protein with putative replication-associated protein (Rep) activity [4]. The ORFs C2, C3 and C4 encodes hypothetical proteins, the biological functions of which remain unclear. To date, some conventional methods have been used to detect the PiCV infection. These include electron microscopy, histological observation and molecular characterization of PiCV, PiCV disease virus (BFDV) [2].

In this study, the PiCV cap gene was fused to a series of different fusion tags in order to improve recombinant Cap (rCap) protein expression. The rCap was then expressed attached to three different expression tags in order to evaluate rCap fusion protein expression and production across a number of different E. coli strains. Three expression vectors were used, one harboring a glutathione-S-transferase (GST) tag, another harboring a 6xHis tag and finally, a third harboring a thioredoxin-6xHis (Trx-His); these were investigated to explore the effect of these very different fusion tags on the expression of rCap protein across various E. coli strains. In addition, optimizations of codon usage for various amino acids within the Cap gene were also carried out to give the rCapopt sequence and then the effect of these changes on expression of rCapopt in the various E. coli strains was assessed. Finally, purified rCapopt protein was examined in order to determine its antigenicity and therefore its usefulness in further serodiagnostic applications. To the best of our knowledge, the yield of E. coli expressed full-length rCapopt in this study after codon optimization of the cap gene is the highest known to date.

Results
The fusion tags and the strain preference facilitate the expression level of recombinant PiCV capsid protein in E. coli
The various fusion tags and the various E. coli strains had a range of effects on the expression level of the two recombinant PiCV capsid proteins in E. coli allowing optimization of the protein purification protocol. To investigate the expression of PiCV capsid protein (Cap) using the prokaryotic expression system, the cap gene
sequence was individually fused with three different tag sequences, His-tag (6xHis), glutathione-s-transferase tag (GST) and thioredoxin-His (Trx-His) in three distinct expression vectors (Figure 1A, a, c and e). All above fusion tags were fused with recombinant rCap at its N-terminus. The resultant constructs, pHis-Cap, pGST-Cap and pTrx-His-Cap were then individually transformed into three distinct E. coli strains in order to address the effect of the fusion tags on the protein expression levels of the cap gene.

As illustrated in Figure 2, when E. coli BL21(DE3) harboring pHis-Cap, pGST-Cap and pTrx-His-Cap were examined, there was no significant amount of rCap fusion protein present in the whole cell lysates after IPTG induction for 4 hrs (SDS-PAGE and Western-blotting of Figure 2A, lane 1–2; Figure 2B, lane 1–2; Figure 2C, lane 1–2, respectively). Proteins at the predicted molecular weights, 32 kDa His-rCap, 58 kDa GST-rCap and 48 kDa Trx-His-rCap, were not detected using anti-His monoclonal antibody and anti-GST antibody as appropriate (Figure 2A, lane 1–2; Figure 2B, lane 1–2; Figure 2C, lane 1–2 of Western blot).

As a result of the above findings, expression of the PiCV rCap fusion protein in E. coli was carried out in two other E. coli strains, BL21(DE3)-pLysS and BL21(DE3)-RIPL and their protein expression levels compared to that of E. coli BL21(DE3). The expression patterns of the rCap fusion protein in E. coli BL21(DE3)-pLysS and in E. coli BL21(DE3)-RIPL containing pHis-Cap, pGST-Cap, and pTrx-His-Cap are shown in Figures 2A, B, C, respectively. The expression patterns for the rCap fusion protein were relatively poor in BL21(DE3)-pLysS. No matter whether the His-rCap or GST-rCap protein was being expressed, the protein products were almost undetectable. In contrast, the 48 kDa specific protein band for Trx-His-rCap could be detected using anti-His monoclonal antibody when the BL21(DE3)pLysS strain containing pTrx-His-Cap was induced with IPTG (Figure 2C, lane 3–4 of SDS-PAGE and Western blot). In addition, when pGST-Cap and pTrx-His-Cap were transformed into BL21(DE3)-RIPL strain, the GST-rCap and Trx-His-rCap protein could be successfully expressed and detected by both SDS-PAGE and Western-blot analysis (Figure 2B, lane 5–6; Figure 2C, lane 5–6 of SDS-PAGE and Western blot). Overall, expression level of the Trx-His-rCap protein, was significant higher than that of the GST-rCap protein in BL21(DE3)-RIPL strain. However, in the BL21(DE3)-RIPL strain containing the pHis-Cap plasmid, expression of His-rCap was still almost undetectable after IPTG induction (Figure 2A, lane 5–6 of SDS-PAGE and Western-blot, respectively). Thus the fusion tags GST and Trx-His seem to be able to facilitate expression of PiCV rCap protein in E. coli and, moreover, the E. coli strain used also plays a crucial role in expression and seems to affect the further application of these strains in large-scale production.

Optimization of the codon usage of the cap gene enhances of recombinant PiCV capsid protein expression in E. coli

Based on the results shown in Figure 2A, B and C, the expression levels of rCap were improved in E. coli when a Trx-His tag or GST tag on the N-terminus of rCap protein was used. The best expression was obtained using the strain BL21(DE3)-RIPL, which harbors extra copies of tRNA^arg(U), tRNA^proL, tRNA^ileY, tRNA^leuW. This is commercial E. coli host strain used for the gene expression of recombinant proteins that contain E. coli’s rare codons. The expression of Trx-His-rCap protein in strain BL21(DE3)-RIPL showing an expression level was significant higher than when the BL21(DE3) and BL21(DE3)-pLysS strains were used (Figure 2B, lane 5–6; Figure 2C, lane 5–6), this suggested that optimization of the codon usage in the rCap protein might further improve the expression level of rCap.

As illustrated in Figure 1B, the cap gene does contain a number of E. coli’s rare codons, which were detected when the sequence was examined by the GeneScript rare codon analysis tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). Approximately 18% rare E. coli codons are presented in PiCV cap gene, and most of rare codons are basic amino acid residues, such as lysine (K) and arginine (R) and are presented near the 5’-end of the cap gene. Using GeneOptimizer software, the codons of the cap gene were optimized without altering the amino acid sequence of the protein to give a gene sequence that had the preferred codon usage of E. coli. As illustrated in Figure 1B, this 819 bp DNA fragment was named as Capopt. Three recombinant constructs were then created using the codon optimized cap gene sequence and then transformed into E. coli to explore expression levels (Figure 1A, panel b, d and f). As shown as Figure 2A, 2B and C, using SDS-PAGE and Western-blot analysis, when E. coli BL21(DE3) or E. coli BL21(DE3)-pLysS were used as host to express the His-rCapopt or the GST-rCap protein, the expression proteins were almost undetectable. However, when the recombinant E. coli BL21(DE3) and BL21(DE3)-pLysS strains harbored the pGST-rCapopt plasmid, rCapopt protein was produced at significant levels after IPTG induction (Figure 2A, panel 7–8 for BL21(DE3) and panel 9–10 for BL21(DE3)-pLysS). The quantitative yield for GST-rCapopt protein production using the BL21(DE3)-pLysS strain was 394.2 ± 26.1 µg/ml, which is higher than that of the GST-rCap protein at 119.2 ± 17.0 µg/ml when the latter protein is expressed in the BL21(DE3)-RIPL strain (right panel of Figure 3A).
The expression level of GST-rCapopt protein in BL21(DE3)-pLysS was even higher than that of BL21(DE3) (Figure 2B, panel 7 – 10 to SDS-PAGE and Western-blot, respectively). This yield of GST-rCapopt protein in the BL21(DE3)-pLysS was 1.3 fold higher than that of the GST-rCapopt protein expressed in the BL21(DE3) strain (right panel of Figure 3A). Furthermore, the pTrx-His-rCapopt plasmid in the BL21(DE3)-pLysS strain also produced a very similar pattern to that of the different E. coli strains expressing GST-rCapopt protein (Figure 2C, panel 7 – 8 for BL21(DE3) and panel 9 – 10 for BL21(DE3)-pLysS to SDS-PAGE and Western-blot, respectively). Furthermore, the quantitative yield for the Trx-His-rCapopt protein in the BL21(DE3)-pLysS strain after IPTG induction for 4 h reached 544.5 ± 33.2 µg/ml at the same optical density (OD) as the cultures, which represents a 5.07 fold increase over Trx-His-rCap in BL21(DE3)-pLysS by densitometric analysis (Figure 2C, panel 3 – 4 for Trx-His-rCap and panel 9 – 10 for Trx-His-rCapopt; right panel of Figure 3B). In addition, the yield of Trx-His-rCapopt protein in BL21(DE3)-pLysS was higher than that of Trx-His-rCapopt protein at 283.3 ± 9.0 µg/ml using BL21(DE3) strain and IPTG induction (right panel of Figure 3C). These findings confirm that the codon-optimized of the cap gene was able to improve protein expression significantly and allowed large amounts of intact rCapopt protein to be produced in E. coli BL21 (DE3) or in BL21(DE3)pLysS with either the GST or the Trx-His fusion tag.

**Figure 1** A schematic diagram of the constructions used in this study and the alignment results for the expressed PiCV capsid gene. **(A)** The full-length wild-type and codon-optimized PiCV capsid protein genes were cloned independently into three expression vectors pET28a, pGEX-4T-1 or pET32a. The PiCV capsid protein with the various different fusion tags, namely a six-histidine (6xHis), a Glutathione-S-transferase (GST) and a Thioredoxin-coupled six-histidine (Trx) at its N-terminus were expressed by T7 or Tac promoter-driving after IPTG induction. **(B)** The nucleotide sequences were compared between the wild-type (WT) and the codon-optimized (OPT) PiCV capsid protein genes. The asterisk (*) represents the fact that the aligned nucleotides are identical.
Chromatographic purification of recombinant PiCV capsid protein

To further characterize and purify the rCap\textsubscript{opt} protein, the solubility of expressed two rCap\textsubscript{opt} fusion proteins, GST-rCap\textsubscript{opt} and Trx-His-rCap\textsubscript{opt} was explored. E. coli BL21(DE3)pLysS cells over-expressing either GST-rCap\textsubscript{opt} or Trx-His-rCap\textsubscript{opt} was separated into the supernatant and pellet fractions after sonication of a suspension of harvested cells. The analysis by SDS-PAGE demonstrated that both GST-rCap\textsubscript{opt} and Trx-His-rCap\textsubscript{opt} protein existed in soluble and insoluble forms (Figure 4A). By densitometric analysis, the solubility of GST-rCap\textsubscript{opt} and Trx-His-rCap\textsubscript{opt} protein were determined (Figure 4B). Approximately 74.58% of the GST-rCap\textsubscript{opt} was soluble, which is higher than the 67.45% solubility obtained for Trx-His-rCap\textsubscript{opt} protein when BL21(DE3)pLysS cells were...
Therefore, purification of the E. coli-expressed GST-rCapopt protein was then carried using a GST affinity column. After affinity chromatography combined with on-column cleavage by thrombin, the presence of collected soluble rCap protein was clearly detectable by SDS-PAGE analysis (Figure 5A, lane 4). The specific 30 kDa band obtained from the column was approximately 90% pure (Figure 5A, lane 4), which indicates that rCap protein had been successful cleaved from GST fusion tag. To further improve the purity of the rCap, the GST-column purified rCap was subjected to cation exchange chromatography. As shown in Figure 5B, the purity of rCap was significantly increased by this process. Once the purification process had been completed, only cleaved GST fusion protein was present at almost homogeneity (Figure 5A, lane 7). When the chromatographic purified rCap protein was examined by MALDI-TOF, five peptides from rCap protein were identified after trypsin digestion and these demonstrated good alignment and a high score when compared to the predicted protein (Figure 5C). The longest peptide fragment, PLGVDITTWKGFGHTVP MYDAR consisted of 22 amino acid residues and, overall, the coverage was 27% of the published amino acid sequence of the PiCV Cap protein (Accession No. AER38484) without any miss-match (Figure 5C). These MALDI-TOF results confirmed that the purified 30 kDa protein is PiCV Cap protein and that the optimization of E. coli’s preferred codon usage within the cap gene had not altered the amino acid sequence (Figure 5C).
Next we investigated whether the PiCV rCap protein expressed by E. coli has antigenic activity when used against PiCV-infected pigeon serum. As illustrated in Figure 6, Western blot analysis using PiCV-infected pigeon serum from five PiCV-infected pigeons showed that the E. coli expressed PiCV rCap<sub>opt</sub> protein had the correct antigenic characteristics in terms of the detection of a specific band of approximately 30 kDa (Figure 6, lane 1–5). In contrast, when PiCV-noninfected serum was used, there were no corresponding bands present on the PVDF membrane (Figure 6, lane 6). These findings support E. coli expressed PiCV rCap<sub>opt</sub> protein as retaining the protein’s original antigenic activity when used against PiCV-infected pigeon serum.

**Discussion**

PiCV infection is associated with development of young pigeon disease syndrome (YPDS). At present, no vaccine is available to prevent PiCV infection. Among circovirus, capsid proteins have been investigated as to their usefulness as immunogens for developing subunit vaccines [13-15]. The Cap protein is the only capsid protein encoded by PiCV. Generally speaking, PiCV Cap protein is thought to be a promising target for the production of a recombinant vaccine or the development of a sero-diagnostic kit [11,12]. Recently, a truncated form of PiCV Cap protein has been shown to have been successful expressed in E. coli [11]. However, expression of full-length recombinant PiCV Cap protein using an E. coli system has remained very difficult.

*E. coli* remains the most attractive expression system when assessing the expression of a heterologous protein for many different purposes [16]. Using an *E. coli* expression system to express a heterologous recombinant protein has several advantages; these include cost-effectiveness, ease of production, time-saving and others. In this study, we have successfully produced for the first time the full-length Cap protein of PiCV using an *E. coli* expression system. Previously, it has been suggested that the Cap protein is likely to be the sole structural protein of PiCV and this protein thus controls viral capsid assembly. Thus, having purified full-length Cap protein will help with the detailed study of PiCV’s structure biology and it will also help with PiCV vaccine development and the production of a sero-diagnostic kit for PiCV detection. The PiCV Cap protein has been demonstrated to have antigenic activity and to be able to recognize by PiCV specific antibodies [11,12]. However, up to the present, a lack of highly purified full-length Cap protein has hindered research in these areas. The main problem with the *E. coli* approach to producing PiCV Cap protein has been poor protein expression and low protein solubility. Previous studies have shown that the production of recombinant Cap protein using an *E. coli* expression system is relatively difficult and therefore this has become a bottleneck [11]. This study surmounts this problem and will allow the efficient production of recombinant PiCV Cap protein for future investigations.

A number of different strategies are available when improving protein expression and enhancing the protein solubility in *E. coli*. These factors include cultivation parameters, the fusing of an affinity tag of one type or another to the target protein and the optimization for *E. coli* of the codon usage of the foreign gene. [13,17,18]. This study explored how three different fusion tags, GST, His-tag and Trx-His tag, affected Cap protein expression;
other fusion tag remain untested and may give further improvement in the future. Both a GST and a Trx-His fusion tag was found to significantly improve the yield of rCap protein compared to a 6 × His tag (Figure 2A, B and C). In a previous study, Liu et al. described how the expression of the Cap protein of porcine circovirus (PCV) was successfully improved in E. coli by fusing the maltose-binding protein (MBP) to the target protein [19], but the mechanism by which a fused MBP-8xHis tag is able to improve protein expression remains unclear. Nonetheless, one possibility is that protein solubility was improved [19].

Similarly, in our previous study, the addition of a GST tag to the CAV VP1 protein also improved expression in E. coli significantly compared to a His × 6 tag [13]. Thus some fusion tags would seem to be able to help improve the solubility of E. coli expressed proteins more than other tags; this occurs perhaps by promoting the correct folding of their selected fusion partner [13,19].

Like porcine circovirus (PCV), beak and feather disease virus (BFDV) and chicken anemia virus (CAV), PiCV is also rich in basic amino acid residues close to the N-terminus of the capsid protein. This region has been predicted to be a nuclear localizing sequence and a nucleic acid binding domain via the DNAbinder software package (http://www.imtech.res.in/raghava/dnabinder/submit.html). In previous studies, this N-terminal regions of capsid proteins have often caused problems with recombinant protein expression using a prokaryotic system [11,13,20]. Deletion of the N-terminus of the capsid protein can often overcome this problem allowing the protein to be expressed successfully in E. coli; nonetheless, the usefulness of the truncated protein for diagnosis or for the development of a subunit vaccine is likely to be hampered. Obviously only the intact capsid protein contains all of the epitopes for elicitation of virus neutralizing antibodies by the host. Thus, it is best to express full-length PiCV capsid protein rather than a truncated form when developing a vaccine or a diagnostic kit.

The present study found that the E. coli strain BL21 (DE3)-RIPL, when used to express Trx-His-rCap, gave the highest level of protein expression, significantly higher than any other combination (Figure 2B, lane 5–6;
This demonstrated that extra copies of tRNA^argU, proL, ileY, leuW are able to improve PiCV Cap protein expression usefully. In addition, it confirms that the E. coli rare codons with the cap gene have a significant effect on its expression. Using the GeneScript rare codon analysis tool, it was found that approximately 18% of the codons in the PiCV cap gene are rare E. coli codons. It had been suggested that when a target gene contains >10% rare codons of E. coli, protein expression efficiency is likely to be decreased [21]. Rosenberg et al. also described how the efficiency of protein translation might be affected by an abundance of rare codons near the 5’-end of the gene. Thus, when a codon-optimized cap gene was used, rCapopt, rather than rCap, expression of the Cap protein was significantly enhanced in E. coli compared to supplying extra copies of the rare tRNA genes via the expression strain.

Figure 2C, lane 5–6). This demonstrated that extra copies of tRNA^argU, proL, ileY, leuW are able to improve PiCV Cap protein expression usefully. In addition, it confirms that the E. coli rare codons with the cap gene have a significant effect on its expression. Using the GeneScript rare codon analysis tool, it was found that approximately 18% of the codons in the PiCV cap gene are rare E. coli codons. It had been suggested that when a target gene contains >10% rare codons of E. coli, protein expression efficiency is likely to be decreased [21]. Rosenberg et al. also described how the efficiency of protein translation might be affected by an abundance of rare codons near the 5’-end of the gene. Thus, when a codon-optimized cap gene was used, rCapopt, rather than rCap, expression of the Cap protein was significantly enhanced in E. coli compared to supplying extra copies of the rare tRNA genes via the expression strain.

We also investigated which of two different recombinant E. coli strains, BL21(DE3) and BL21(DE3)pLysS, was able to improve protein production and yield. With both GST-Capopt and Trx-His-Capopt, expression was better with BL21(DE3) and produced more Cap protein than BL21(DE3)-RIPL (right panel of Figure 3A and 3B). It is worth noted that BL21(DE3)pLysS has a higher growth rate than BL21(DE3) or BL21(DE3)-RIPL when expressing Trx-His-Capopt or Trx-His-Cap. (left panel of Figure 3B). This discrepancy may involve either higher protein stability or Trx-His-Capopt having a less cytotoxic nature when present in BL21(DE3)pLysS. However, these effect were not present during the production of GST-Capopt protein using E. coli BL21(DE3)pLysS at high expression levels. The superiority of Trx-His-Capopt expression in BL21(DE3)pLysS may be due to the presence in the strain of the pLysS plasmid during protein induction. The cytotoxicity tolerance of BL21(DE3)pLysS might be associated with the expression of T7 lysozyme which attenuates transcription leakage by T7 RNA polymerase. However, this phenomenon was not significant when GST-Capopt was expressed grown using BL21(DE3)pLysS. GST-Capopt was decreased when there was induction by IPTG. One possibility is that the cytotoxicity of GST-Capopt may be higher that that of Trx-His-Capopt in BL21(DE3)pLysS. In other words, a “protein burden” may not yet have been encountered when BL21(DE3)pLysS was used to express Trx-His-Capopt. Overall, we concluded that BL21(DE3)pLysS is the preferred choice for expressing Trx-His-Capopt.

The solubility of recombinant Cap protein is a potential problem and in this context the solubility of GST-Capopt protein is superior to that of Trx-His-Capopt. Furthermore, it is very easy to apply the protein to a GST-affinity column in order to carry out a protein purification. Moreover, the Cap protein contains basic amino acid residues rich at its N-terminus. Such highly positive charge amino acids within the recombinant Cap protein allow easy polishing by cation exchange column as part of downstream processing. Therefore, it seems likely that, a higher purity of Cap protein can be obtained when an affinity column is combined with an ion-exchange chromatography during vaccine development.

In this study, positive PiCV-infected pigeon sera were used to evaluate the antigenic activity of the E. coli-expressed recombinant PiCV Cap protein. All tested PiCV-infected pigeon sera were able to demonstrate that E. coli-expressed recombinant PiCV Cap protein has the correct antigenic activities. This might be a result of the E. coli-expressed recombinant Cap protein displaying all of the appropriate protein antigenic regions on the protein surface for recognition by the pigeons’ antibodies. However, rCap protein when used against some pigeon sera did not show very strong antigenicity. This perhaps suggests that the titers of antibodies against PiCV Cap protein have various levels in pigeons and such variation might explain in lower performance of antigenicity in certain birds.

**Conclusions**

In conclusion, using a prokaryotic system, the optimal expression of recombinant full-length PiCV Cap protein

![Figure 6](image-url)
was established successfully during this study. Furthermore, by fusing the Cap protein to an affinity tag, by using the appropriate preferred E. coli and by optimizing the codon usage of the polypeptide, it was possible to increase the yield of Cap fusion protein significantly. In this context, a convenient and cost-effective strategy for increasing the expression of Cap protein, which was used herein, was the direct engineering of the codons of the Cap protein to fit the E. coli codon preferences. This approach paves the way for the large-scale efficient production of the PiCV Cap protein. In the future, this will also allow recombinant Cap protein to be used as a potential antigen for the development of a PiCV diagnostic test.

**Methods**

**Bacterial strains and cell inoculation**

Three commercial E. coli strains, BL21(DE3) (Invitrogen, Carlsbad, CA), BL21(DE3)CodonPlus-RIPL (Stratagene, La Jolla, CA) and BL21(DE3)pLysS (Stratagene, La Jolla, CA) were used and maintained at 37°C in the Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0). First, 0.5 mL of an overnight culture was inoculated into 50 mL LB medium to allow strain activation by growth at 37°C for around 3 hours, by which time the optical density of culture had reached 0.5 of OD$_{600}$. These bacterial cells were then used for transformation.

**Construction of the recombinant plasmids**

A 822 bp of cDNA fragment consisting of the cap gene that encodes the full-length PiCV capsid protein was synthesized by Genemark Biosci & Tech Co. (Taichung, Taiwan) based on the published sequence (Columbid circovirus, isolate 9030; Accession No. AJ298229). This cDNA was cloned into either pET28a, pET32a (Novagen, Madison, WI) or pGEX-4T-1 (GE Healthcare, Piscataway, NJ) individually using EcoR1 and XhoI (Takara, Japan) restriction sites. The resulting recombinant plasmids were designated pHis-Cap, pTrx-His-Cap and pGST-Cap, respectively (Figure 1, panel a, e and c). To improve the codon usage of the cap gene from PiCV, a second cDNA sequence was synthesized by Genemark Biosci & Tech Co that contained the codons that were optimized for E. coli; this was also ligated individually into the same three E. coli expression vectors using the same restriction sites; these constructs were designated pHis-Cap$_{opt}$, pTrx-His-Cap$_{opt}$ and pGST-Cap$_{opt}$ respectively (Figure 1, panel b, f and d). The six constructs were then individually transformed into One Shot® Top10 (Invitrogen, CA) chemically competent E. coli for maintenance of the recombinant plasmids. Transformants that containing a insert of the correct size were then confirmed as correct by restriction enzyme digestion and by DNA sequence analysis.

Expression of recombinant Cap protein (rCap) and codon optimized Cap protein (rCap$_{opt}$) in E. coli

To express the recombinant rCap or rCap$_{opt}$ protein, all the constructed recombinant plasmids carrying either the cap gene or the codon-optimized cap gene, as described in Figure 1, were transformed into various E. coli strains to allow evaluation of protein expression. Three commercial E. coli host strains, BL21(DE3), BL21(DE3)CodonPlus-RIPL and BL21 (DE3)pLysS, each with a different recombinant construction, were used for protein induction and expression. The culture conditions, the composition of the LB medium and the protein induction condition of these recombinant strains have been described previously [13]. After IPTG induction, samples of the cells were harvested and analyzed for protein expression. The total protein was measured by the procedure described in a previous study [22]. Samples containing the expressed Cap or Cap$_{opt}$ proteins were analyzed by 12.5% SDS-PAGE and Western-blotting using a monoclonal anti-His antibody (Invitrogen, Carlsbad, CA) or a monoclonal anti-GST antibody (GE Healthcare, Piscataway, NJ).

**Purification of recombinant Cap$_{opt}$ protein using GST affinity chromatography with on-column cleavage by thrombin**

Recombinant rCap$_{opt}$ protein was purified from cells expressing the GST-rCap$_{opt}$ protein. This was carried out by spinning down 50 mL of culture supernatant and resuspended the pellet in GST resin binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.3). The mixture was then sonicated on ice three times for 3 minutes using a 20% pulsed activity cycle (MISONIX Sonicator® 300). Next, the lysate was centrifuged for 10 min at 10,000 rpm to remove the cell debris. The resulting cell supernatant was loaded onto a GSTrap FF affinity column (GE healthcare, Piscataway, NJ) for protein purification using the standard procedure described in a previous study [13]. The total protein concentration of each collected fraction from the column was determined using a Micro BCA kit (Pierce, Rockford, IL) with bovine serum albumin acting as the reference protein. The purity of the protein from each fraction was analyzed by 12.5% SDS-PAGE and then the resulting gels were Western blotted using monoclonal anti-GST antibody (GE Healthcare, Piscataway, NJ).

**Mass spectrometry**

To confirm the identity of the recombinant Cap$_{opt}$ protein, E. coli expressed GST-rCap$_{opt}$ protein that had been purified by GSTrap FF column was used. The rCap$_{opt}$ protein that had been eluted from the GSTrap FF column was loaded onto a SP cation exchange chromatography column (GE Healthcare) for further purification. The cation exchange column-purified rCap$_{opt}$ protein
was then analyzed by 12.5% SDS-PAGE. The relevant band was then cut out from the 12.5% SDS-PAGE gel after coomassie blue staining and digested with trypsin. The resulting peptides were subjected to the MALDI-TOF-MS mass spectrometry (ESI-QUAD-TOF) to allow amino acid sequence identification of the protein, as described in a previous study [22].

Competing interests
The author declares that they have no competing interests.

Authors’ contributions
MSL participated in this study design, performed the experiments and in the writing of the manuscript. GHL performed the experiments, study design and participated in the construction of the plasmids. YYL participated in the experiments on protein antigenicity and MKL, YCL, and YLT participated in the protein purification step and determining protein solubility. JTCT participated in the data analysis and the writing of the manuscript. HJC and WTC coordinated the study and participated in performing ELSA assay. All authors read and approved the final manuscript.

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References
1. Raue R, Schmidt V, Freick M, Reinhardt B, Johna R, Kamphausen L, Kaleta EF, Müller H, Kautzwald-Junghanns ME: A disease complex associated with pigeon circovirus infection, young pigeon disease syndrome. Avian Pathol 2005, 34:418–25.
2. Todd D, McNulty MS, Mankertz A, Lukert PD, Dale JL, Randles JW: Family Circoviridae. In “Virus Taxonomy: classification and nomenclature of virus. Academic press: New York, San Diego; 2000.
3. Mankertz A, Hattermann K, Ehlers B, Solke D: Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. Arch Virol 2000, 145:2469–79.
4. Todd D, Weston JH, Solke D, Smyth JA: Genome sequence determinations and analysis of novel circoviruses from goose and pigeon. Virology 2001, 286:354–62.
5. Solke D, Hattermann K, Albrecht K, Segall J, Domingo M, Schmitt C, Mankertz A: A diagnostic study on columbid circovirus infection. Avian Pathol 2001, 30:505–11.
6. Todd D, Duchatel JP, Weston JH, Ball NW, Borthmanns BJ, Moffett DA, Smyth JA: Evaluation of polymerase chain reaction and dot blot hybridisation tests in the diagnosis of pigeon circovirus infections. Vet Microbiol 2002, 89:1–16.
7. Roy P, Dhillon AS, Lauerman L, Sh bvaprasad HLN: Detection of pigeon circovirus by polymerase chain reaction. Avian Dis 2003, 47:218–22.
8. Franciosini MP, Fringuelli E, Tarhuni O, Guelfi G, Todd D, Casagrande Proietti P, Falconi N, Asdrubali G: Development of a polymerase chain reaction-based in vivo method in the diagnosis of subclinical pigeon circovirus infection. Avian Dis 2003, 49:540–3.
9. Freick M, Müller H, Raue R: Rapid detection of pigeon herpesvirus, fowl adenovirus and pigeon circovirus in young racing pigeons by multiplex PCR. J Vet Viral Methods 2008, 148:226–31.
10. Smyth JA, Weston J, Moffett DA, Todd D. Detection of circovirus infection in pigeons by in situ hybridization using cloned DNA probes. J Vet Diagn Invest 2001, 13:475–82.
11. Daum I, Finsterbusch T, Härtele S, Gobel TW, Mankertz A, Korbel R, Grund C: Cloning and expression of a truncated pigeon circovirus capsid protein suitable for antibody detection in infected pigeons. Avian Pathol 2009, 38:135–41.
12. Duchatel JP, Todd D, Smyth J, Costes B, Jauniaux T, Farnir F, Losson B, Vanderplasschen A: Pigeon circovirus: baculovirus expression of the capsid protein gene, specific antibody and viral load measured by real time polymerase chain reaction. Israel J Vet Med 2011, 66:26–31.
13. Lee MS, Hseu YC, Lai GH, Chang WT, Chen HJ, Huang CH, Lee MS, Wang MY, Kao JJ, You BJ, Lin W, Lien YL, Lin MK: High yield expression in a recombinant E. coli of a codon optimized chicken anemia virus capsid protein VP1 useful for vaccine development. Microb Cell Fact 2011, 10:56.
14. Tu Y, Wang Y, Wang G, Wu J, Liu Y, Wang S, Jiang C, Cai X: High-level expression and immunogenicity of a porcine circovirus type 2 capsid protein through codon optimization in Pichia pastoris. Appl Microbiol Biotechnol 2013, 97:2867–75.
15. Bonne N, Shearer P, Sharp M, Clark P, Raidal S: Assessment of recombinant bead and feather disease virus capsid protein as a vaccine for psittacine bead and feather disease. J Gen Virol 2009, 90:640–7.
16. Jonasson P, Liljequist S, Nygren P, Stahl S: Genetic design for facilitated production and recovery of recombinant proteins in Escherichia coli. Appl Biochem 2002, 35:91–105.
17. Lee MS, Sun FC, Huang CH, Lien YY, Feng SH, Lai GH, Lee MS, Cao J, Chen HJ, Tseng JTC, Cheng HY: Efficient production of an engineered apoptosis from chicken anemia virus in a recombinant E. coli for tumor therapeutic application. BMC Biotechnol 2012, 12:27.
18. Trundova M, Celer V: Expression of porcine circovirus 2 ORF2 gene requires codon optimized E. coli cells. Virus Genet 2007, 34:199–204.
19. Liu Q, Tikko SK, Babiuk LA: (Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. Virology 2001, 285:91–99.
20. Johne R, Raue R, Grund C, Kaleta EF, Muller H: Recombinant expression of a truncated capsid protein of bead and feather disease virus and its application in serological tests. Avian Pathol 2004, 33:328–336.
21. Rosenberg AH, Goldman E, Dunn JJ, Studier FW, Zubay G: Effects of consecutive AGG codons on translation in Escherichia coli, demonstrated with a versatile codon test system. J Bacteriol 1993, 175:76–22.
22. Lee MS, Chou YM, Lien YY, Lin MK, Chang WT, Lee HZ, Lee MS, Lai GH, Chen HJ, Haung CH, Lin WH: Production and diagnostic application of a purified, E. coli-expressed, serological specific chicken anemia virus antigen VP3. Transbound Emerg Dis 2011, 58:232–9.