Research in Molecular Medicine

Molecular Cloning of DARPins G3 in pET28b Expression Vector and Optimization of the Expression of This Protein in *Escherichia Coli*

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ABSTRACT

Background: Human epidermal growth factor receptor 2 (HER2) is over-expressed in breast, ovarian, gastric, and prostate cancers and is used as a tumor marker in the diagnosis of cancer. Monoclonal antibodies have been used as a diagnostic and therapeutic tool against HER2. Because of the difficulties associated with the stability and complexity of the construct and the high cost of antibody production, we aimed to investigate, cloning, and expression of HER2-binding DARPins genes to identify, HER2-positive tumor markers, we aimed to investigate.

Methods: After synthesis, the DARPins peptide gene was cloned into the M13 vector and sub-cloned into the TOP10 pet28b bacterial vector. After culturing the bacteria on an agarose plate containing antibiotics, the transfected bacteria expressing the DARPins gene were selected. To ensure gene cloning, we used enzymatic digestion and recombinant plasmid delivery for sequencing. Isopropyl β-d-1-thiogalactopyranosideIPTG was used for the induction of recombinant protein expression and the SDS-PAGE method and Western blot for expression confirmation.

Results: The polymerase chain reaction (PCR) amplification product of DARPins was analyzed using agarose gel electrophoresis. Plasmid was purified from the positive clone by PCR cloning, sequenced and gene cloning was confirmed. After culturing from competent cells, protein expression was obtained from positive colonies. SDS- PAGE results showed the effect of different conditions including temperature, IPTG concentration, and time on the pET-DARPins expression.

Conclusion: We were succeeded to express a new codon-optimized DARPins gene in *Escherichia coli* and HEK293t system.

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Introduction

Human epidermal growth factor receptor 2 (HER2) is highly expressed in breast, ovarian, gastric, and prostate cancers. Overexpression of HER2 is found in 25% of breast cancer cases, which is associated with a high tumor invasion phenotype that is highly resistant to endocrine therapy. The lifetime of people with HER2-positive breast cancer is shorter than patients without the over-expression of this protein [1]. Gastric cancer, the second leading cause of death among cancers in the world, has a high expression of HER2 [2, 3]. The diagnostic and therapeutic value of this protein in breast cancer is well established. Epidermal growth factor receptor or HER2 is a 185 kDa protein called ErbB2, c-erbB2, or HER2/neu, which has tyrosine kinase activity in the intracellular domain and has the second ligand binding outside the cell that can interact with other members of the family. HER2 plays an important role in cell growth, survival, and differentiation. However mutation or overexpression directly plays a role in tumor formation and metastasis, high expression of this protein and its availability at the extracellular level make HER2 an ideal target for the targeted delivery of anticancer drugs.

Since the discovery of the role of HER2 in tumorigenesis, this protein has been the focus of cancer research [4]. The successful discovery of the monoclonal anti-HER2 antibody called trastuzumab has encouraged scientists to produce various types of anti-HER2 antibodies [5]. Monoclonal antibodies, due to their half-life in serum, dual antigen binding, and immuno-protein function, are used well in medicine [6]. However, their monoclonal antibodies face a wide range of limitations in use. Antibodies are complex proteins, with several domains consisting of two different chains (heavy and light) and a total of six different domains (VH, VL, CH1, CH2, CH3, and CL) [7].

The correct formation of the antibody molecule, and more importantly its structural stability, relies heavily on disulfide bonding and post-translational modifications involving glycosylation of the constant molecule domains. On the other hand, the expression levels of these molecules in human cells are low and have a high cost [8]. In general, the above conditions complicate the conditions and lead researchers to produce non-antibody constructs for treatment and imaging as well as detection. One of the molecules under investigation is duplicate proteins designed based on ankyrin or DARPins [9]. DARPins, designed ankyrin repeat proteins, are very stable due to the tight and regular packing of the repeat segments and conserved hydrogen bonding networks [10]. DARPins are a new class of binding molecules capable of overcoming the limitations of monoclonal antibodies that can be used to bind to any target protein with high specificity and affinity. These features have made them ideal drug candidates for inhibitory, antagonistic, and agonistic properties. Besides, these synthetic peptides have a high binding affinity and specificity to their target proteins.

DARPins are small single-domain proteins (14 kDa) primarily consisting of repeated motifs capable of binding to any kind of biological receptors. DARPins can be engineered to produce efficacious or a combination of several proprietary junctions, and the ability of these drug forms provides new ones [9]. DARPins has no experimental and technical limitations for their action in different hosts [11]. In this study, the DARPins peptide gene was cloned into *E. coli* and then the protein expressed in that purification was examined for the detection and identification of HER2 antigens.

Materials and Methods

Bacterial strains, reagents, and plasmids

The following bacteria were used in this research: *E. coli* strain TOP10 F and *E. coli* (BL21). Antibiotics were used for bacterial culture ampicillin and kanamycin (Merck, Germany) and restriction enzymes for plasmid cleavage BamH1 and Xho1 (TAKARA, Japan). The chemicals needed to build the culture medium for the growth of *E. coli* were Agar-Agar, peptone, Yeast extract (Micromedia, English), and NaCl (Merck, Germany). Protein and DNA electrophoresis buffer materials were supplied from Merck and Sigma (Germany) and the antibody Anti-His-Taq for Western blotting from Santa Cruz (America).

Gene synthesis, primer design, and pcr method

We optimized the gene sequence from the articles and it was synthesized by Gene Script Company and sent to us in vector PUC 57. DARPins as synthetic genes as templates were amplified using forward (5-ATGTCTATGAGGGTTCGGCGC-3′) and reverse (5′-CTATCTTCCCCTTTTAATGGTCAG-3′). The Polymerase chain reaction (PCR) was carried out under the following conditions: initial denaturation temperature of 95°C for 2 minutes followed by 35 cycles of denaturation of the 30s at 95°C, 30s at 55°C, 45s at 72°C, with final extension for 5 minutes at 72°C using a thermocycler (Bio-Rad, USA). The PCR products were analyzed by electrophoresis on 1% agarose gel and visualized by Gel Doc (Biotium, England). Then, the PCR product was purified using a Vivantis nucleic acid extraction kit.

Bacterial culture on LB medium

The target bacteria were grown on Luria-Bertani (LB) Agar plate and LB broth medium containing 100 µg/mL kanamycin for the Top10 strains and ampicillin 50 µg/mL ampicillin for *Escherichia coli* BL21(DE3). Then, the LB agar plate was incubated at 37°C overnight and LB broth media to put in a 37°C for 2-4 hours at 170 rpm in a shaking incubator until the cell density reached an optical density (OD=2) in 630 nm.

Extraction and purification of plasmids

Plasmid extraction was performed according to Favor Prep's instructions (Minikit) from 16h cultured cells. Plasmids were then subjected to electrophoresis on 1% agarose gel using the standard protocol. The gels were visualized on an ultraviolet transilluminator (UVP, high-performance transilluminator; USA). A particular plasmid band was excised from the gel and purified with the Favor Prep™ plasmid DNA extraction mini kit (Favorgen Biotech Corp., Taiwan).

Enzymatic digestion and ligation reaction

The PCR product of the DARPins gene and pET28b plasmid were double-digested in a 20 µL volume consisting of 12 µL PCR product, 2 µL 1X K common buffer,1 µ Xho1 (10 U μL^{-1}), 1 µL BamH1(10 U μL^{-1}), 4 µL ddH20 at 37°C for overnight (12-16 hours). The ligation of the amplified DNA product (Insert DNA) and the vectors (pET28b) was carried out in a molar ratio of 3:1 following the Takara protocol, by the T4 DNA ligase an hour at 22°C and the mixture was transformed in expression bacteria *E. coli* (BL21) (DE3).

Competent cell preparation, plasmid transformation

Competent cells of *E. coli*, TOP10, and BL21 strains were prepared as described by Sam brook et al. [12]. For transformation, 10 µL of plasmid DNA was added to a tube containing 0.2 mL of competent cells. The mixture was placed on the ice for 20 minutes, and then exposed to heat shock at 42°C for 1 minute and kept immediately in ice for 10 minutes. The sample was then centrifuged at 3800 rpm for 20 minutes and the resulting precipitate was dissolved in 300 of calcium chloride solution or water, distilled and sterilized 4 times on the ice, and divided into 100 microliters into 1.5 mL microtubes for transformation. Transformed bacteria were cultured on plates containing ampicillin, Isopropyl β-d-1-thiogalactopyranoside (IPTG), and 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) due to the presence of X-gal substance inside these plates [13].

Identification of recombinants

The DARPin genes were amplified from the PUC57 vector and BsitesamH1 and Xho1 cloning sites were inserted into the amplified gene sequence. Then, the fragment was cloned in M13 by the TA cloning method and transformed into the TOP10 strain. After that, the transformed cells were cultured in the LB agar medium containing IPTG/Xgal and the white colonies were selected containing the desired plasmid for more confirmation colony PCR [13].

The plasmid was extracted from the positive colony and double-digested with specific restriction enzymes. After sequencing the plasmid was digested, and the fragment was cloned into pET28b and cultured on Kanamycin containing LB agar medium. The plasmid extraction and sequencing were performed on the bacteria which formed a colony for final confirmation.

Optimization of recombinant protein expression

After confirming the DARPins-pET28b construct, the plasmid was transformed into the competent BL21 strain of *E. coli*, prepared by the calcium chloride method [14]. The transformed bacteria were grown in an LB broth containing 100 µg/mL ampicillin at 37°C in a shaker (200 rpm) until the optical density (OD600) at 600 nm reached 0.5-0.6. Then, the T7 promoter was induced by 1.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 4-6 hours (to increase the expression). Three factors were analyzed for optimization of recombinant protein expression including the effects of temperature on the induction (20, 25, 30, and 37°C in overnight), time (4, 6, 8, 10, and 16 hours at 37°C), and inducer concentration (0.2, 0.5, and 1, 2 mM in 37°C, and overnight) were investigated using the Taguchi statistical method (Table 1).

Western blot analysis

Total protein was extracted from transformed bacteria using a lysis buffer containing the protease inhibitor cocktail (Roche, Germany). Briefly, bacteria were washed twice with ice-cold PBS and pelleted by centrifugation at 3000×g for 10 minutes. Lysis buffer was added to the cells in a microfuge tube and incubated on ice for 20 minutes with shaking. Cell debris was precipitated by centrifugation at 13000 rpm for 20 minutes and the upper phase was collected to use as a total protein sample. The quantity of total proteins was calculated using the Bradford assay. Ten µg of extracted protein was boiled for 5 minutes in 1 µg

Table 1. Parameter effects on the Taguchi method

Factors	Range				
Time	4 h	6 h	8 h	10 h	16 h
Temperature	18°C	20°C	25°C	30°C	37°C
Inducer Contraction (IPTG)	o.2 mM	0.5 mM	1 mM	1.5 mM	2 mM

of 10X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS) sample buffer and run on 10% polyacrylamide gel for 2 hours. Protein fragments were transferred to the nitrocellulose membrane under the constant current of 300 mA for 2 hours and the membrane was blocked in 5% non-fat dry skim milk in PBS for 2 hours by shaking. The membrane was washed in PBS and probed overnight with HRP-conjugated anti-His tag antibody to detect DARPins G3 protein. After 3 times of washing with PBST, the desired band was detected using ECL solution (Pars Toos Teb co., Iran).

Results

Amplification of DARPins gene

The gene sequence related to DARPins was selected from the articles [15], and after optimization for expression, both eukaryote and prokaryotic cells were synthesized by Gene Script Company. To amplify the DARPins gene, specific primers were designed based on the DARPins gene sequence and the genetic map of the pET28b expression vector and restriction sites for BamH1 and Xho1 enzymes. The BamH1 enzyme restriction site sequence was added to the forward primer and the Xhol enzyme restriction site sequence was also added to the reverse primer (Table 2). The DARPins were amplified using PCR. The PCR product was analyzed using 1% agarose gel electrophoresis (Figure 1).

Cloning of DARPins peptide gene fragment at the desired location of vector PET28b

To clone the DARPins in pET28b, the PCR product of the previous step and pET28b were digested by Xho1 and BamH1 restriction enzymes, and the product was ligated into PET28b. Then, the ligation product was transformed into the competent cell and growth on LB containing IPTG-X-gal medium in the positive colony (Figure 2). Colonies were randomly selected, colony PCR was performed (Figure 3), and the plasmid from the

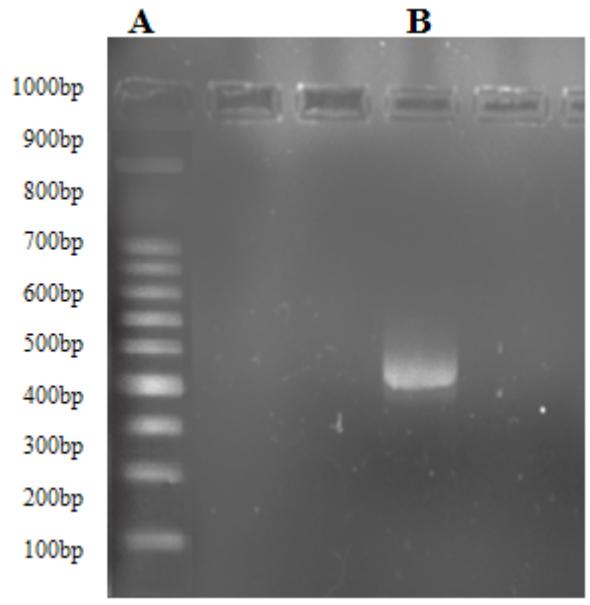

Figure 1. Agarose gel electrophoresis of DNA from DARPins peptide amplification by PCR
Lane A: 100bp DNA ladder; Lane B: 400bp DARPins band.

Table 2. List of primers applied for PCR and cloning

Construct	Templet	Primer-Sequence
DARPins-Forward	DARPins-synthetic	5-ATGTCTATGAGGGTTCGGCGC-3
DARPins-Reverse	DARPins-synthetic	5-CTATCTTCCCCTTTTAATGGTCAG-3
DARPins-F-Bamh1	Restriction site-Bamh1	5-CGGGATCCGGGAGCGATCTGGGTAAG-3
DARPins-R-Xho1	Restriction site-Xho1	5-CCGCTCGAGGCTACCGCCGTTGAGTTTC-3

Two set primers were designed to generate recombinant DARPins constructs; DARPin forward and reverse were designed for PCR, and DARPin BamH1 and DARPin Xho1 were designed for gene cloning.

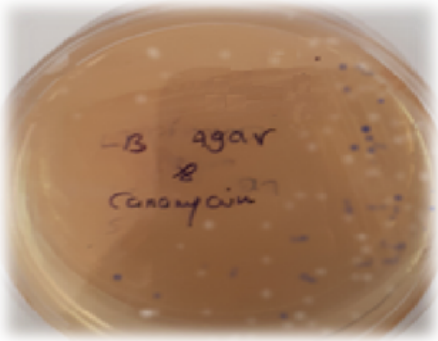

Figure 2. Agar plate with blue and white bacterial colonies
The white colonies could not digest the X-gal (had desired gene) but the blue colonies had this ability.

positive clone was purified, sequenced, and confirmed the gene cloning.

Confirmation of recombinant protein expression

The protein expression from positive colonies was obtained after culturing from the competent cell. The positive clones which had pet-DARPins were grown at 37°C at 170 rpm in 50 mL of LB medium containing100 µg/mL Kanamycin. The protein was expressed by the addition of 1.5 mM IPTG when the culture reached an OD 600~0.6-0.8. In this research, the expression of recombinant pET-DARPins protein using Anti-His Tag HRP-conjugated by western-blotting after repeated SDS-PAGE reaching an optimal temperature of 37°C for 6 hours and IPTG concentration of 1.5 mM was confirmed (Figure 4).

Optimization of gene expression

Optimization of gene expression was performed by the Taguchi method and the induction was continued for 4 to 6 hours at 37°C. However the sample with concentrations of 0.5, 1, 1.5 mM IPTG in a volume of 5 mL of LB broth, incubated in 4 ranges of temperature consisting of 20, 25, 30, 37°C, and different times of 2, 4, 6, 8, 10, and 16 hours were examined many times, due to the weak band in the SDS-PAGE, after reviewing the related articles, the best expression at 37°C and induction time of 4 to 6 hours in the presence of 1.5 mM IPTG was obtained and confirmed by SDS-PAGE and

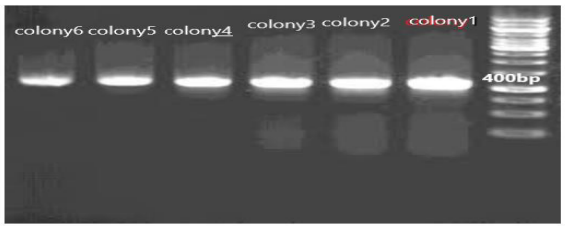

Figure 3. Electrophoresis images of positive colonies after preparation of competent cell and colony PCR confirmation competent cell preparation step and detect of 400bp protein in comparison with 100-1000 bp DNA ladder

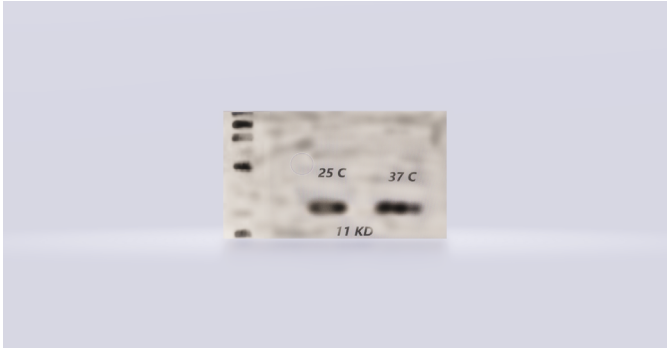

Figure 4. Western blotting image related to the expression of recombinant Pet-darpins protein

Notes: The expression of the recombinant pET-DARPins protein was examined in the best expression conditions by the Taguchi method, which was confirmed by the HRP-conjugated anti-His Tag antibody. The 11 kDa band of the recombinant target protein was confirmed.

Western blotting methods. This protein size is 11-kD. The results of recombinant protein optimization using the Taguchi method are shown in Table 2. SDS-PAGE results show the analysis of variance for the responses of recombinant protein production carried out according to the factor's contribution by the Taguchi method. Also, the optimal conditions were obtained by analyzing the final concentration of the recombinant protein and the variance of productivity (Figure 5).

The average effect of the investigated three factors showed that changes to each factor influence the amount of produced protein. Hence, it can be concluded that different times and IPTG concentrations had the greatest influence on the production of the recombinant protein. Also, the induction temperature had the greatest effect impact on protein production. Furthermore, the results showed the optimum conditions for the production of PET28b- DARPins recombinant protein in *E. coli*. Figure 5 shows the effect of different conditions including temperature, IPTG concentration, and time on the pET-DARPins expression. After optimizing the production conditions, recombinant protein western blotting was done. Western blotting may be a powerful technique for quantifying macromolecule levels; but, it is typically not well-optimized.

Discussion

Designed ankyrin repeated protein is a new class of synthetic peptide witch template from Ankyrins, the family of proteins that mediate the attachment of integral membrane proteins to the spectrin-actin-based membrane cytoskeleton. DARPins can recognize targets with specificities and high affinities that equal those of antibodies; but, because of their robustness and extreme stability, they allow a multitude of more advanced formats and applications.

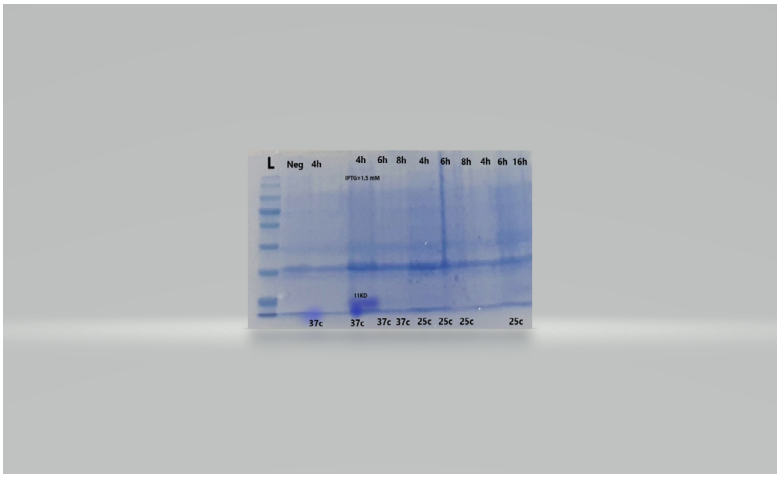

Figure 5. SDS-Page recombinant pET-DARPins protein, expression optimization, and investigation of three variables of Time, temperature, and IPTG concentration on the expression quality of pET-DARPins recombinant protein.

The DARPins library was built based on a study of thousands of DARPins molecules found in nature. Each DARPins molecule consists of 33 amino acid sequences that have been repeated at least 3 times. In the structure of this repetition, there are 2 alpha helix motifs along with the beta-screw structure. This amino acid molecule does not contain cysteine and propylene. So far, peptides have been designed to bind to EGFR, EpCAM, HER2, and β amyloids.

In 2006, the Pulkham group selected the G3 DARPins molecule against the HER2 molecule and expressed it in bacteria. In 2007, the group was able to improve and isolate 90-picmular DARPins by error-prone PCR technique for HER2 with a high-level expression as much as 10 mg/L in bacteria [16]. In 2010, this molecule, called G3, was used as a chimera with adenovirus and lentivirus envelope proteins to target and transfer genes to breast cancer cells. In 2015, this molecule with technetium 99 was labeled for use in imaging cancer cells [17]. This protein has a high affinity to HER2, the same as an antibody. Because of the robust property, we resigned, synthesized, and cloned this peptide gene in pet28b with some modification in the primary gene.

In a previous report on DARPins gene expression systems, Zahang et al. used pQE-30 (Qiagen) system and the QIA express system using *E. coli* strain M15 [pREP4] which permits high-level expression [16]. Robert Goldstein et al. cloned and expressed this gene in P. pastoris [17]. DARPins constructs were expressed in *E. coli* XL1-Blue by Zahnd et al. [18]. Evgeniya Sokolova cloned DARPins in the pet22b vector and expressed it in the *E. coli* BL21 (DE3) strain cells [19]. We used the pET28b expression system in the BL21 (DE3) strain of *E. coli*. For some purposes, we need one gene for expression in two different systems: prokaryotic and eukaryotic cells. Then, we resigned the basic gene with some modifications. Firstly, we optimized the codon-based on the selections of specific codons with minimum limiting expression in dual pro and eukaryotic cells. Therefore, we chose the most suitable rare codon from those works in both systems. Secondly, we designed a gene based on the expression in HEK293t and optimized it by gene script company tools, and then we altered some moderate rare codons manually, based on the codon probability table witch compatible with two different systems.

DARPins G3 was applied to target exosome-derived HEK and IDC cells to transfer siRNA to HER2-positive cancer cells [20].other group of researchers expressed darpin in bacteria by codon optimization, DARPins-functioning liposomes that carry large amounts of fluorescent or toxic proteins can be promising candidates for tumor diagnosis and treatment [21]. Anti-EGFR DARPins are targeted molecules that are effectively produced in bacteria and can inhibit A431 cell proliferation and receptor recycling, as EGFR is a valuable target for tumor treatment. [22]. In another study, *E. coli* BL21 (DE3) was used to produce and express the recombinant DARPins-LOPE protein that [11].

Based on literatures, DE3 is the best choice for darpins expression, therefore we applied it for gene expression, After cloning, 1st, we determined the probability expression of the new gene in the origami strain and cultured bacteria in 1 ug/mL IPTG, when the OD of the bacterial population was 0.6 overnight at 37°C. Fortunately, the protein expression was confirmed by western blot. Then, we optimized gene expression by 4 effective variable factors including temperature, inducer concentration, and bacterial x optical density (OD) before inducing and induction time by the Taguchi method. We determine the best temperature, between 18, 25, 30, and 37. In 37 best concentration of inducer is 1.5 mg/mL and the best induction time is 8 hours.

Conclusion

Codon optimization can help researchers to express a gene in two different systems, and we succeeded to express a new codon-optimized DARPins gene in the *E. coli* and HEK293t system.

Ethical Considerations

Compliance with ethical guidelines

This article is extracted from a thesis entitled "Molecular cloning of G3 DARPin in pET28a expression vector and optimization of this protein expression" (Code: IR.MAZUMS.REC.1397.187).

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Authors' contribution

All authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

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