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Immunization with outer membrane vesicles of avian pathogenic *Escherichia coli* O78 induces protective immunity in chickens

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) typically causes colibacillosis and is a major concern for the poultry industry and public health. As a vaccine platform, the outer membrane vesicles (OMVs) derived from various gram-negative bacteria and even some gram-positive bacteria have been reported to be immunogenic in laboratories or upon commercial usage worldwide. Here, we purified OMVs from APEC serotype O78 strain by ultracentrifugation and gradient isolation. By SDS-PAGE and LC–MS/MS analysis, the 20 most abundant proteins located on OMVs were identified and analyzed; the lipopolysaccharide (LPS) profiles of OMVs were not different from those of the bacteria. Moreover, three groups of chickens were immunized with OMV-, outer membrane protein (OMP)- and PBS, with the latter two serving as positive and negative controls, respectively. By analyzing the anti-OMP and anti-LPS IgG titers stimulated by the tested vaccine candidates, the macrophage opsonophagocytic activity and the bactericidal activity mediated by serum antibodies in vaccinated chickens, we found that the OMV-vaccinated chicken group was superior to the two other groups. These findings were confirmed by additional chicken challenge tests, in which all OMV-vaccinated group chickens obtained complete protection but those of the other two groups were barely protected. Our data demonstrate that native APEC O78 OMVs can induce protective immunity in chickens and therefore be used as a candidate vaccine for APEC serotype O78 strain infections.

1. Introduction

Avian colibacillosis caused by avian pathogenic *Escherichia coli* (APEC) has been considered one of the most serious poultry bacterial infectious diseases worldwide. This bacterium infects all age groups of chickens, ducks, geese and wild birds (Nagano et al., 2012). Birds infected by APEC may suffer some acute and chronic clinical manifestations, such as septicemia, airsacculitis, enteritis, polyserositis, cellulitis, salpingitis, pericarditis, perihepatitis, arthritis, yolk sac infection, and so on. In addition, this disease is always accompanied by multiple lethal secondary infections, such as avian influenza virus, infectious bursal disease, mycoplasmosis (Umar et al., 2018). Transmission routes of this disease are varied, from breathing feces-contaminated dust around the hatchery to eating infected food or hatching through a contaminated egg shell. As a foodborne pathogen, APEC also causes intractable human colibacillosis through contaminated poultry products (Singer, 2015). Furthermore, those strains may transfer their antibiotic resistance genes and/or plasmids to human endogenous flora and human pathogens (Moulin-Schouleur et al., 2007; van den Bogaard et al., 2001). Therefore, APEC has been a major concern due to the substantial economic loss in the global poultry industry and the threat to public health.

APEC is notoriously difficult to control in poultry due to its outstanding diversity of serogroups. Mainly three serotypes, APEC O1, O2 and O78 (according to the O-antigens), contribute to more than 80% of colibacillosis outbreaks (Ebrahimi-Nik et al., 2018). The major treatment against APEC currently depends on the administration of antibiotics in developed or developing countries. Nevertheless, the excessive usage of antibiotics has resulted in both ineffectiveness against multidrug-resistant APEC strains and unsafe antibiotic residues in poultry products (Cavicchio et al., 2015; Subedi et al., 2018; Weiss et al., 2018). Scientists worldwide have been seeking vaccination solutions for several decades. The surface structures and some virulence factors of APEC, such as outer membrane proteins (OMPs), O-antigen polysaccharide, flagella, P fimbriae, the iron-regulated aerobactin receptor *iutA*, and the CS31A surface antigen adhesin, have been separately proven to provide immunogenic protection against different serotypes of APEC (Ebbensgaard et al., 2018; Han et al., 2018).

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Numerous experimental or commercial vaccines for APEC, including inactive vaccines, live attenuated mutant vaccines, recombinant multiantigen vaccines, and bacterial ghost vaccines, have been prepared and evaluated in research labs (Ebrahimi-Nik et al., 2018; Frommer et al., 1994; Han et al., 2018; Kariyawasam et al., 2004; La Ragione et al., 2013; Van Goor et al., 2017). Those vaccines were effective in protecting against their corresponding serogroup; however, it is still necessary to develop an effective and broad-spectrum vaccine for all serotypes of APEC involved in field outbreaks worldwide.

Bacterial outer membrane vesicles (OMVs), known as nanosized (20–200 nm) spherical phospholipid bilayer vesicular structures, are usually released into the extracellular environment by nearly all species of gram-negative bacteria and even some gram-positive bacteria and archaea. The function of OMVs includes long-distance signaling and toxin transferring, biofilm formation, defense against or killing of competing microbes, antibiotic response and envelope stress relaxation (Macdonald and Kuehn, 2013; Schooling and Beveridge, 2006). Naturally budding from bacterial outer membranes, OMVs entrap many of the materials of the underlying periplasm from their parent bacteria, including OMPs, periplasmic proteins, phospholipids, lipopolysaccharide (LPS), DNA and RNA (Acevedo et al., 2014). The capacity of OMV-based vaccines to stimulate a protective immune response has already been exploited against several bacterial pathogens, such as *Neisseria meningitidis* (Tunheim et al., 2014), *Salmonella enterica* Typhimurium (Muralinath et al., 2011), *Pseudomonas aeruginosa* (Ellis et al., 2010), and *Vibrio cholerae* (Bishop et al., 2010). In particular, vaccines based on wild-type OMVs have shown efficacy against serogroup B meningococcal disease in many countries. Therefore, the OMV-based vaccine has been used as an ideal vaccine platform for various pathogens because of its similarity to its parental bacteria in regards to immunogenic and self-adjuvant properties and its reduced virulence compared to that of its parent bacteria (Aguilera et al., 2014; Liu et al., 2016b; Sevestre et al., 2017).

OMVs derived from various species of *E. coli*, such as enterohemorrhagic *E. coli* (EHEC) and extraintestinal pathogenic *E. coli* (ExPEC), have been investigated and proven to be effective against their corresponding bacteria in animal models (Lee et al., 2007); however, the characteristics and immunogenic properties of APEC OMVs in chicken models have not been reported. In this study, the serotype APEC O78, the major bacterial serogroup responsible for colibacillosis in domestic poultry, was chosen as our research object. The main components of OMVs purified from APEC O78 were characterized and analyzed. The immunogenicity and protective activity of the OMVs were tested in a chicken model, and an effective OMV vaccine against serotype O78 was expected. The research strategy and methods of this study could be used to develop multivalent OMV vaccines against all serogroups of APEC in the future.

2. Materials and methods

2.1. Bacterial strains, cells, experimental animals and ethical statement

The APEC O78 strain used in this study was grown at 37 °C in Luria-Bertani (LB) broth or on LB agar without any antibiotics. Murine macrophage RAW264.7 cells were maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, HyClone, USA). Lohmann chickens were purchased from Muxing Poultry Co., Ltd. (Chengdu, China) and fed under standard conditions until they were 10 days old. All experiments involving animals were conducted in compliance with the Animal Welfare Act and associated regulations of Sichuan Agricultural University related to animal experiments (Ya'an, China; Approval No. 2011-028). All animal studies were approved by the committee of Sichuan Agricultural University. The principles stated in the Guide for the Care and Use of Laboratory Animals were followed. All efforts were made to minimize animal suffering during the experiments.

2.2. Purification and characterization of outer membrane vesicles (OMVs)

Native OMVs were prepared from APEC O78 culture supernatants, as described previously (Lee et al., 2007; Muralinath et al., 2011). A Polara G2 Tecnai cryo-transmission electron microscope (FEI, Hillsboro, USA) was used to confirm the morphology and integrity of the purified OMVs, as described previously (Hu et al., 2015). Quantification of OMVs was based on the protein concentration of purified OMVs, which was measured by a bicinchoninic acid assay (BCA) protein assay kit (Thermo Pierce, Rockford, IL, USA). To determine the protein profile of OMVs, 10 μg of purified OMVs was analyzed on 12% SDS-PAGE gels and stained by GelCode™ Blue Stain Reagent (Thermo Pierce, Rockford, IL, USA), as previously described (Liu et al., 2017). Protein components were identified through LC–MS/MS analysis with an Easy-Nano LC II HPLC and a C18 reversed-phase column (75 m ID, 15 cm length). Following SDS-PAGE, each protein lane in the polyacrylamide gel was cut into five slices, and each slice was then cut into 1 mm^3 cubes for use as the LC–MS/MS analysis samples. All of the procedures of LC–MS/MS analysis were described in our previous study (Liu et al., 2017). To determine LPS profiles, LPS in the purified OMVs was separated by 12% SDS-PAGE gels and visualized using silver staining methods, as previously described.

2.3. Database search and bioinformatic analysis

Briefly, raw MS files for APEC O78 were searched in the UniProt database (https://www.uniprot.org/). The bioinformatic analysis was performed by MaxQuant software, as described previously (Neuhauser et al., 2012). The following input parameters were set: two missing trypsin cleavage sites allowed, precursor mass tolerance of 7 ppm and fragment mass tolerance of 0.5 Da. At least two unique peptides were required to identify proteins. Oxidation of methionine and N-terminal acetylation of proteins were taken as variable modifications in database searching. The identified proteins were classified by Gene Ontology (GO) tools, and the subcellular location was predicted by the PSORTb online tool (version 3.0.2). Finally, the molecular function was obtained based on the databases provided by STRAP software (Boston University, Boston, MA, USA).

2.4. Immunization and challenge

Before the animal experiments were initiated, specific PCR was performed to exclude the chickens infected with APEC O78 for the animal work (Wang et al., 2014). Three injections were prepared as follows: 75 μg of OMVs suspended in 100 μl of phosphate-buffered saline (PBS) or 75 μg of OMPs in 100 μl of PBS, as potential immune-protective injections, and 100 μl of PBS as a negative control. Three groups of 10-day-old Lohmann chickens (20 per group) were intramuscularly immunized twice with one of the above three injections at a two-week interval, according to modified procedures (Liu et al., 2016a). The first immunization day was marked as day 0, and the booster day was marked as week 2. During the period of immunization, 1–2 ml of blood was aseptically drawn from the chicken brachial vein after the first and second immunization (marked as week 1, week 3, or week 4). Serum samples for further experiments were prepared as follows: incubating the collected blood to clot at room temperature for 30 min, removing the blood clots with a sterile toothpick, centrifuging at 1000 × *g* for 15 min, and gently removing the supernatant. The prepared serum samples were stored at −80 °C. All of the chickens were challenged by the air sac route with a lethal dose of the wild-type APEC O78 strain, which was suspended in 20 μl of PBS with 0.01% gelatin, at three weeks after booster administration (week 5). The health status and mortality of the chickens were monitored daily for three weeks after challenge (Fig. 1).

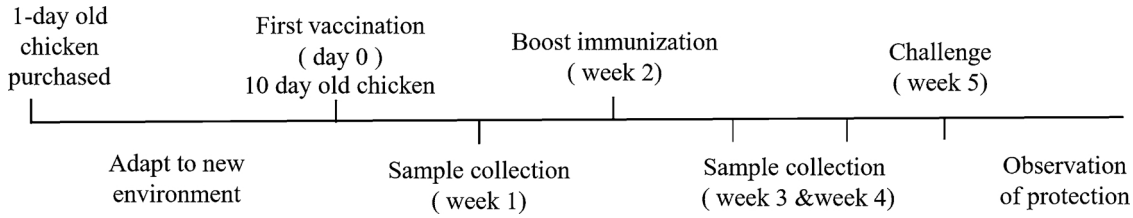

Fig. 1. Timeline of immunization and challenge in a chicken experiment to evaluate outer membrane vesicles (OMVs) from APEC O78. Three groups of chickens were separately immunized with OMVs, OMPs or PBS (as a control) on day 0, boosted at week 2, and challenged at week 5. Each group consisted of 20 chickens. Blood samples were collected at weeks 1, 3 and 4. Before immunization, all chicken blood was drawn for testing whether the blood was contaminated by bacteria. PBS-vaccinated chickens served as a control group.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The titers of anti-OMP IgG and anti-LPS IgG in serum samples were measured by enzyme-linked immunosorbent assay (ELISA), as described previously (Muralinath et al., 2011). Briefly, 96-well plates were coated, washed and blocked as follows: coating plates overnight at 4 °C with 100 ng of purified OMP or LPS in 100 µl of coating buffer (0.016 M Na_2CO_3, 0.034 M $NaHCO_3$ [pH 9.6]), removing the coating solution, washing twice with 350 µl of washing buffer (PBS + 0.05% Tween 20), and blocking for 30 min at room temperature with 200 µl of blocking buffer (washing buffer + 3% bovine serum albumin [BSA]). A total of 100 µl of serum samples was serially diluted 2-fold in 100 µl of dilution buffer (PBS + 3% BSA) from 1:200 to 1:25,600 and incubated in wells for 1 h at room temperature, with 200 µl of dilution buffer as a negative control. Then, 100 µl of 1:10,000 anti-chicken IgG-horseradish peroxidase (HRP) conjugate (Abcam, England) in dilution buffer was added to the wells and further incubated for 1 h at room temperature. Subsequently, 100 µl of TMB substrate was transferred to the wells and reacted for 1 h at room temperature. Then, 100 µl of stop solution (1 M HCl) was added to terminate the reaction. The OD_{450} was measured immediately in an ELISA plate reader (BMG Labtech). The IgG titer was defined as the highest serum dilution factor for which the OD_{450} of the tested serum was less than 0.1 and 2.1 times larger than that of the negative control. All samples were independently run in triplicate, and the logarithmic IgG titers were calculated for further analysis.

2.6. Opsonophagocytosis uptake assay (OPA)

To determine the functional capacity of vaccine candidate-raised antibodies in the serum samples, opsonophagocytic assay (OPA) was performed as previously described, with some modifications. Macrophages RAW264.7 (5×10^5 cells/well) were seeded in 24-well plates and incubated with RPMI complete medium without antibiotics (500 µl/well, Gibco, USA) at 37 °C in a 5% CO_2 incubator for 2 h. To perform opsonization with the antibody and complement system, 10^5 CFU of APEC O78 bacterial suspensions were incubated with serum samples (100 times dilution) at 37 °C for 10 min. To test phagocytosis, the above opsonized bacteria were added to macrophage monolayers in 24-well plates at a multiplicity of infection of 1:10 (cells:bacteria). These 24-well plates were incubated for 30 min at 37 °C in a 5% CO_2 incubator to allow phagocytosis to occur between the cells and bacteria. The unattached extracellular bacteria were removed by gentamicin treating for 1 h, and later washing three times with PBS. Then, the bacteria inside the macrophages were obtained by immediately lysing with 1% Triton X-100 for 10 min at 37 °C. The lysates were 10-fold serially diluted and plated onto LB plates at 37 °C overnight. The number of phagocytosed bacteria was calculated by enumerating the colonies on the plates.

2.7. Serum bactericidal activity (SBA) assay

To determine the bactericidal ability of antibodies in serum samples in vitro, a modified serum bactericidal activity (SBA) assay was performed as previously described. The wild-type APEC O78 strain was cultured to log-phase (OD_{600} of 0.7) and then suspended in PBS to a concentration of 10^6 colony-forming units (CFU)/ml for the following assay. The serum samples were diluted 1:10 with LB medium. In round-bottom 96-well plates, 90 µl of these diluted sera and 10 µl of the previously adjusted bacteria were mixed and then incubated at 37 °C with shaking at 100 rpm for 1 h. The reaction mixture was 10-fold serially diluted and plated at 37 °C overnight until viable colonies were counted. To calculate the survival rate of O78 in the different serum samples, the value for bacterial CFU with the serum treatment was divided by the value for the original bacterial CFU. All serum samples were independently tested three times.

2.8. Statistical analysis

All data were statistically analyzed using GraphPad Prism version 8.0.2 (GraphPad Software package, San Diego, USA). The data were expressed as the mean ± standard deviation (SD). The differences between the immunized group and the PBS control group were compared using Student's *t*-test. *P* values of < 0.05 were considered significant.

3. Results

3.1. Purification, visualization and characterization of APEC O78 OMVs

OMVs purified from APEC O78 were observed by cryo-electron microscopy (cryo-EM). The diameter of the OMVs ranged from 50 nm to 200 nm, and their appearance was visualized as shown (Fig. 2A). The LPS profiles between the purified OMVs and their parental bacteria showed no obvious difference (Fig. 2B). The protein profiles of OMVs were evaluated by SDS-PAGE and LC–MS/MS analysis. On the basis of molecular weight, some OMPs (e.g., OmpA, OmpC and OmpD), lipoproteins, and periplasmic proteins were observed by SDS-PAGE (Fig. 2C). Analysis of the OMVs by LC–MS/MS confirmed the protein composition, which was consistent with the results of SDS-PAGE in this study. By performing a blast search at www.uniport.org, 110 proteins were identified from the native OMVs of APEC O78 and ranked according to their intensity. The 20 most abundant proteins, along with their gene, subcellular localization, function, molecular weight and intensity, are listed in Table 1. The high enrichment of lipoproteins, such as major outer membrane lipoprotein (Lpp), many OMPs (OmpA, OmpC, OmpX, and OmpD) and flagellin, suggested the vesicular formation process of OMVs and the high similarity of the outer membrane structure between OMVs and their parental bacteria. Moreover, the abundance of nucleoside-specific channel-forming protein TSX, long-chain fatty acid transport protein and ferrochrome transporter proteins, which are associated with the transmembrane transport of small molecules (nucleoside, fatty acid, and iron), suggested active functions of the OMVs involved in heredity, signaling, virulence, etc. Notably, two proteins, carbamoyl-phosphate synthase and formate dehydrogenase-N subunit alpha, were unique to APEC O78 and not reported previously in the OMVs of *E. coli* DH5α (Lee et al., 2007).

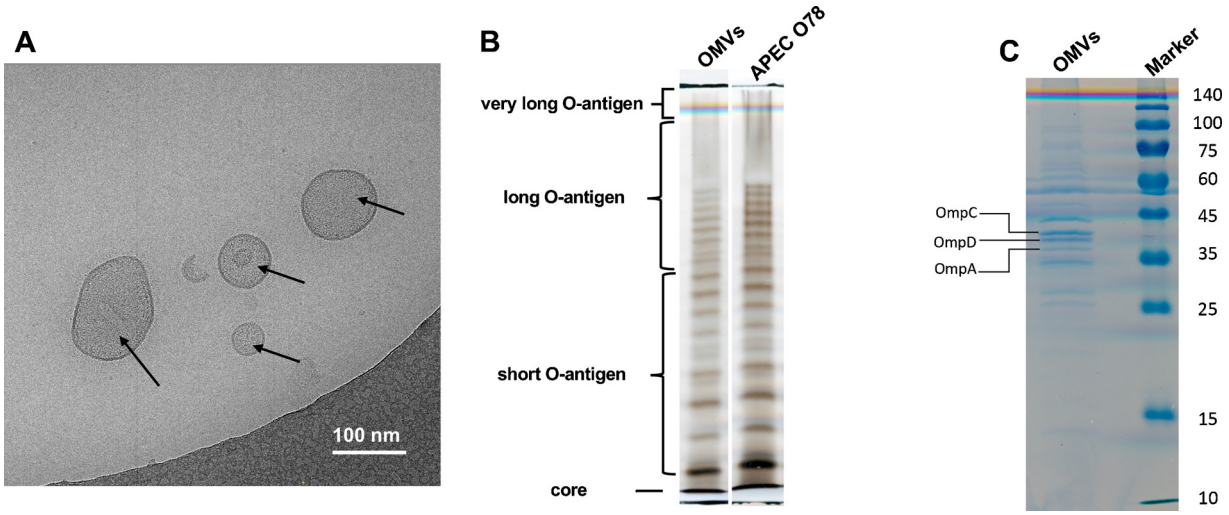

Fig. 2. Visualization and characterization of OMVs from APEC O78. (A) Cryo-EM imaging of OMVs. OMVs derived from APEC O78 were visualized using cryo-EM. The visible OMVs indicated by the black arrows ranged from 50 nm to 200 nm. (B) LPS profiles of OMVs. LPS of the purified OMVs and wild-type strain were separated using 12% SDS-PAGE and visualized using silver staining. (C) Protein contents of APEC O78 OMVs analyzed by SDS-PAGE. Ten micrograms of OMVs were separated in a 12% SDS-PAGE gel and stained with GelCode™Blue Stain Reagent. A protein marker and molecular mass standards (kDa) are shown on the right. Outer membrane protein OmpC, OmpD and OmpA are marked on the left.

3.2. IgG immune responses induced by APEC O78 OMVs in chickens

To determine the immunogenicity of OMVs from APEC O78, the titers of anti-OMP and anti-LPS IgGs in serum samples were measured by ELISA. At week 1, both the OMV and OMP groups had significantly higher anti-OMP IgG production than the PBS control groups, whereas for the anti-LPS IgG production, the OMV group had highly significantly higher anti-OMP IgG production than both the OMP and PBS control groups ($P < 0.001$). At week 3, the OMP group produced a booster effect on only the anti-OMP IgG titer, while the OMV group had a booster effect on both anti-OMP and anti-LPS IgG titers. At week 4, the trends of ani-OMP and anti-LPS IgG titers in both the OMP and OMV group remained constant (Fig. 3A, B). The results suggested that the OMPs induced anti-OMP IgGs in chickens while the OMVs induced both anti-OMP and anti-LPS IgGs in chickens. Obviously, the composition and structure of OMVs could explain the above results. PBS group showed weak responses to OMP and LPS of APEC O78, indicating non-specific antibodies were present in the chicken serum.

3.3. Opsonophagocytosis enhanced by APEC O78 OMVs in chickens

To test the opsonophagocytic capability of antibodies in the experimental chickens, the serum samples from the OMV, OMP, and PBS treatment groups at different time points were tested by a modified OPA in which the percent uptake of phagocytosed bacteria were measured by comparing the number of bacterial uptake by macrophage RAW264.7 to the inoculum, and are shown in Fig. 4A. Compared to the PBS and OMP treatment groups, the OMV group showed a significantly higher capability of opsonophagocytosis at all time points (week 1, week 3 and week 4). Notably, the capacity of phagocytosed bacteria reached a peak in the OMV-boosted chicken group at week 4 while the capacity of phagocytosed bacteria in OMP-immunized group was not changed much compared to that at week 3 in the OMP-immunized group.

3.4. SBA induced by APEC O78 OMVs

To test the bactericidal activity of antibodies in the OMV-immunized chickens at different time points, a modified SBA assay was utilized. The serum samples derived from PBS-, OMP- and OMV-immunized chickens at week 1, week 3 and week 4 were incubated with APEC O78 bacteria at 37 °C for one hour, and then the bacterial survival in different treatments was statistically analyzed and is shown in Fig. 4B. The bacterial survival in serum samples from the PBS-immunized group was not significantly changed between weeks 1, 3 and 4 (survival mean ± SD, 150 ± 25%, $P > 0.05$). The increased bacterial survival in the PBS-immunized serum at different time points suggested that those serum samples did not suppress the growth of bacteria but rather enhanced their growth by providing nutrition. Compared to PBS control group serum samples, both OMP and OMV group serum samples from one week after the first immunization (week 1) slightly suppressed the growth of bacteria (110 ± 32%, $P > 0.05$ and 101.9 ± 30%, $P < 0.05$, respectively). The SBA reached the highest level with OMV-immunized group sera one week after booster immunization (49.1 ± 10%, $P < 0.001$, week 3) and remained similar at week 4 (50.7 ± 9%, $P < 0.001$). These results indicated that the OMV booster immunization may significantly enhance SBA and displayed a long-term effect on bacterial killing or growth suppression. In addition, the SBA presented by OMV-immunized group sera was significantly higher than that presented by OMP-immunized group sera at the same time points (81.6 ± 15% survival, $P < 0.05$ for week 3; 85.6 ± 7%, $P < 0.05$ for week 4).

3.5. Protection against virulent APEC O78 challenge

To evaluate protective efficiency, immunized chickens in different groups were challenged via the air sac route with 1×10^{11} CFU (approximately $100 \times LD_{50}$) of APEC O78 at three weeks after the booster immunization (week 5). Immunization with OMVs derived from wild-type APEC O78 provided chickens with complete protection against a challenge with APEC O78, while the OMP-immunized group chickens had 30% survival (6 out of 20 survived), and all of the chickens in the PBS control group succumbed to the infection with the wild-type strain within 7 days (Fig. 5). The OMV-immunized group had a significantly higher survival rate than the PBS control group ($P < 0.01$) and the OMP-immunized group ($P < 0.05$). Therefore, the OMVs of APEC O78 may provide highly significant protection against a virulent dose of wild-type bacteria.

4. Discussion

Currently, there are mainly two types of commercial APEC vaccines,

Table 1
Major proteins identified from native OMVs of APEC O78.

Rank	UniProt accession	Gene	Protein name	Subcellular localization	Function	MW (kDa)[a]	Intensity[b]
1	P69776	*lpp*	Major outer membrane lipoprotein (Lpp)	Outer membrane	Cell wall/membrane biogenesis	8.3234	3.35E+07
2	A0A0E0TX86	*ompA*	Outer membrane protein A (OmpA)	Outer membrane	Cell wall/membrane biogenesis	37.478	2.01E+07
3	C6K7M3	*ompC*	Outer membrane porin protein C (OmpC)	Outer membrane	Cell wall/membrane biogenesis	40.408	6.09E+06
4	P0A917	*ompX*	Outer membrane protein X (OmpX)	Outer membrane	Cell wall/membrane biogenesis	18.602	5.37E+06
5	A0A029LW22	*ompD*	Outer membrane porin protein (OmpD)	Outer membrane	Cell wall/membrane biogenesis	39.619	2.93E+06
6	A0A023Z4F6	*slp*	Starvation lipoprotein (Slp)	Outer membrane	Cell wall/membrane biogenesis	20.948	1.68E+06
7	J7RU84	*fliC*	Flagellin	Extracellular	Cell motility	68.132	1.41E+06
8	P0A905	*slyB*	Outer membrane lipoprotein (SlyB)	Outer membrane	Cell wall/membrane biogenesis	15.601	9.37E+05
9	P0A908	*mipA*	MltA-interacting protein	Outer membrane	Cell wall/membrane biogenesis (peptidoglycan biosynthesis)	27.831	6.94E+05
10	P0A927	*tsx*	Nucleoside-specific channel-forming protein (tsx)	Outer membrane	Cell membrane biogenesis/ transporter	33.589	6.43E+05
11	P0A915	*ompW*	Outer membrane protein W (OmpW)	Outer membrane	Cell wall/membrane biogenesis	22.928	4.38E+05
12	P0A912	*pal*	Peptidoglycan-associated lipoprotein	Outer membrane	Cell wall/membrane biogenesis	18.824	4.13E+05
13	P0A937	*bamE*	Outer membrane protein assembly factor (BamE)	Outer membrane	Cell wall/membrane biogenesis	12.302	3.07E+05
14	A7ZHR7	*bamA*	Outer membrane protein assembly factor (BamA)	Outer membrane	Cell wall/membrane biogenesis	90.552	2.84E+05
15	A0A023YRP9	*carB*	Carbamoyl-phosphate synthase	Periplasmic	Membrane biogenesis/ synthesize carbamoyl phosphate	117.87	2.54E+05
16	A0A023L4W8	*fdnG*	Formate dehydrogenase-N subunit alpha	Periplasmic	Cellular respiration/ metabolism	89.568	1.71E+05
17	A0A023YT96	*ECRM12581_2890*	Protease VII (Omptin)	Outer membrane	Cell membrane/ membrane biogenesis	35.547	1.66E+05
18	P69411	*rcsF*	Outer membrane lipoprotein (RcsF)	Outer membrane	Cell wall/membrane biogenesis	14.163	1.66E+05
19	B6I6Q7	*fadL*	Long-chain fatty acid transport protein	Outer membrane	Fatty acid transport and metabolism	48.74	1.60E+05
20	A0A026V742	*fhuA*	Ferrochrome transporter	Outer membrane	Inorganic ion transport and metabolism	80.858	1.05E+05

[a] The values represented as MW (kDa) indicate the calculated molecular weight of the identified proteins.
[b] Ranking by intensity of proteins in the OMVs of APEC O78.

inactivated and attenuated live vaccines, both of which have drawbacks. Inactivated vaccines have low immunogenic activities and provide low protection, while attenuated live vaccines have difficulty in achieving minimum toxicity with optimal immunogenicity. OMVs provide several potential advantages: 1) better safety than that of attenuated live vaccines due to a lack of replication; 2) substantial immunogenicity and intrinsic adjuvant effects that are provided by LPS, OMPs and other immune-stimulating molecules; 3) effective induction of migration to lymph nodes and efficient uptake by antigen-presenting cells due to their typical nanoparticle structure; 4) easy preparation because they are naturally secreted, also called “blebbing” or “vesiculation”, during bacterial growth (Ellis et al., 2010). The OMV vaccine derived from *Neisseria meningitidis* has already been commercially produced in several countries (van der Pol et al., 2015). OMVs, as novel potential animal and human vaccines against pathogenic strains, have become a research hotspot worldwide.

Our study is the first to disclose that OMVs of APEC were purified from cell-free culture supernatant. Moreover, we comprehensively identified the vesicular proteins of purified OMVs using LC–MS/MS analysis. Among the 20 most abundant, ranked by the intensity, proteins, the majority were OMPs, such as Lpp, OmpA, SlyB, OmpW, OmpX, and Slp, which are mainly involved in the mechanism of vesicle biogenesis. The others were less abundant, including one extracellular protein (flagellin), which functions in cell motility; two periplasmic proteins (carbamoyl-phosphate synthase and formate dehydrogenase-N subunit alpha), which are metabolic enzymes involved in nutrient and energy metabolism; and two transmembrane transport proteins (long-chain fatty acid transport protein and ferrochrome transporter), which transport fatty acid and inorganic ions across the membrane, respectively. The protein patterns of APEC O78 OMVs are similar to those of native OMVs derived from *E. coli* DH5α reported previously (Lee et al., 2007). Notably, two proteins, carbamoyl-phosphate synthase and formate dehydrogenase-N subunit alpha, were unique to APEC O78 and were not reported previously in the OMVs of *E. coli* DH5α, suggesting that those two proteins were potentially related to poultry infection.

Due to the similarity between the structures of OMVs and the bacterial outer membrane, we speculate that OMVs have a similar level of immunogenicity as their bacterial parents. Previous reports have shown that OMVs derived from ETEC, EHEC, and *E. coli* DH5α can specifically stimulate the host immune system to produce a humoral immune response due to the presence of specific OMPs, including OmpA, OmpW, and OmpX (Lee et al., 2007). In our study, administration of both OMVs and OMPs of APEC O78 induced significantly higher anti-OMP IgG titers than administration of PBS, which indicated that OMVs derived from APEC also may stimulate a substantial humoral immune response in chickens, consistent with previous studies (Lee et al., 2007). Moreover, OMVs induced high anti-LPS IgG titers in chickens, whereas OMPs did not, indicating that the LPS antigen located in OMVs also stimulates a high humoral immune response. APEC infection is a systemic disease, indicating cell-mediated immunity may also play an essential role in conferring protection against challenge (Stromberg et al., 2018). As both antigens and adjuvants such as LPS and lipoprotein are coexisting in OMVs, after immunization, OMVs directly interact with various immune cell populations, including neutrophils, macrophages and dendritic cells (DCs) to enhance antibody production and T cell responses (Kaparakis-Liaskos and Ferrero, 2015). Therefore, we believe that OMVs induced a higher level of humoral immune response and T-cell mediated immunity than either protein or LPS subunit vaccines.

Opsonophagocytic activity includes opsonization (the process through which the pathogen is bound and marked by antibodies and complement) and subsequent phagocytosis (the process of ingestion and elimination by macrophages and neutrophil granulocytes). An OPA and SBA assay have been used to measure the functional capacities of serum antibodies and to determine the effectiveness of vaccine candidates. We performed an OPA and SBA assay in vitro to test the functions of antibodies induced by OMVs of APEC O78 in this study. The results

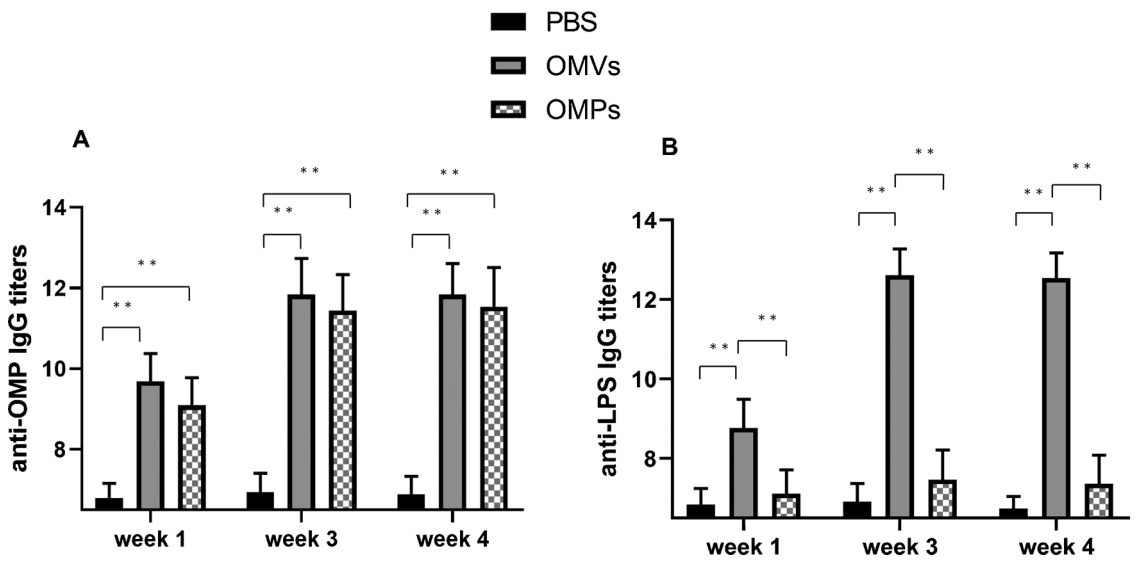

Fig. 3. IgG immune responses in chickens. (A) The titers of anti-OMP IgG in sera from chickens immunized with OMVs, OMPs or PBS. (B) The titers of anti-LPS IgG in sera from chickens immunized with OMVs, OMPs or PBS. A two-tailed *t*-test was performed for statistical analysis. $P < 0.05$ refers to statistically significant, annotated as ∗; $P < 0.001$, highly statistically significant, marked with ∗∗.

showed that OMV-vaccinated chicken sera enhanced opsonophagocytosis of the *E. coli* O78 strain by macrophages and promoted bacterial killing by serum antibodies (Fig. 4). Additionally, an animal experiment confirmed the results of these two in vitro tests, in which the chickens immunized with *E. coli* O78 OMVs obtained complete protection while the chickens immunized with OMPs provided partial protection against a lethal dose of the corresponding serotype bacteria (Fig. 5). This enhanced protection observed in group immunized with OMVs of *E. coli* O78 compared with group immunized with OMPs should ascribe to the O78 O-antigen polysaccharide in OMVs because OMV induced significantly higher immune responses against LPS while OMV induced a similar level of response against OMP as did OMP, and only serum antibody directed against bacterial surface could lead to bacteriolysis and opsonophagocytosis, because O-antigen on the surface of bacteria may restrict most of access of antibodies to OMP epitopes that are proximal to the membrane surface.

As avian colibacillosis is caused by multiple APEC serotypes, broadly cross-protective vaccines are required. While live-attenuated

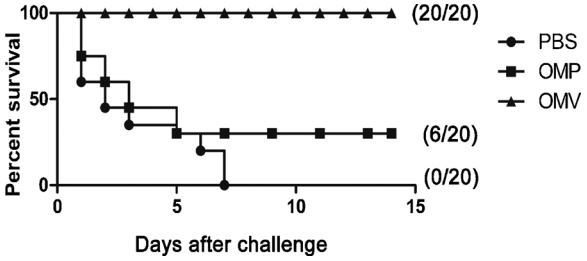

Fig. 5. Survival of immunized chickens after challenge with wild-type APEC O78. Immunization with OMVs derived from wild-type APEC O78 provided complete protection against a lethal-dose (10^{11} CFU) APEC O78 strain challenge in chickens. All of the chickens were monitored daily for 3 weeks after challenge for morbidity and mortality. The OMV-immunized group had a significantly higher survival rate than the PBS control group ($P < 0.01$) and the OMP-immunized group ($P < 0.05$).

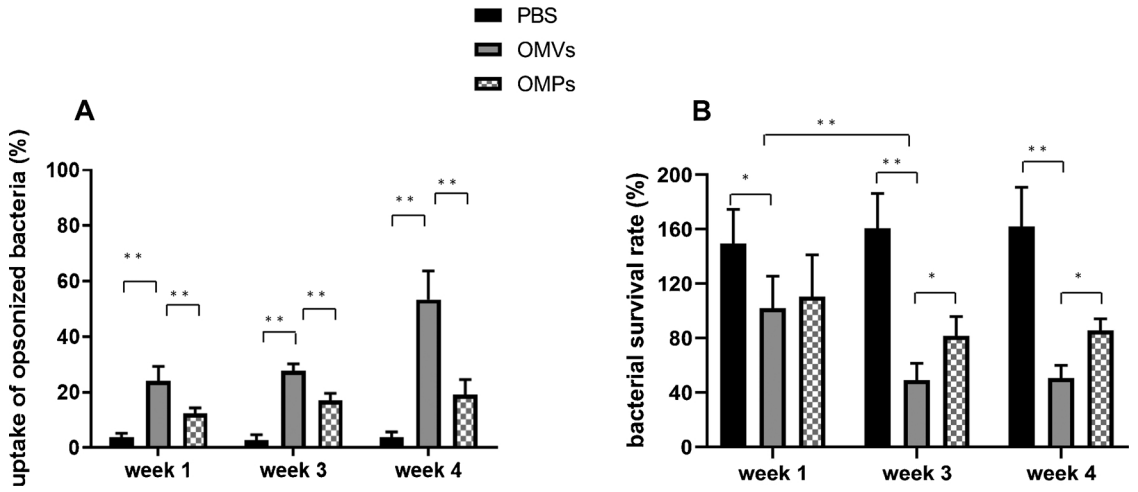

Fig. 4. Opsonophagocytic and bactericidal ability of immunized chicken serum. (A) OMV-, OMP- and PBS-immunized chicken serum samples were allowed to opsonize APEC O78 at 37 °C for 10 min. Then, macrophages were inoculated at a multiplicity of infection of 10:1 for 30 min, the percent uptake of phagocytosed bacteria were measured by comparing the number of bacterial uptakes by macrophage RAW264.7 to the inoculum. (B) Bacterial survival after incubation with OMV-, OMP-, and PBS-immunized serum samples at 37 °C for 1 h. A two-tailed *t*-test was performed for statistical analysis. $P < 0.05$, annotated as ∗, was considered as significant diff ;erence; $P < 0.001$ annotated as ∗∗, was considered as a highly significant difference.

APEC vaccines were observed to confer a certain degree of cross protection against different sero-group (Peighambari et al., 2002), however, the immunological basis of this cross protection has not been properly elucidated. OMV-based vaccine may provide an option to confer cross-protection after expressing heterologous O-antigen polysaccharide or other conserved protective antigen such as common pilus (ECP) (Stromberg et al., 2018).

In this study, we purified and characterized OMVs from APEC serotype O78 strain, and identified the 20 most abundant proteins located on OMVs by HPLC-MS. We also investigated the immunogenicity induced by OMVs of *E. coli* O78 in chicken animal model and demonstrated that OMVs was a super antigen complex, which could confer complete protection against lethal challenge of wild-type E. coli O78. This work provided us an alternative way for developing a promising vaccine based on OMVs, and our future efforts will focus on developing a trivalent vaccine against APEC O1, O2 and O78, which are three predominant serotypes responsible for colibacillosis.

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