Vaccine 31 (2013) 2947–2953

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Chaperonin GroEL: A novel phylogenetically conserved protein with strong immunoreactivity of Avian Pathogenic *Escherichia coli* isolates from duck identified by immunoproteomics

Yinli Bao, Zhipeng Zhai, Shaohui Wang, Jiale Ma, Wei Zhang*, Chengping Lu*

Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, China

ARTICLE INFO

Article history:
Received 17 July 2012
Received in revised form
13 December 2012
Accepted 17 April 2013
Available online 30 April 2013

Keywords:
Avian Pathogenic *Escherichia coli* (APEC)
Immunoproteomics
Two-dimensional electrophoresis (2-DE)
GroEL
Immunogen

ABSTRACT

Avian Pathogenic *Escherichia coli* (APEC) is one of the most important bacterial pathogens of poultry. The lack of suitable vaccines and the emergence of multi-resistant strains have hampered the control of avian colibacillosis. To identify immunogenic proteins of APEC as vaccine candidates, immunoproteomics and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) were applied. Proteins from total cell lysates of APEC DE205B isolated from the brain of a duck with septicemia and neurological symptom in China were separated by two-dimensional electrophoresis (2-DE) and reacted with hyperimmune duck serum against DE205B. Fourteen immunoreactive spots were found, representing 11 distinct proteins. These included two predominant immunogenic components, outer membrane protein A (OmpA) and flagellin (FliC). GroEL, which is a member of the molecular chaperone family and identical structurally to eukaryotic heat shock protein 60 (Hsp60), and the other eight antigens are reported here as immunoreactive proteins of APEC for the first time. Subsequently, nine genes encoding the identified proteins were successfully cloned and expressed in *E. coli* BL21 (DE3). Seven of the recombinant proteins were able to react with hyperimmune duck serum and three of them, GroEL, OmpA and FliC, showed stronger immunoreactivity. Challenge studies revealed that, just like OmpA and FliC, recombinant GroEL stimulated a strong antibody response and supported protective efficacy against APEC infection in ducks. With high phylogenetic conservation, it is considered that GroEL would be an ideal immunogen of APEC for vaccine development.

1. Introduction

Avian Pathogenic *Escherichia coli* (APEC) is the causative agent of avian colibacillosis, an illness associated with systemic infection of internal organs and a diversity of symptoms [1]. It is a predominant bacterial disease of poultry in China, the largest commercial duck breeding country [2]. Since intensive breeding has been widely applied in the poultry industry, outbreaks of acute mortality in flocks due to avian colibacillosis have frequently been observed, and are responsible for the significant economic losses [3]. As a type of extra-intestinal pathogenic *E. coli* (ExPEC), APEC strains share many common genetic characteristics with human uropathogenic *E. coli* (UPEC) and neonatal meningitis-causing *E. coli* (NMEC), indicating zoonotic potential [4,5]. Furthermore, the phenomena of the emergence of multi-resistant strains and risk of residues are becoming increasingly severe [6,7]. Currently, the control of APEC has become not only an urgent issue but also a great challenge.

Vaccination is an effective method for controlling infectious diseases [8]. To date, a number of experimental vaccines have been developed to prevent colibacillosis [9,10]. The traditional whole-cell inactivated vaccines can provide satisfactory protection to the homologous serotypes [11,12] but do not evoked cross-protection against non-homologous strains, which is also the disadvantage of gene deleted vaccines [13,14]. Gyimah et al. developed a multivalent pilus vaccine to overcome the problem of cross-protection [15]. Subunit vaccines and recombinant living vector vaccines based on fimbriae, flagellum or LPS have also been developed [16–19]. However, the large heterogeneity of APEC, including a large number of different serotypes and virulence-associated genes, hampers the development of vaccines for APEC [6,20,21]. Therefore, it is necessary to identify more proteins, which are immunogenic and conserved among different APEC strains, for vaccine development.

The identification of the predominant APEC phenotypes is critical for rational design of vaccines due to the variety of serotypes [6,20]. Epidemiological studies showed that O1, O2 and O78 are

* Corresponding author. Tel.: +86 25 84396517.
E-mail addresses: 2010107071@njau.edu.cn (Y. Bao), 365zzp@163.com (Z. Zhai), shwang0827@126.com (S. Wang), 2011107079@njau.edu.cn (J. Ma), vszw@njau.edu.cn (W. Zhang), lucp@njau.edu.cn (C. Lu).

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http://dx.doi.org/10.1016/j.vaccine.2013.04.042

still the most popular common in China, especially in the eastern regions [22–24]. Furthermore, the APEC strains serotyped to O2:K1 are by far the most important bacterial pathogens in developing colibacillosis in poultry [25].

In this study, immunoproteomics, consisting of immune serological techniques and proteomics [26], was used to identify immunogenic proteins of the APEC O2:K1 virulent strain DE205B, in order to screen suitable vaccine candidates for APEC. The identified proteins were then expressed *in vitro* and the protective efficacy of selected recombinant proteins was demonstrated.

2. Materials and methods

2.1. Duck

Healthy seven-day-old ducks, tested negative for antibodies to DE205B or recombinant proteins by indirect enzyme linked immunosorbent assay (ELISA), were used in this study [27,28]. All the animals were allowed free access to sterile water and animal feed and handled according to the guidelines of the Laboratory Animal Monitoring Committee of Jiangsu Province.

2.2. Bacterial strains, plasmids and culture conditions

The APEC strain DE205B was isolated from the brain of a duck with septicemia and neurological symptoms in Anhui, China in 2007 [29]. It was serotyped as O2:K1 and belonged to the phylogenetic *E. coli* reference (ECOR) group B2 analyzed by multiplex PCR [30]. APEC O1 (O1:K1) is a highly virulent strain isolated from a chicken with colisepticemia. The APEC strain EC942 (O78) was isolated from the brain of a duck with colibacillosis.

In addition, another 70 APEC strains (listed in Table S1), isolated from ducks with clinical signs of colibacillosis at different times and in different areas of China, were used for detecting the genes encoding candidate immunogens. The *E. coli* strain DH5α (TaKaRa) was used for cloning procedures and BL21 (DE3) (TaKaRa) was used for protein expression.

All *E.coli* strains were cultured in Luria–Bertani broth (LB broth; Oxoid, UK) at 37 °C with aeration. When necessary, LB broth was supplemented with ampicillin (50 μg/ml).

2.3. Hyperimmune serum preparation

The formaldehyde-killed DE205B cells were washed in phosphate buffer saline (PBS) twice and emulsified with the adjuvant of Montanide ISA 206 VG (SEPPIC, France). Ducks were administered three doses (1.0×10^9 cells/duck) by intramuscular injection at 2-week intervals. To monitor the potential influence of rearing environment on immunization, another five healthy ducks were reared under identical conditions without immunization. All ducks were bled twice, just before immunization and at 7 days after immunization. All sera were prepared by centrifugation and stored at −20 °C for later analysis. Serum titers were evaluated by DE205B whole-cell indirect ELISA [27].

2.4. Immunoproteomics

Protein sample extraction, isoelectric focusing (IEF), SDS-PAGE and Western blotting were performed according Zhang [31,32] with some modifications and described in supplemental files. Horseradish peroxidase-rabbit anti-duck serum (Epbio, China) (1:4000) was added after the reaction with hyperimmune serum (1:200). Each sample was analyzed thrice. Additionally, pre-immune serum was introduced to determine the presence of spots detected before immunization. Sera collected from ducks reared under identical conditions were tested to monitor the potential influence of rearing environment on immunization.

MALDI-TOF-MS and bioinformatics analysis were used to identify and analyze proteins.

2.5. Expression and purification of recombinant proteins

According to the gi number of the identified proteins listed in Table S2, the primers (Table 1) were designed to express the recombinant proteins identified as potential immunogens. The construction of recombinant plasmids, proteins expression and purification were manipulated according to Zhang [32] and described in supplemental files. Western blotting was carried out with hyperimmune serum to confirm the immunoreactivity of the recombinant proteins.

Table 1
Primers used for the cloning of candidate immunogens.

Spot no.	Gene expressed	Primer (5′–3′)	Annealing temperature (°C)	Product length (bp)
E1	*groEL*	CCGGAATTCATGGCAGCTAAAGACGTA CCGCTCGAG TTACATCATGCCGCCCAT	57.0	1647
E2	*atpD*	CGCGGATCCATGGCTACTGGAAAGATT CCGCTCGAG AAGTTTTTTGGCTTTTTC	53.9	1383
E3	*fliC*	CCGGAATTCATGGCACAAGTCATTAAT CCGCTCGAGTTAACCCTGCAGCAGAGA	53.9	1497
E4&E14	*malE*	CCG GAATTC ATGAAAATAAAAACAGGT CCG CTCGAGTTACTTGGTGATACGAGT	49.2	1191
E5	*ydeN*	CCG GAATTCATGAAGTCTGCATTAAAG CCGCTCGAGTTATTTCGCTTCGCTTAG	48.0	1683
E6&E12	*ompA*	CCGGAATTCATGAAAAAGACAGCTATC CCGCTCGAGTTAAGCCTGCGGCTGAGT	53.9	1041
E7&E9	*prs*	CCGGAATTCGTGCCTGATATGAAGCTT CCGCTCGAG TTAGTGTTCGAACATGGC	55.0	948
E8	*tsf*	CGCGGATCCATGGCTGAAATTACCGCA CCGCTCGAG TTAAGACTGCTTGGACAT	55.0	852
E10	*cpdB*	CCG GAATTCATGATTAAGTTTAGCGCA CCGCTCGAGTTACTTACTCAAATCCAC	45.0	1944
E11	*aceF*	CCGGAATTCATGGCTATCGAAATCAAA CCGCTCGAG TTACATCACCAGACGGCG	48.0	1893
E13	*pnp*	CCGGAATTCTTGCTTAATCCGATCGTT CCGCTCGAGTTACTCGCCCTGTTCAGC	53.4	2136

Ecor I (or BamH I) and Xhol restriction sites are underlined.