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Design and optimization of primer for the cloning of the mouse Fd immunoglobulin M for antibody phage display technology

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KEYWORDS
Opisthorchis viverrini; Antibody; Phage display technology; Primer.

ABSTRACT
Hybridoma technique is worldwide used for antibody production. However, there are many issues of using this technique such as hybridoma instability, chromosome loss, mutation and short haft-life that lead to loss of hybridoma genome and loss of antibody production. The technique which displays antibody on phage particle can solve this problem. Therefore, antibody phage display was used to sustain the hybridoma technique to produce antibodies. In this technique, it is needed to amplify antibody sequence by a primer that is specific to antibody isotype and have cloning site to clone antibody sequence into a phagemid vector. Unfortunately, there was no reverse primer for amplifying mouse Fd immunoglobulin M (IgM) and clone into the pComb3HSS phagemid vector. The aim of this study was to design the new reverse primers to amplify mouse Fd immunoglobulin M antibody gene by polymerase chain reaction (PCR) using KKU505 hybridoma as a model. The KKU505 hybridoma can produce anti-Opisthorchis viverrini (O. viverrini) monoclonal antibody (mAb). The results demonstrated that this new primer could amplify the Fd immunoglobulin M of anti-Opisthorchis viverrini mAb and construct into pComb3HSS phagemid vector. This technology could preserve antibody gene and use for more stable antibody production.

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Introduction

Antibody phage display technology is a technique that displays antibody fragments that still have the antigen-binding ability on the phage particles. This technique was introduced in 1990 by George P. Smith who won the 2018 Nobel Prize in chemistry[1-3]. This technique can be used for monoclonal antibody-producing, selection, and purification of the specific antibody and improvement of affinity antibody using mutagenesis[4-6]. This technique is more effective and stable in monoclonal antibody production than conventional hybridoma techniques[1, 2, 4, 7]. Since, this technique needs to clone the antibody coding gene in the phagemid vector; half-life of the vector is longer and more stable than a cell line such as the hybridoma cell line[8, 9]. This study focuses on the Fab antibody format which is a stable format to construct a phage vector with anti-*O. viverrini* mAb sequence. This vector can be used for producing anti-*O. viverrini* mAb using phage display technology.

One of the steps in antibody phage display technology is to amplify the antibody fragment gene for cloning into a phagemid vector[10, 11]. The forward primer should be variety like degenerate primers (Zhongde Wang, 2000)[12]. The reverse primer, which binds in the 3' end constant region, should be specific to antibody fragment format, isotype, and species of the interested genome. Both forward and reverse primers have to contain enzyme restriction sites used in processing to clone antibody genes into a phagemid vector. Unfortunately, there has no reverse primer to amplify mouse Fd immunoglobulin M for pComb3HSS phagemid vector construction nowadays[10, 11].

The aim of this article was to design and optimize the optimal condition for new reverse primers, which was applied for combination with degenerate forward primers from the Zhongde Wang's study. Both forward and reverse primers were used to amplify mouse Fd immunoglobulin M antibody gene by polymerase chain reaction (PCR), constructed to pComb3HSS phagemid vector with antibody gene for antibody phage display technology. KKU505 hybridoma cell line which produces IgM specific to *Opisthorchis viverrini* was used as model.

Materials and methods

Cell culture

KKU505 hybridoma cells producing anti-*O. viverrini* mAb were validated isotype antibody using a mouse monoclonal antibody isotyping using by Isostrip kit test (mouse monoclonal antibody isotyping kit, Roche, cat. 1493027) according to the manufacturer's instructions, which provided by the liver fluke and cholangiocarcinoma research center, faculty of medicine, Khon Kaen University, Khon Kaen, Thailand. The cell was cultured in RPMI 1640 medium (Gibco) with 10% fetal bovine serum (FBS) (Gibco) and streptomycin (100 µg/ml), and was maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Escherichia coli and vectors

E. coli strains, XL-1 Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F' proAB+, LacIq, ZdelM15, Tn10]) (Stratagene, USA) and TG-1 (K-12 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, (rK-mK-)) were used as host for phage display. The pGEM®- T easy vector (Promega, USA) was used for the pGEM®- T easy vector (promega) system and the pComb3HSS phagemid vector, which was kindly provided from Carlos Barbas, was used for the pComb3HSS system.

Primer design

The new reverse primers were designed with a restriction site using IgM isotype mouse 3' end from the constant region (sequence ID are AJ851868.3, S79401.1, AH003148.2, and V00818.1) as templates. The heavy chain Fd fragment amplification using these new reverse primers is combined with degenerate forward primers from the Zhongde Wang's study. This primer set also was optimized annealing temperature and magnesium concentration for an optimal condition of heavy chain Fd fragment amplification. The design of this primer was the following characteristics of population-based methods from Li-Yeh Chuang's study[13].

Amplification of KKU505 Fab gene

Total RNA was extracted from KKU505 hybridoma cells using TRIzol® (invitrogen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by cDNA synthesis kits (Promega, USA) to the manufacturer's instructions from 2 µg total RNA using a random forward primer and reverse primer. Resulting in the first-strand cDNA was used as a template for further PCR amplification of Fab fragments. IgM-specific variable heavy (VH) and light (VL) chain Fab gene from purified KKU505 cDNA was amplified using PCR system (Bio-active, Thailand) for 30 cycles in the first round (at 94°C for 60 sec, at 45°C for 60 sec, at 72°C for 90 sec and 10 min at 72°C for final extension), with each forward and reverse oligonucleotide primers set.

Results

Primer design and alignment

IgM isotype mouse 3' end from the constant region (sequence ID are AJ851868.3, S79401.1, AH003148.2, and V00818.1) as a template for design new reverse primers as MUHCM1-4 with cloning enzyme restriction sites for pComb3HSS phagemid vector construction (Figure 1). The specific sequence of IgM constant region was in the 3' end of these primers. The cloning enzyme restriction site is in the 5' end of these primers that have no effect for template-primer binding and all primer sequences in this study are shown in Table 1.

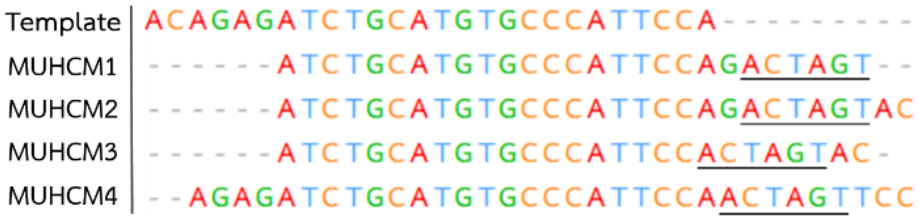

Figure 1 Alignment of new primer sequences of different 4 heavy chain reverse primers compared with the template. The single-letter nucleotide codes were colored. Adenine (A), thymine (T), cytosine (C) and guanine (G) are red, blue, yellow and green, respectively. The template was IgM isotype mouse 3' end from the constant region. The letters with underline represent cloning enzyme restriction sites.

Polymerase chain reaction using these reverse primers

All four new reverse primers (MUHCM1-4) combined with two heavy chain high degenerated forward primers (MH1-2) which are shown in Table 1 were used to amplify the heavy chain Fd sequence of KKU505 cDNA. The specific band is 600-700 bp. The specific band was shown in all reactions. The intensity of the interaction bands from MUCHM4 was highest and clearest combined with two heavy chain high degenerated forward following MUCHM2, MUCHM1 and MUCHM3, respectively (Figure 2).

Table 1 Sequences of PCR primers used in this study

Name	Sequences
Heavy chain high degenerated forward primers (ref 10)	
MH1	5'-CTT ctc gag SAR GTN MAG CTG SAG SAG TC-'3
MH2	5'-CTT ctc gag SAR GTN MAG CTG SAG SAG TCW GG-'3,
Heavy chain low degenerated forward primers (ref 10)	
MH3	5'-ctt ccg gaa ttc CAG GTT ACT CTG AAA GWG TST G-'3
MH4	5'-ctt ccg gaa ttc GAG GTC CAR CTG CAA CAR TC-'3
MH5	5'-ctt ccg gaa ttc CAG GTC CAA CTV CAG CAR CC-'3
MH6	5'-ctt ccg gaa ttc GAG GTG AAS STG GTG GAA TC-'3
MH7	5'-ctt ccg gaa ttc GAT GTG AAC TTG GAA GTG TC-'3
Heavy chain reverse primers (New primers from this study)	
MUHCM1	5'-act agt CTG GAA TGG GCA CAT GCA GAT-'3
MUHCM2	5'-GTa cta gtC TGG AAT GGG CAC ATG CAG AT-'3
MUHCM3	5'-GTa cta gtG GAA TGG GCA CAT GCA GAT-'3
MUHCM4	5'-GGA act agt TGG AAT GGG CAC ATG CAG ATC TCT-'3
Light chain forward primers (ref 8)	
MULC1	5'-CCA GTT CCg agc tcG TTG TGA CTC AGG AAT CT-3'
MULC2	5'-CCA GTT CCg agc tcG TGT TGA CGC AGC CGC CC-3'
MULC3	5'-CCA GTT CCg agc tcG TGC TCA CCC AGT CTC CA-3'
MULC4	5'-CCA GTT CCg agc tcC AGA TGA CCC AGT CTC CA-3'
MULC5	5'-CCA GAT GTg agc tcG TGA TGA CCC AGA CTC CA-3'
MULC6	5'-CCA GAT GTg agc tcG TCA TGA CCC AGT CTC CA-3'
MULC7	5'-CCA GTT CCg agc tcG TGA TGA CAC AGT CTC CA-3'
Light chain reverse primer (ref 8)	
MULK3	5'-GCG CCG tct aga ATT AAC ACT CAT TCC TGT TGA A-3'

Note: The reverse primers for heavy chain sequence were designed in this study. Small letters represent cloning or enzyme restriction sites, *XhoI* (ctcgag), *XpeI* (actagt), *SacI* (gagctc) and *XbaI* (tctaga).

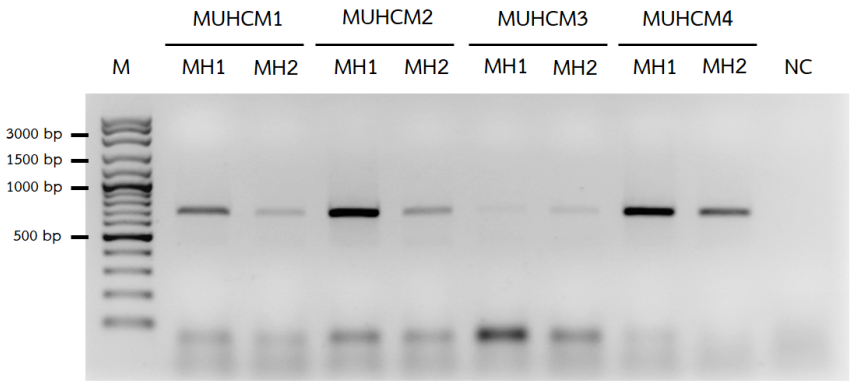

Figure 2 Gel analysis of PCR of difference 4 reverse primers with degenerated forward primers. MUHCM1-4 was new designed reverse primers. MH1-2 was degenerated forward primers. The heavy chain Fd fragments were 600-700 bp, M was DNA marker, Bioline 100 bp.

The optimal condition of MUCHM4 reverse primer

MUCHM4 reverse primer combined with two heavy chain high degenerated forward primers (MH1-2) were used to optimize the condition. Annealing temperature and magnesium concentration were optimized for heavy chain Fd fragment amplification. The result suggested that the optimal annealing temperature was 48 ºC and optimal magnesium concentration was 2.5 mM. This optimal condition was used to amplify the heavy chain Fd sequence of KKU505 cDNA (Figure 3).

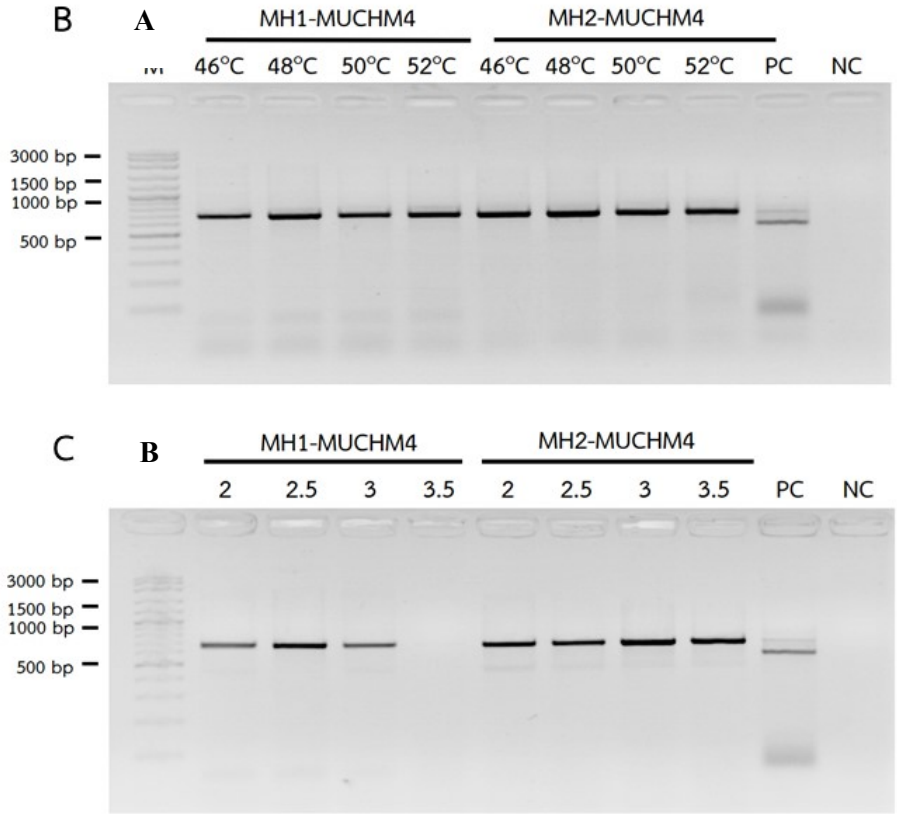

Figure 3 Optimization for heavy chain Fd fragment amplification. **A.** The heavy chain Fd fragment amplification was optimized annealing temperature and the optimal temperature was 48 ºC. **B.** The heavy chain Fd fragment amplification was optimized magnesium concentration and optimal magnesium concentration was 2.5 mM. M was DNA marker, Bioline 100 bp. PC was positive control. NC was negative control.

Construction of the pComb3HSS phagemid vector carrying KKU505 Fab sequence

MUCHM4 reverse primer with all heavy chain degenerated primers (MH1-7) and light chain reverse primer (MULK3) with all light chain forward primers (MULC1-7) which are provided in Table 1 were used to amplify heavy chain Fd and light chain sequence of KKU505, respectively. The specific band for heavy chain Fd and light chain fragments were about 600-700 bp. The KKU505 heavy chain Fd sequence was successfully amplified using MUCHM4 and degenerated forward

primers No. 1 and 2 (MH1 and MH2). The light chain was successfully amplified using forward primers No. 3, 4, 5, 6 and 7 (MULC3-7) (Figure 4). Heavy chain Fd and light chain fragment were eluted from electrophoresis gel before ligated into pGEM®- T easy vector and then heavy chain Fd and light chain fragments were digested from pGEM®- T easy vector to insert into pComb3HSS phagemid vector. The simulation diagram of construction the pComb3HSS phagemid vector with heavy chain Fd and light chain fragment is provided in Figure 5. After that, inserted pComb3HSS phagemid vector was transformed to *E.coli*. The colonies from transformed *E.coli* were selected to extract phagemid vector and validated by restriction enzyme to ensure that the vector was carrying the KKU505 heavy chain Fd and light chain Fab sequences. Heavy chain Fd fragment was digested with XhoI/SpeI and Light chain fragment was digested XbaI/SacI. The result showed that colony number C.HM.LC.1, 2, 3, 4 and 8 were successful to demonstrate the heavy chain Fd and light chain Fab fragment (Figure 6). The sequences from C.HM.LC.1, 2, 3, 4 and 8 were confirmed again by sequencing analysis. The result showed that all of these sequences were heavy chain Fd and light chain sequence and found regions of similarity between biological sequences. This suggested that the heavy chain Fd and light chain Fab sequences were carried in pComb3HSS phagemid vector.

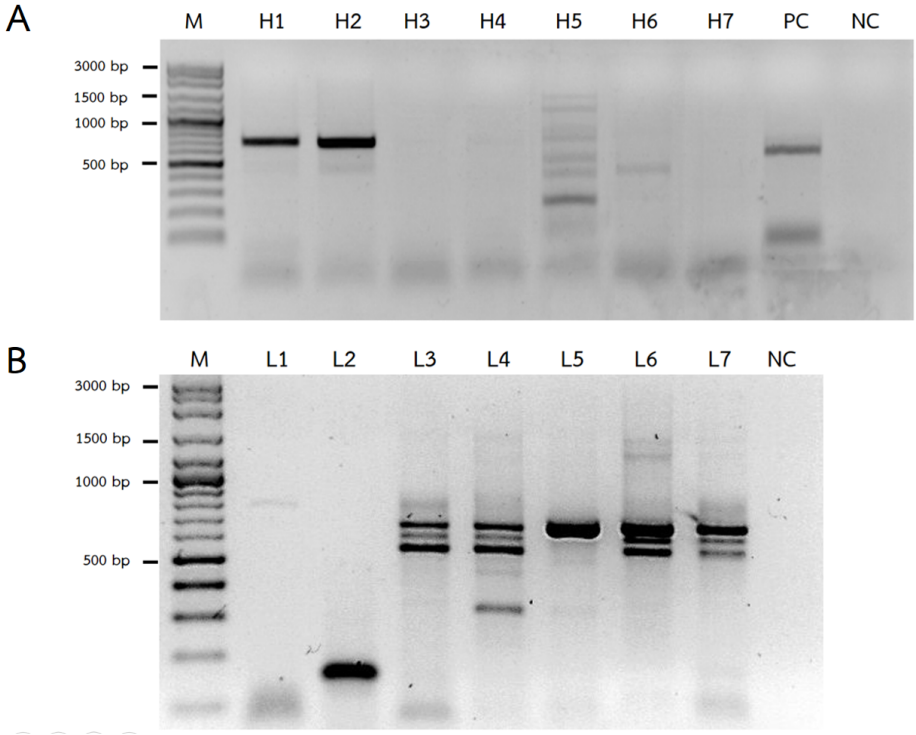

Figure 4 Gel analysis of PCR of KKU505 heavy chain Fd and light chain fragment. The heavy chain Fd and light chain fragments were amplified by different primers and the specific band was shown at 600-700 bp. **A.** For heavy chain Fd fragment amplification, the primers that could amplify this fragment were reverse primer; MUHCM4 with forward primers; MH1 and 2. **B.** For light chain fragment amplification, the primers that could amplify this fragment were reverse primer; MULK3 with forward primers; MULC3, 4, 5, 6 and 7. M was DNA marker, Bioline 100 bp. NC was negative control (PCR buffer without DNA template.

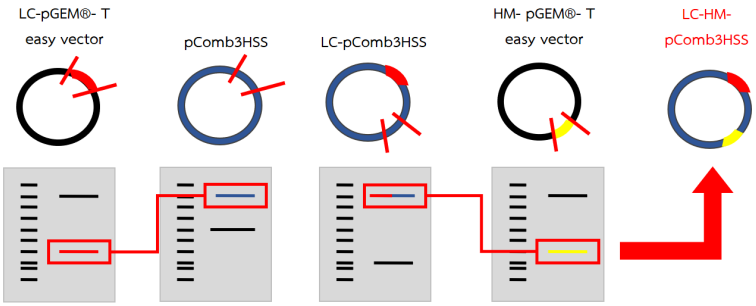

Figure 5 Simulation diagram and experiment for respectively construct the pComb3HSS phagemid vector with KKU505 Fab sequence. Simulation diagram showed the construction method for the pComb3HSS phagemid vector with KKU505 Fab sequence.

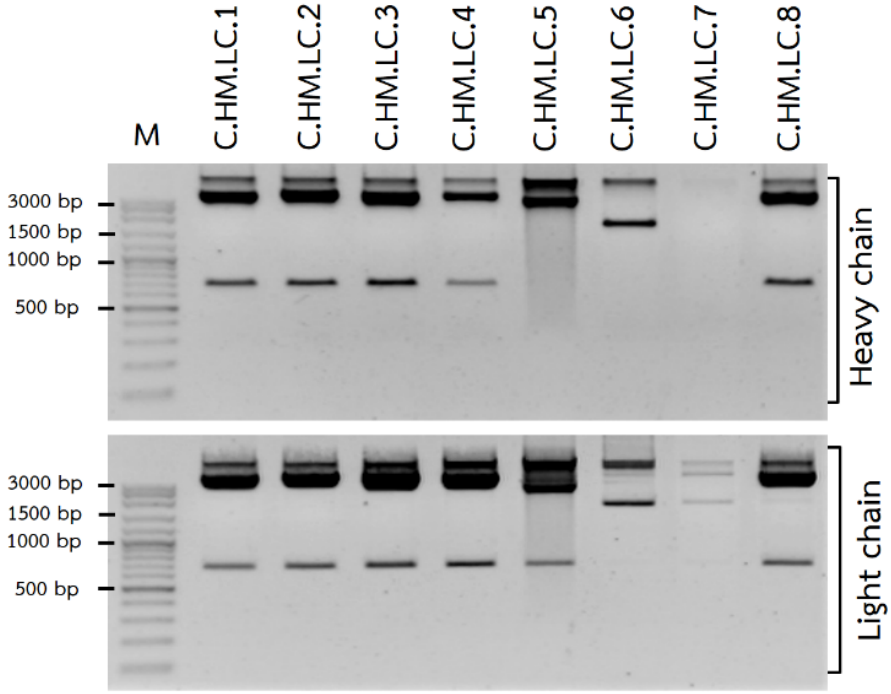

Figure 6 Verification of KKU505 Fab sequences insertion into the pComb3HSS vector. The heavy chain Fd and light chain sequences, which were inserted into the expression vector pComb3HSS were confirmed by restriction method. *XhoI*/*SpeI* restriction site for heavy chain Fd fragment and *XbaI*/*SacI* restriction site for light chain fragment. The specific band size is 600-700 bp. M was 100 bp Bioline DNA marker.

Discussion

An initial objective of the project was to preserve anti-*O. viverrini* antibody sequence from KKU505 hybridoma cell line, which can be translated to antibody-protein which can be utilized for *O. viverrini* infection detection in a urine sample for diagnosis[14]. In Chanika study, the anti-*O. viverrini* antibody was produced using hybridoma technique. The antibody from this technique is not stable. Several studies have

reported that the hybridoma technique had many limitations; there are no practical ways to alter the properties or improve antibody produced, and the cell line has a short half-life that can lead to cell death and genome loss[15, 16]. This study was designed to preserve the antibody sequence and maintain antibody production. For solving the hybridoma cell half-life issue, Phage display has been introduced as a technology to sustain hybridoma technology for antibody production[1, 2, 4, 7], because this technique has to construct phagemid harboring antibody sequence. Antibody sequence preservation in phagemid or nucleotide form has longer half-life and is more stable than hybridoma cell because nucleic acid is more stable than cell line[8].

We have developed the new reverse primers for using with degenerate forward primers for amplifying mouse IgM to the construction of phagemid vector carrying anti-*O. viverrini* Fab. For reverse primer design, the new primers were designed with a restriction site using mouse 3' end from constant region templates (sequence ID are AJ851868.3, S79401.1, AH003148.2, and V00818.1). The new primers were combined with degenerated forward primers[12], which were used for binding the complementary sequences of mouse heavy chain FR1 region or the 5' end of the variable region. This study successfully designed new reverse primers that is specific to amplify mouse IgM and had restriction site to the construction of phagemid vector carrying anti-*O. viverrini* Fab. In addition, the PCR products from this amplification were successfully constructed into pComb3HSS phagemid vector that might be used to produce the antibody specific to *O. viverrini* antigen. However, the antibody from this technique needs to be characterized in the future.

The expression of antibody on the surface of phage particles represents a powerful way for the isolation of monoclonal antibody with bio-panning that can serve for design and construct of antibody fragments[3, 17]. Therefore, antibody phage display can be applied to improve the affinity of antibody using mutagenesis technique and is easy to isolate a single clone from the mutagenesis technique to select the better phage display antibody. Several experiments could explain this idea, antibody against major histocompatibility complex class I[18] and a human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus were improved for binding activity by phage display technology[19]. This finding broadly supports that the anti-*O. viverrini* antibody affinity improvement can be increased *O. viverrini* detection in urine for better diagnosis with more sensitivity and specificity in the future.

Conclusion

The present study has demonstrated new reverse primers for using with degenerate forward primers for amplify mouse IgM to construction of phagemid vector carrying anti-*O. viverrini* Fab.

Take home messages

Antibody phage display was used to produce the antibody and sustain the antibodies from hybridoma cell line. However, there was no reverse primer for amplifying mouse Fd immunoglobulin M (IgM) and clone into the pComb3HSS phagemid vector. This study could design and demonstrate the efficiency of the primer for mouse IgM amplification for phage display technology.

Conflicts of interest

The authors declare no conflict of interest.

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