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Original Research Article

Immunoprotective evaluation of *Escherichia coli* outer membrane protein A against the main pathogens of animal mastitis

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Abstract

Purpose: *To evaluate prokaryotic expression of the Escherichia coli (E. coli) outer membrane protein A (OmpA) and its immunoprotective function against the main pathogens of animal mastitis.*
Methods: *A molecular cloning method was used to develop a prokaryotic strain expressing OmpA protein, which was purified by Ni-affinity chromatography. Polyclonal antiserum was generated in mice immunized with OmpA protein. Enzyme-linked immunosorbent assay (ELISA) and western blotting were used to determine the titer and verify anti-OmpA serum specificity, respectively. Interaction between OmpA antiserum and main pathogens of animal mastitis was verified by ELISA and a pull-down method. The immune protective function of OmpA protein was evaluated in mice challenged with pathogens of animal mastitis. Optimal fermentation conditions to produce OmpA protein were determined by the L9(34) orthogonal test.*
Results: *A prokaryotic strain expressing OmpA protein was developed, and purified OmpA was used to develop a mouse polyclonal antibody. The anti-OmpA serum exhibited high specificity and a titer of 1:1600. Anti-OmpA serum directly interacted with E. coli and Staphylococcus aureus (S. aureus). OmpA demonstrated a significant immune protective function of 58.33 % against E. coli and 46.15 % against S. aureus. The optimal conditions for expressing fermentation OmpA were a strain absorbance of 0.5 at a wavelength of 600 nm, IPTG final concentration of 0.3 mmol/L, induction time of 12 h, and induction temperature of 28 °C.*
Conclusion: *OmpA possesses selective immunogenicity and a significant immune protective effect against the main pathogens of animal mastitis. The results suggest that OmpA may potentially be used as a vaccine for animal mastitis.*

Keywords: *E. coli, OmpA protein, Immunoprotection, Animal mastitis, Protein fermentation*

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INTRODUCTION

Mastitis is a common disease in the animal breeding industry, especially in cows and dairy goats [1]. Its main pathogens are *E. coli*, *S. aureus*, and *Streptococcus* [2,3]. Repeat infections resulting in animal mastitis occurs easily, seriously affecting milk production in cows and goats and causing huge economic losses to the dairy industry [4]. At present, the treatment for animal mastitis mainly consists of antibiotics, which inevitably leads to drug resistance, drug residues, and environmental pollution [5,6]. In addition, *E. coli* and *S. aureus* also affect human health, which are zoonotic pathogens [7]. Therefore, it is necessary to develop a new type of drug to cure animal mastitis.

Outer membrane proteins (OMPs) are the main extracellular proteins of *E. coli.* They play an important role in drug resistance, substance transport, immune recognition of the bacteria and host, and improve pathogenicity [8]. Outer membrane protein A (OmpA) is an important outer membrane protein of *E. coli*. OmpA protein has a β-barreled transmembrane structure, and is a mutual recognition protein between *E. coli* and host cells [9]. Thus, OmpA protein improves the pathogenicity of *E. coli* and is a target recognition protein of the immune system. Studies have shown that OmpA protein has a preferential immunogenicity, could activate the immune response in animals [10], and improves the ability of animals to resist bacterial infection. Taken together, these observations suggest that OmpA protein may be a good candidate for a possible vaccine.

EXPERIMENTAL

Chemicals and reagents

E. coli, *S. aureus*, and pET-32a plasmids were obtained from the Shaanxi University of Technology bacterial conservation center. Primer synthesis and gene sequencing were completed by the Beijing Oak Science and Technology Corp, China. Endonuclease, HRP secondary antibodies, and TMB were obtained from Sigma-Aldrich, USA.

Animals

Mice were obtained from the College of Medicine, Xian Jiaotong University, China. All animal procedures were performed in accordance with the guidelines prescribed in Guide for the Care and Use of Laboratory Animals [11] and were approved by the ethics committee of the Shaanxi University of Technology, China (approval ref no. 20170907).

Construction of prokaryotic expression strain of *ompA* gene

Primers for the *ompA* gene primers were designed based on the *E. coli ompA* gene sequence in the GenBank database (accession number LN832404.1): sense primer5'-CGGGAATTCATGAAAAAGACAGCTATC-3'; anti-senseprimer: 5'-CCCAAGCTTTTAAGCCTGCGGCTGAG-3'. (The underscore represents the site of *Eco*R I and *Hin*d IIIenzyme cleavage.) The *E. coli* genome was extracted using a genomic Extraction Kit (TaKaRa, Japan). The PCR system consisted of 2.5 μL buffer, 2 μL dNTP (10 mmol/L), 1.5 μL primers (25 μmol/L), 5 μL template DNA, and 0.2 μL Taq enzyme (TaKaRa, Japan). The PCR parameters consisted of 32 cycles of pre-denaturing for3 min at 94°C, denaturing (30 s at 94°C), annealing (45 s at 55°C), and extension (90 s at 72°C), followed by full extension at 72°C for 10 min. PCR samples were separated and recovered with 0.8% agarose gel electrophoresis. After the PCR product and pET-32a plasmid vector was digested, the recombinant plasmid pET32a-*ompA*was developed by ligase ligation (TaKaRa, Japan). The recombinant plasmid was identified with double enzyme digestion analysis and sequencing. Then, the recombinant plasmid of pET32a-*ompA* was transformed into *E. coli* BL21 strain to create the OmpA protein-expressing strain.

Prokaryotic expression and purification of OmpA protein

Expression and purification were performed as described previously. Briefly, OmpA recombinant strains were cultured overnight and transferred to fresh LB medium. At an OD_{600}value of 0.5, IPTG was added to a final concentration of 0.5 mmol/L; the strains were then cultured at 37°C for 5 h. The expression of OmpA was assessed by SDS-PAGE electrophoresis. The OmpA protein was loaded onto a Ni-affinity chromatography column and purified using a Ni-NTA flowresin method (Sigma-Aldrich, USA) [12].

Preparation of mouse anti-OmpA polyclonal antiserum

At 4-5 weeks of age, Kunming mice were randomly selected and purified OmpA protein was intraperitoneally injected three times. The experimental and control groups were immunized with OmpA protein (50 μg per mouse) and PBS

solution, respectively. OmpA protein and the control of PBS solution were emulsified with Freund's Complete Adjuvant (Sigma-Aldrich, USA) for the first immunization. After 14 days, the mice were boosted using Freund's incomplete adjuvant (Sigma-Aldrich, USA) [12]. After 7 days, a third immunization was performed. Then, the eyeballs of mice were dissected under anesthesia to harvest OmpA antiserum, which was then stored at -80°C.

Specificity and titer detection of OmpA protein antiserum

Specificity of OmpA antiserum was evaluated by western blot analysis. Briefly, *E. coli* lysates were resolved by SDS-PAGE and transferred to nitrocellulose (NC) membrane (TaKaRa, Japan) by electrotransfer. After incubation in a skim milk solution for blocking, the membrane was incubated with mouse anti-OmpA serum. Then, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (TaKaRa, Japan). The NC membrane was then incubated with a DAB solution (Sigma-Aldrich, USA) coloration system to visualize bands [12]. OmpA serum specificity was determined according to the color of NC membrane bands.

Antiserum titer was determined by the ELISA method. Briefly, the OmpA protein was diluted to 0.5μg/μL, and 100 μL solution was added to 96-well plate at 37°C for 3 h. After incubating in skim milk solution for blocking, 100 μL of anti-OmpA serum was added to each well and the plate was incubated at 37°C for 30 min. After rinsing, 100 μL of horseradish peroxidase-conjugated anti-mouse secondary antibody was added to each well. Then, each well was incubated with a coloration solution (Sigma-Aldrich, USA) at 37°C under dark conditions for the color reaction. Finally, a stop solution was added, and the absorption at OD_{450} was determined using a microplate reader (ThermoFisher Scientific, USA).

Interaction between OmpA antiserum and pathogens of animal mastitis by ELISA and pull-down assay

Pull-down and ELISA methods were performed as described previously. Pathogens of animal mastitis of *E. coli* and *S. aureus* were collected at logarithmic growth phaseby centrifugation and washed two times with a 0.85% NaCl solution. Pathogens were inactivated and immobilized with oxymethylene at 80°C for 90 min. Then, the samples were dissolved in 0.85% NaCl and adjusted to an OD_{600} of 0.2. Samples (1 mL) were transferred into 1.5 mL tubes with 10^8 CFU bacterial cells. OmpA antiserum (100 μL) was added to each tube and 1.5 μg/μL of bovine serum albumin was used as the negative control. After rinsing, 100 μL of horseradish peroxidase-conjugated anti-mouse antiserum was added to each tube. Coloration liquid was added to every tube to avoid light reaction. After a stop solution was added to each tube, a microplate reader was used to detect the absorbance value at OD_{450} [12].

Immune protective function of OmpA protein

SPF Kunming mice were divided to the experimental group and the control group. Briefly, purified OmpA protein (50 μg per mouse) was injected three times into the experimental group while the control group received a PBS solution. OmpA protein and the control of PBS solution were emulsified with Freund's Complete Adjuvant (Sigma-Aldrich, USA) for the first immunization. After 14 days, the mice were boosted using Freund's incomplete adjuvant (Sigma-Aldrich, USA). After 7 days, a third immunization was performed. Primary immunizations were performed with Freund's complete adjuvant, while booster doses were immunized with Freund's incomplete adjuvant. After the third immunization, mice were intraperitoneally challenged with 1.0×10^8 *E. coli* and 1.5×10^9 *S. aureus*, respectively. After 15 days, the relative percentage survival of mice was measured. The immune protection rates were expressed as a formula of 1 -(OmpA immunity mortality/non-OmpA immunity mortality) × 100%. The statistical software package Social Science (SPSS) was used for statistical significance analysis [11,12].

Optimization of induced OmpA protein expression conditions

The $L_9(3^4)$ orthogonal design model, which is a four-factor and three-level orthogonal design, was used to determine the optimum expression conditions of OmpA protein. The factors of orthogonal design were strain OD_{600} value, IPTG final concentration, induction time, and induction temperature; these factors were represented as A, B, C, and D, respectively (Table 2). Briefly, according to the orthogonal design model, when the OD_{600} concentration of OmpA expression was reached, corresponding concentrations of IPTG were added to the culture to induce OmpA protein expression with an appropriate time and temperature. One milliliter of bacterial liquid was harvested, and boiled for 5 min with 300 μL buffer solution. After centrifugation, samples (10 μL) were resolved by SDS-PAGE. G-250 dye

liquor (Sigma-Aldrich, USA) was used to visualize the OmpA protein band. Finally, Phoretix 1D software was used to analyze the optical density of OmpA protein bands, and SPSS software was used to analyze the statistical significance for each factor [12].

RESULTS

Development of a prokaryotic strain expressing OmpA protein

A fragment of approximately 1041 bp, which was consistent with the expected size, was amplified from the *E. coli* genome by PCR (Figure 1). The target gene obtained by PCR was ligated to pET-32a plasmid. The size of the target gene obtained by double enzyme digestion was consistent with the prediction (Figure 1). In addition, sequencing confirmed that the target gene was the same as the *ompA* gene sequence published by the NCBI database. Finally, the *ompA* gene recombinant plasmid was transformed into *E. coli* Bl-21 strain to create the OmpA protein-expressing strain.

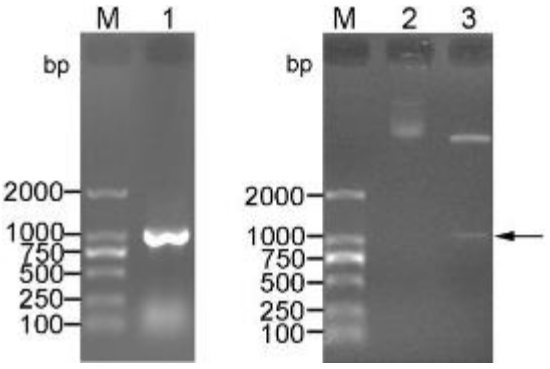

Figure 1: PCR and restriction enzyme analysis of the *E. coli ompA* gene. M, DNA-Marker. 1, PCR of the *ompA* gene. 2, recombinant plasmid of the *ompA* gene. 3, recombinant plasmid digested with *Eco*R I and *Hin*d III. A fragment of approximately 1041 bp was amplified by PCR, which was consistent with the size of the *ompA* gene. The size of the double enzyme digestion product was consistent with the predicted recombinant gene

Prokaryotic expression and purification of OmpA protein

To verify the expression of the recombinant target protein, bacteria containing the recombinant plasmid were induced with IPTG. The presence of OmpA protein was confirmed by visualization of a 58.4 kDa protein band representing the 38 kDa OmpA protein and a 20.4 kDa fusion protein label of the pET-32a plasmid, which was consistent with the expected size (Figure 2). Purified OmpA protein was obtained with Ni-affinity chromatography (Figure 2).

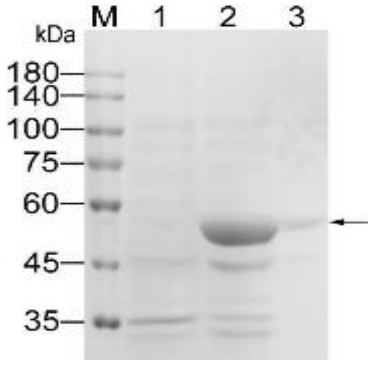

Figure 2: Prokaryotic expression and purification of OmpA protein. M, Protein marker. 1, no induction with IPTG. 2, induction with IPTG. 3, purified OmpA protein. Recombinant protein-expressing bacteria were induced with IPTG. The band corresponding to purified OmpA protein exhibited a molecular weight of approximately 58.4 kDa, representing the 38 kDa OmpA protein and a 20.4 kDa fusion protein. Our results demonstrate that purified OmpA protein was obtained and exhibited as one band of the expected size

Specificity and titer detection of OmpA protein antiserum

Western blot analysis was used to verify the specificity of the OmpA mice antiserum. We detected a single band corresponding to the expected molecular weight of OmpA protein, indicating that the OmpA antiserum was specific (Figure 3 A). In addition, the antiserum titer of OmpA protein reached 1: 1600 with ELISA (Figure 3 B).

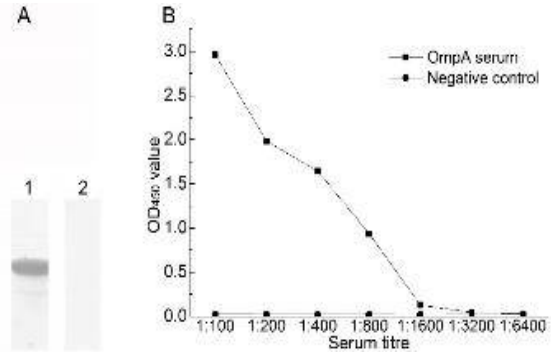

Figure 3: Specificity (A) and titer (B) determination of OmpA protein antiserum. A. Western blot analysis was performed to verify the specificity of OmpA protein antiserum. Lanes 1 and 2 show results from the OmpA protein antiserum and negative control, respectively. One band was visualized, indicating that OmpA antiserum had good specificity. B. ELISA was conducted to determine the OmpA antiserum titer. As the titer increased, the OD_{450} value decreased, showing that the OmpA antiserum titer was 1:1600

Interaction between OmpA antiserum and main pathogens of animal mastitis

The interaction between the OmpA antiserum and main pathogens of animal mastitis was assessed by ELISA and a pull-down method. Compared to the control group, an interaction between OmpA antiserum and *E. coli* was observed until a titer of 1:600 (Figure 4-A). The interaction between OmpA antiserum and *S. aureus* was detected up to a titer of 1:400 (Figure 4-B). These results suggest that OmpA antiserum and pathogens of animal mastitis formed antigen-antibody complexes, which likely led to antigen presentation. Thus, OmpA protein may have a preferential immunogenicity.

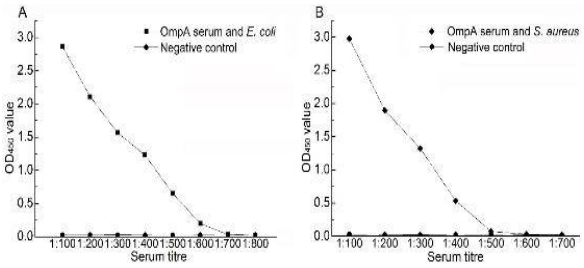

Figure 4: Analysis of the interaction between OmpA antiserum and the main pathogens of animal mastitis, namely *E. coli* (A) and *S. aureus* (B). As the OmpA antiserum titer increased, the OD_{450} value decreased. The interaction between OmpA antiserum and *E. coli* was detected up to 1:600, while interaction between OmpA antiserum and *S. aureus* was observed up to 1: 400

Immune protective function of OmpA protein

Mice were immunized with OmpA protein and then challenged with the enteric pathogens *E. coli* and *S. aureus* to evaluate the immunological protective effect of OmpA protein. The mice developed severe toxic symptoms, such as fluffy folds, sluggish activity, listlessness, and lethargy. Moreover, a large number died within 48 h. After 4 days, death was controlled, and the mice gradually recovered. OmpA protein demonstrated an immune protective function of 58.33% against *E. coli* and 46.15% against *S. aureus*. These results were statistically different from the control group, which was immunized with a PBS solution (Table 1).

Optimized prokaryotic expression conditions of OmpA protein

To detect the expression of OmpA protein, orthogonal design experiments were carried out. The expression map of OmpA protein was obtained by SDS-PAGE, which showed that the quantity of OmpA expression varied under different induction conditions (Figure 5). The optical density value of the OmpA protein band was obtained by Phoretix 1D software, and the range analysis was carried out (Table 2). Comparison of K1, K2, and K3 led us to determine that the optimal expression conditions of OmpA protein were A1, B2, C3, and D1, corresponding to a strain absorbance value of 0.5 at a wavelength of 600 nm, IPTG final concentration of 0.3 mmol/L, induction time of 12 h, and induction temperature of 28 °C. Variance analysis of the optical density data showed that two factors were statistically significant, including the strain absorbance value and induction time (Table 3).

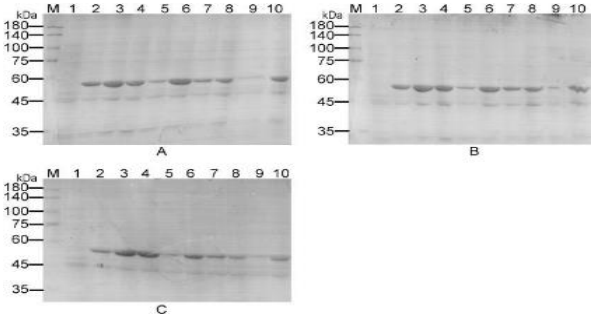

Figure 5: Results from the orthogonal experiment assessing OmpA-expressing strain induced by IPTG. A, B, and C represent three independent experiments. M, Protein marker. Lane 1: non-induced strain. Lanes 2, 3, 4: strains with absorbance of 0.5, IPTG final concentration of 0.1, 0.3, and 0.5 mmol/L, induction temperature of 28, 32, and 37 °C, induction time of 3, 8, and 12 h. Lanes 5, 6, 7: strains with absorbance value of 0.8, IPTG final concentration of 0.1, 0.3, and 0.5 mmol/L, induction temperature of 37, 28, and 32°C, induction time of 8, 12, and 3 h. Lanes 8, 9, 10: strains with absorbance value of 1.0, IPTG final concentration of 0.1, 0.3, and 0.5 mmol/L, induction temperature of 32, 37, and 28 °C, induction time of 12, 3, and 8 h

Table 1: Active immunity of OmpA in mice following challenge with pathogens of animal mastitis

Bacterium	OmpA				Control			
	Nos	Alive	ADR (%)	RPS (%)	Nos	Alive	ADR (%)	RPS (%)
E. coli	15	10	33.33	58.33*	15	3	80	----
S. aureus	15	8	46.67	46.15*	15	2	86.67	----

ADR means accumulating death rates. RPS means relative percent survivals. RPS (%) = 1 - (OmpA immunity mortality/non-protein immunity mortality) × 100%. *P < 0.05 (compared to the control group which received PBS only)

Table 2: Optimized expression conditions for OmpA strains

Number	A	B (mmol/L)	C (h)	D (°C)	Optical density/SD (×10^4)
1	0.5	0.1	3	28	3.556 ± 0.803
2	0.5	0.3	8	32	4.207 ± 0.248
3	0.5	0.5	12	37	4.367 ± 0.203
4	0.8	0.1	8	37	2.644 ± 0.634
5	0.8	0.3	12	28	4.265 ± 1.310
6	0.8	0.5	3	32	2.950 ± 0.365
7	1.0	0.1	12	32	2.733 ± 0.442
8	1.0	0.3	3	37	2.134 ± 0.907
9	1.0	0.5	8	28	3.238 ± 0.558
K1 (Mean value 1)	**4.043**	2.978	2.880	**3.686**	
K2 (Mean value 2)	3.286	**3.536**	3.363	3.297	
K3 (Mean value 3)	2.702	3.518	**3.789**	3.048	
Range analysis	1.341	0.54	0.909	0.638	

A, B, C, and D represent the strain OD_{600} value, IPTG induction concentration, induction time, and induction temperature, respectively. K1, K2, and K3 indicate the mean value of the analysis size, while the optimal induction expression conditions of OmpA protein are represented by A1, B2, C3, and D1

Table 3: Variance results for OmpA protein expression

Factor	Absorbance analysis		
	Mean square	***F-value***	***P-value***
A	4.070	8.467	0.003
B	0.906	1.884	0.181
C	1.858	3.865	0.040
D	0.931	1.936	0.173

Note: *A, B, C, and D represent the strain absorbance value at a wavelength of 600nm, IPTG concentration, induction time, and induction temperature, respectively. P < 0.05 was considered statistically significant compared to control group*

DISCUSSION

OmpA protein exhibits good immunogenicity, can stimulate an immune response, and has a potential application for use in a vaccine [10]. In this research, we developed an OmpA prokaryotic expression strain from which we purified OmpA protein. In addition, an anti-OmpA mouse polyclonal serum was prepared with a preferential specificity and titer of 1:1600. Compared with monoclonal antibodies, polyclonal antibodies are more convenient and economical to prepare [13,14]. Moreover, they are widely used to analyze immune function. With this study, we developed an OmpA antiserum, which laid the foundation for assessing the immunological function of OmpA protein. In this research, we found an interaction between OmpA antiserum and *E. coli* and *S. aureus*, suggesting that the anti-OmpA serum and pathogens of animal mastitis formed antigen-antibody complexes. These antigen-antibody complexes may be involved in antigen presentation, enabling easy identification by the immune system for eliminating pathogenic bacteria [15]. Thus, OmpA protein may display a selective immunogenicity. It was found that OmpA protein could activate the immune function of animals. This research showed that the immune protection of OmpA protein was significant at 58.33% against *E. coli* and 46.15% against *S. aureus*. Since *E. coli,* and *S. aureus* are the primary mastitis pathogens in cows and goats, this study lays a practical foundation for the development of an OmpA protein vaccine for animal mastitis.

The L_9 (3^4) orthogonal experimental test was used to examine the feasibility of large-scale fermentation to produce OmpA protein. We found that the optimum expression conditions for OmpA protein are an OD_{600} value of 0.5, a final IPTG concentration of 0.3 mmol/L, induction time of 12 h, and induction temperature of 28°C. Bacteria exhibit vigorous metabolism in the logarithmic growth phase, which is conducive to protein expression [16]. In this study, we found that induction of OmpA protein expression was optimal during the logarithmic growth phase. IPTG has some cytotoxicity and high concentrations can inhibit protein expression [16,17]. Consistent with this fact, our results demonstrated that a low concentration of IPTG (0.3 mmol/L) was advantageous for OmpA expression. Some research studies found that low temperature is also beneficial for protein expression [18,19], which supports our results. Thus, our data suggest that optimal fermentation production conditions involve strain induction at the logarithmic growth period, low IPTG concentration, and low temperature.

CONCLUSION

A novel prokaryotic strain expressing *E. coli* OmpA protein has been developed, from which OmpA protein has been purified to generate an antiserum. Optimized conditions for large-scale fermentation production of this protein have also deen developed. OmpA possesses selective immunogenicity and a significant immune

protective effect against the mastitis pathogens, *E. coli* and *S. aureus*. Thus, a new member has been suggested for addition to the group of vaccines used to treat animal mastitis.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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