Molecular Cloning and Comparative Analysis of Variable Regions of Monoclonal Antibody against Enrofloxacin Clone 48

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ABSTRACT
Enrofloxacin is fluoroquinolone antibiotic which prohibited approved for treatment in animals. However, their residues in animal products can pose adverse side effects to consumer. Therefore, the maximum residue limit of these drugs has been enforced in many countries. In this study, the cDNA encoding VH and VL genes was amplified from monoclonal antibody which was specific to enrofloxacin clone 48, cloned and sequenced. The obtained sequences were compared in the NCBI databases by using blastp program. The results found that VH nucleotide was composed of about 399 bps and theirs deduced amino acids showed 80-85% degree identities to the Ig superfamily group. A totally 356 bps of VL nucleotide was found and showed the degree of identities of 97-100% with an immunoglobulin kappa light chain. Moreover, the CDR I, CDR II and CDR III of the VH and VL sequences were specified. The results indicated that the highest degree of VH sequence homology (highest relationship) found in accession no. B26471 with 80% homology. However, 100% of VL sequences were found similarly to accession no. AHJ10945.1 than the others. The obtained results provided the useful and important information for the further recombinant antibody construction and production against enrofloxacin antibiotic.

CCS Concepts
• Applied computing →Life and medical sciences → Computational biology → Molecular sequence analysis

Keywords
Monoclonal antibody; enrofloxacin; variable region; fluoroquinolone.

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1. INTRODUCTION
Enrofloxacin is an antibacterial agent that widely used for growth promotion, as well as for therapeutic treatment (pulmonary, urinary and digestive infections) particularly in poultry husbandry [1]-[3]. However, the extensive abuse of this antibiotic caused severe food safety problems. Meanwhile, some research indicates that low-level doses for long periods could result in bacteria resistance [4]. In addition, contaminated food sources can cause long-term anomalies such as allergic reactions and hypersensitivity [5], [6]. As a direct consequence, in September 2005 the American Food and Drug Administration (FDA) banned the use of the fluoroquinolones (FQs) compound enrofloxacin (Baytril®) in poultry production in the USA [7]. In many countries, the maximum residue limits (MRLs) have been established to protect customers [8], [9]. In the case of enrofloxacin, the MRLs were set to 100-300 ng/g, depending on the types of targeting tissues [1], [10]-[12].

In order to monitor enrofloxacin residue levels in livestock products, simple and rapid analytical methods are required. Various chromatography methods have been developed for the determination of enrofloxacin in different biological food matrixes [13], [14]. However these instrumental methods are time-consuming and costly and samples preparation are demanding [15]. During the last two decades, many immunoassay methods have been developed to detect enrofloxacin residue based on polyclonal antibody (PAb) [2],[16] and monoclonal antibody (MAb) [10],[17]. However, PAbs sometimes experience nonspecific reactivity and MAb preparation is extremely complicated, unstable hybridoma clone, requires expertise, respectively. Moreover, the preparation of high quality antibodies is still a bottleneck issue when establishing immunoassay methods [4], [5], [18].

Currently, recombinant antibody technology (RAb) has currently provided an alternative approach for engineering low-cost antibodies with desirable affinities and specificities [19]. With RAb, it is possible to manipulate immunoglobulin genes for the heterologous production.

Therefore, in the present study, we cloned and analyzed the variable region of heavy (VH) and light (VL) chains of MAb specific for enrofloxacin clone 48. The genetic information of

desired sequences will be useful for further anti-enrofloxacin antibody production by methylotrophic yeast *Pichia pastoris*.

2. MATERIALS AND METHODS

2.1 Preparation of First-Strand cDNA

Total RNA extraction from 5×10^6 hybridoma cells against enrofloxacin (Enro48) using a NucleoSpin® RNA II (Macherey-Nagel) was carried out according to the manufacturer's instruction. First strand cDNA coding for the VH and VL chains was synthesized from the total RNAs extract (approximately 1 μg RNA) by using a first-strand cDNA synthesis kit (Fermentas).

2.2 Construction of VH and VL genes

Here, the VH and VL genes were mainly constructed by PCR amplification and ligation-reaction. For amplification of VH and VL genes, the 1st strand cDNA fragments encoding the VH gene were amplified by using VHFwMH1 as forward primers and VHRwIgG1 as a reverse primer while those encoding the VL gene were amplified by using VLFwMk as a forward primer and VLRwKc as a reverse primer (Table 1). The PCR amplification was performed as descripted by [4]. Then, the amplicons were electrophoresed through 1.5% low-melting point agarose gel and visualized by staining with ethidium bromide. Each of the amplified DNA fragments corresponding to the predicted size was excised from the gel and was purified by using QIAquick PCR Purification Kit (QIAGEN). The purified fragments then were ligated individually into the pGEM® T-easy vector (Promega) and subsequently introduced into competent *E. coli* TOP10F' (Invitrogen). The blue and white screening method was used to select the positive clones (Amp^R) on Luria-Bertani (LB)(5 g/L yeast extract, 10g/L tryptone peptone and 10 g/L NaCl) agar plates containing 100 μg/mL ampicillin, 50 μL X-gal (20mg/ml) and 30 μL IPTG (0.1 M) respectively.

Table 1. The Universal Primers [20] for VH and VL amplification. The underline letters represent cloning sites, *Eco*RI (GAATTC) and *Sac*I (GAGCTC), respectively

Primer ID	Sequence (5'-3')	Purposes
VHFwMH1	GAATTCSARGTNMAGCTGSAGSAGTC	Amplified V_H
VHRwIgG1	GAATTCAATGACAGATGGGGGTGTCGTTTTGGC	
VLFwMk	GGGGAGCTCGAYATTGTGMTSACMCARWCTMCA	Amplified V_L
VLRwKc	GGGGAGCTCGGATACAGTTGGTGCAGCATC	

Degenerate bases: R= (A/G), Y=(C/T), M= (A/C), S=(C/G), W= (A/T), N= (A/G/C/T)

2.3 Verification of Sequence

To confirm the ligation-reaction of constructed VH- (or VL-) pGEM® T-easy plasmid, the following methods were carried out.

I) Restriction enzyme digestion: The Amp^R clones that individually harbored the VH or VL gene were digested with either *Eco*RI or *Kpn*I, respectively.

II) Sequencing analysis: To consider the VH and VL gene were cloned in frame with the T base overhang sequence in the pGEM® T-easy vector, the Amp^R clones which showed the expected size from I) were sequenced by Macrogen, Korea.

2.4 Identification of CDRs and FRs

The complementarity determining regions (CDRs) and framework sequences (FRs) sequences were identified by comparison between the obtained VH and VL sequence with IgG1 and light chain sequences from the database of GenBank.

3. RESULTS AND DISCUSSION

3.1 Characterization of Monoclonal Antibody (MAb) Clone Enro48

In this study, the anti-enrofloxacin-producing hybridoma clone 48 (Enro48) was selected, since it poses good antibody properties for the further development of an ELISA for enrofloxacin detection. It revealed the best several characterizations over the other clones. As reported in [21], the limit of detection (LOD) and the half maximal inhibitory concentration (IC50) were 1.01 ηg/mL and 0.42 μg/mL, respectively. Moreover, the cross-reactivity was ranged from 47% - 2100% with other FQs which is suitable for a kit test. It isotype was identified to be IgG1 and the light chain was a kappa type. Therefore, Enro48 was used as the source of genetic information for study the sequence of its variable region of heavy and light chains clone.

3.2 RNA Extraction and 1st Strand cDNA Synthesis

The total RNAs of MAb Enro48 were isolated by using NucleoSpin® RNA II kit. The concentration and purity of the extracted RNA were estimated by spectrophotometry. The absorbance at 260 nm indicated the concentration of 2.19 ± 0.07 μg/μL which the absorbance ratio of 260/280 nm estimated the purity of 2.03 ± 0.09. The RNA integrity was assessed prior to cDNA synthesis. The quality of extracted RNA was observed via 1.5% formaldehyde agarose gel electrophoresis followed by ethidium bromide staining (data not shown).

For the synthesis of 1st strand cDNA coding for the VH and VL genes, an approximately 1 μg of isolated RNA was used as the template in the reaction. In this experiment, the oligo $(dT)_{18}$ primer was selected to synthesize 1st strand cDNA with a reverse transcriptase enzyme. After completion of the reaction, the cDNA reaction mixture was examined by running through 1% agarose gel electrophoresis (data not shown).

3.3 Amplification of VH and VL Genes

The VH and VL genes were amplified separately by PCR using the 1st strand cDNA template. After separation, the DNA fragment through 1% agarose gel electrophoresis and subsequent ethidium bromide staining, the sharp band of VH DNA fragment was observed at approximately 399 bp (Figure 1A; lane1-3). The lengths of VL DNA fragment were approximately 356 bp (Figure 1C, lane 1-2). The agarose gel bands containing the desired DNA fragments were carefully cut and separately purified. After elution with TE buffer pH 8.0, the purified VH and VL DNA fragments

were then examined by 1% agarose gel electrophoresis (Figure 1B; lane 1 and Figure 1D; lane1-2).

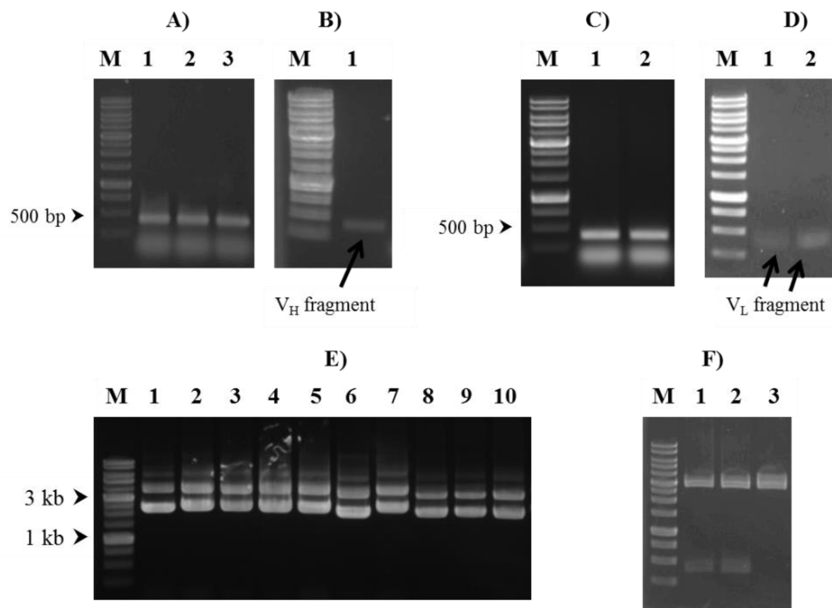

Figure 1. A) PCR product of VH amplification (lane 1-3). B) Lane 1: the purified VH fragment. C) PCR product of VL amplification (lane 1-2). D) Lane 1-2: the purified VL fragment. E) The recombinant pGEM®T Esay vector-VH fragment: lane 1-10; the obtained plasmid of 10 white colonies. F) Plasmid digested with EcoRI. Lane M: 1 Kb plus DNA ladder (Invitrogen). The expected band was indicated by an arrow.

The purified VH and VL DNA fragments were individually cloned into pGEM® T-easy vector by using T4 DNA ligase enzyme, then transformed to freshly competent *E. coli* TOP10F'. After 16 hours cultivation in LB plates containing ampicillin, IPTG and x-gal, the blue and white recombinant colonies appeared. Over 50 white colonies of each VH and VL fragments plasmids were randomly picked up and cultured in LB/amp liquid medium at 37℃ on the shaker, overnight. The plasmid was extracted by using alkaline lysis solution and the obtained plasmids were run through 1% agarose gel electrophoresis as shown in Figure1E.

3.4 Verification of Sequence

By restriction enzyme digestion, Figure 1F showed the linear DNA fragment after using *Eco*RI digestion. Lane 1 and lane 2 indicated that VHEnro48 and VLEnro48 clones were completely digested and the DNA fragment of VH was released with the size of about 400 bp while lane 3 represented pGEM® T-easy vector which extracted from a blue colony on LB/amp plate that no insert DNA fragment (negative control).

After confirmation with *Eco*RI digestion, the VHEnro48 and VLEnro48 clones were sent to analyze the nucleotide sequence by Macrogen, Korea. By using T7 primers and SP6 primers for sequencing, the nucleotide sequences of VHEnro48 showed 399 nucleotides and their deduced 133 amino acid sequence was shown in Figure 2. In addition, the nucleotide sequences of VLEnro48 clones revealed 356 nucleotides and their 118 amino acid sequence was shown in Figure 3. The VHEnro48 sequences have been submitted to the NCBI GenBank with accession no. AJG06889.1 and the VLEnro48 sequence will be being performed.

```
1   GAATTCGAGGTCGAG CTGGAGAAGTCTGGG GCAGAACTTGTGAAG CCAGGGGCCTCAGTC AAGTTGTCCTGCACA
1    E  F  E  V  E   L  E  K  S  G   A  E  L  V  K   P  G  A  S  V   K  L  S  C  T
76  GCTTCTGGCTTCAAC ATTAAAGACACCTAT CTACACTGGGTGAGG CTGAGGCCTGAACAG GGCCTGGAGTGGATT
26   A  S  G  F  N   I  K  D  T  Y   L  H  W  V  R   L  R  P  E  Q   G  L  E  W  I
151 GGAAGGACCGCGAAT GGTAATACTAGATAT GACCCGAAGTTCCAG GTCGAGGCCACTATA ACAACAGACACATCC
51   G  R  T  A  N   G  N  T  R  Y   D  P  K  F  Q   V  E  A  T  I   T  T  D  T  S
226 TCCAACACAGCCTAC CTGCAACTCAGCCGC CTGACGTCTGAGGAC ACTGCCCTCTATTAC TGTGCTAGATCCGAG
76   S  N  T  A  Y   L  Q  L  S  R   L  T  S  E  D   T  A  L  Y  Y   C  A  R  S  E
301 GGGATCTACTATAGC TACGCCTGGTTTGCT TACTGGGGCCAAGGG ACTCTGGTCACTGTC TCTGCAGCCAAAACG
101  G  I  Y  Y  S   Y  A  W  F  A   Y  W  G  Q  G   T  L  V  T  V   S  A  A  K  T
376 ACACCCCCATCTGTC TATGAATTC
126  T  P  P  S  V   Y  E  F
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Figure 2. The coding sequence (CDS) and amino acid sequence of VHEnro48 (accession no. AJG06889.1).

```
  1 GACATTGTGATGACA CAGTCTCCTGCTTCC TTAGCTGTATCTCTG GGGCAGAGGGCCACC ATCTCATACAGGGCC
  1  D  I  V  M  T   Q  S  P  A  S   L  A  V  S  L   G  Q  R  A  T   I  S  Y  R  A
 76 AGCAAAAGTGTCAGT ACATCTGGCTATAGT TATATGCACTGGAAC CAACAGAAACCAGGA CAGCCACCCAGACTC
 26  S  K  S  V  S   T  S  G  Y  S   Y  M  H  W  N   Q  Q  K  P  G   Q  P  P  R  L
151 CTCATCTATCTTGTA TCCAACCTAGAATCT GGGGTCCCTGCCAGG TTCAGTGGCAGTGGG TCTGGGACAGACTTC
 51  L  I  Y  L  V   S  N  L  E  S   G  V  P  A  R   F  S  G  S  G   S  G  T  D  F
226 ACCCTCAACATCCAT CCTGTGGAGGAGGAG GATGCTGCAACCTAT TACTGTCAGCACATT AGGGAGCTTACACGT
 76  T  L  N  I  H   P  V  E  E  E   D  A  A  T  Y   Y  C  Q  H  I   R  E  L  T  R
301 TCGGAGGGGGGACCA AGCTGGAAATAAAAC GGGCTGATGCTGCAC CAACTGTATCC
101  S  E  G  G  P   S  W  K  *  N   G  L  M  L  H   Q  L  Y
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Figure 3. The coding sequence (CDS) and amino acid sequence of VLEnro48.

3.5 CDRs and FRs Identification

The CDRs and FRs were identified by comparison with database from GenBank (NCBI) or published data. CDRs are part of the variable chains of immunoglobulins (antibodies), where these molecules bind to their specific antigens. As the most variable parts of the molecules, CDRs are crucial to the diversity of antigen specificities. Within the variable domain, CDR I, CDR II, and CDR III are found in the variable region of a polypeptide chain, and CDRIII is the most variable [4].

The CDR I, II and III of the variable region of both VH and VL were identified. As depicted in Figure 4, the predicted of CDRI (L1 hypervariable region), CDR II (L2 hypervariable region) and CDR III (L3 hypervariable region) of VH domain was located at position 26-35, 74-80, and 101-115 in the primary protein sequences, while the prediction of CDRI, CDR II and CDR III of VL domain was specified at position 23-30, 45-52, and 84-96, respectively.

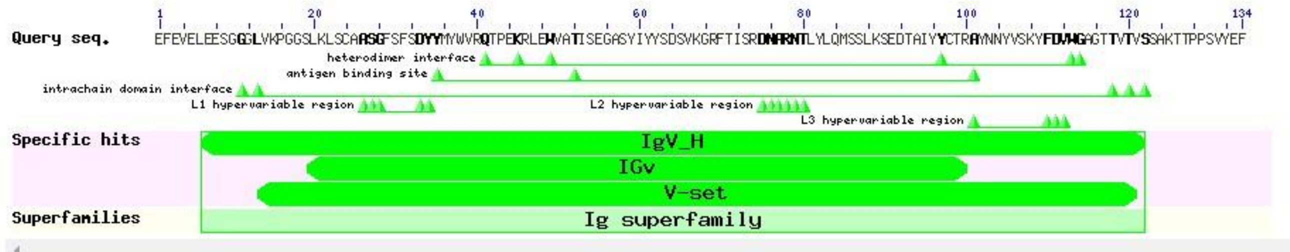

Figure 4. The primary protein sequences of VHEnro48 through the blastp program.

The primary protein sequences were aligned by searching against the GenBank database for sequence homology analysis. Through the BLASTP program (BLASTP programs search protein databases using a protein query), the result showed the similarity of VHEnro48 sequence with others. Over 50 different homologous sequences, each first-five of these sequences were selected and demonstrated. The comparative analysis of the amino acid sequence with the five known sequences in the database was shown and summarized in Table 2. According to the Table, it was found that the amino acid sequence of VHEnro48 gene was closely related to the Ig heavy chain precursor V region (MAK33) (accession no. B26471) with 80% identity. Moreover, the VHEnro48 sequence was found to be immunoglobulin IgG.

Table 2. Comparative analysis of amino acid sequence of VHEnro48 with sequences in the GenBank database using BLASTp program

No.	Accession	Description	Max score	Total score	Query cover (%)	Ident. (%)
1	B26471	Ig heavy chain precursor V region (MAK33) - mouse	214	214	98	80
2	5DO2_H	Chain H, Complex Structure Of Mers-rbd Bound With 4c2 Antibody	213	213	98	80
3	2J4W_H	Chain H, Structure Of A *Plasmodium Vivax* Apical Membrane Antigen 1- Fab F8.12.19 Complex	213	213	98	81
4	AGS48040.1	immunoglobulin mu heavy chain variable region [*Mus musculus*]	208	208	89	85
5	3VRL_H	Chain H, Crystal Structure Of Bmj4 P24 Capsid Protein In Complex With A10f9 Fab	211	211	98	80

Table 3 showed the result of amino acid sequence comparison between VLEnro48 and the most identical sequence that found in the GenBank database by using blastp program. The result indicated that VLEnro48 sequence was found to be an immunoglobulin kappa light chain (Figure 5). Moreover, the VLEnro48 sequence was most closely related to the anti-3ABC nonstructural protein ScFv immunoglobulin gamma light chain [synthetic construct] with the percentage of identity 100%.

Figure 5. The primary protein sequences of VLEnro48 through the blastp program.

Table 3. Comparative analysis of amino acid sequence of VLEnro48 with sequences in the GenBank database using BLASTp program

No.	Accession	Description	Max score	Total score	Query cover (%)	Ident. (%)
1	AHJ10945.1	anti-3ABC nonstructural protein ScFv immunoglobulin gamma light chain [synthetic construct]	226	226	91	100
2	ACV40677.1	anti-chloramphenicol immunoglobulin kappa light chain variable region [*Mus musculus*]	224	224	91	100
3	AFR11387.1	anti-Staphylococcal enterotoxin K immunoglobulin light chain variable region [*Mus musculus*]	224	224	93	98
4	AEX96941.1	anti-aflatoxin immunoglobulin alpha light chain variable region [*Mus musculus*]	224	224	94	97
5	ACJ05306.1	truncated immunoglobulin light chain variable region [*Mus musculus*]	223	223	91	99

4. CONCLUSION

We have successfully cloned and sequenced the VH and VL genes of the monoclonal antibody against enrofloxacin clone 48 (Enro48). The CDRs of amino acids that contribute the binding activity of VH and VL antibodies were predicted. Various results have been supported that the genetic information of these genes is possible to be used for construction and production of recombinant antibody which specifics for the enrofloxacin antibiotic in the future.

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