The level of decoy epitope in PCV2 vaccine affects the neutralizing activity of sera in the immunized animals

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ABSTRACT

Viral pathogens have evolved a wide range of tactics to evade host immune responses and thus propagate effectively. One efficient tactic is to divert host immune responses toward an immunodominant decoy epitope and to induce non-neutralizing antibodies toward this epitope. Therefore, it is expected that the amount of decoy epitope in a subunit vaccine can affect the level of neutralizing antibody in an immunized animal. In this study, we tested this hypothesis by generating an antibody specific to the decoy epitope on the capsid protein of porcine circovirus type 2 (PCV2). Using this antibody, we found that two commercial vaccines contained statistically different amounts of the decoy epitope. The vaccine with lower levels of decoy epitope induced a significantly higher level of neutralizing antibody after immunization. This antibody can be used as an analytical tool to monitor the quality of a vaccine from batch to batch.

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1. Introduction

Viral pathogens have evolved a wide range of tactics to evade host immune responses and propagate effectively [1]. One such tactic is to divert the host immune response with a decoy. Decoy epitopes have been reported in a wide variety of viruses, including human immunodeficiency virus (HIV) [2], feline immunodeficiency virus (FIV) [3], hepatitis C [4], foot and mouth disease [5], middle-east respiratory syndrome coronavirus [6], severe fever with thrombocytopenia syndrome virus [7], porcine reproductive and respiratory syndrome virus (PRRSV) [8], murine gammaherpesvirus-68 (MHV-68) [9], and porcine circovirus type 2 (PCV2) [10]. Because a recombinant mutant capsid protein (CP) with a deleted decoy epitope induced a higher level of virus-neutralizing antibody than wild type proteins [11], it was expected that higher levels of decoy epitope in a prepared batch of subunit vaccine would decrease its efficacy. However, it was not possible to test this hypothesis by measuring levels of decoy

epitope in a vaccine because an antibody specific to the decoy epitope was not readily available.

PCV2 has a spherical structure with icosahedral symmetry [12]. The only structural protein of this virus is a 28-kD CP, which includes major antigenic determinants that can be used in subunit vaccines [13]. A previous study showed that an epitope composed of amino acid residues 169–180 ($CP_{169-180}$) is the most immunodominant among these antigenic determinants [11]. Because this epitope is buried inside the virus-like particle (VLP) structure (Fig. 1A), antibodies to this epitope cannot react with the intact virus, and therefore are non-neutralizing [14]. As expected, PCV infection elicited a humoral response to the $CP_{169-180}$ epitope and drove the production of non-neutralizing antibodies [15]. Therefore, previous infection with PCV decreased the vaccines prevention of the future infections.

Since the first vaccine against PCV2 was introduced to the global market in 2006, nine PCV2 subunit vaccines have become available [16]. However, they were expected to contain differing amounts of incomplete VLPs, which are non-uniformly aggregated CPs with an exposed $CP_{169-180}$ epitope [17]. Incomplete VLPs can induce production of non-neutralizing antibodies and reduce vaccine efficacy [14,18]. As there was no analytical tool to measure the amount of $CP_{169-180}$ epitope in a prepared batch of vaccine, it was not possible
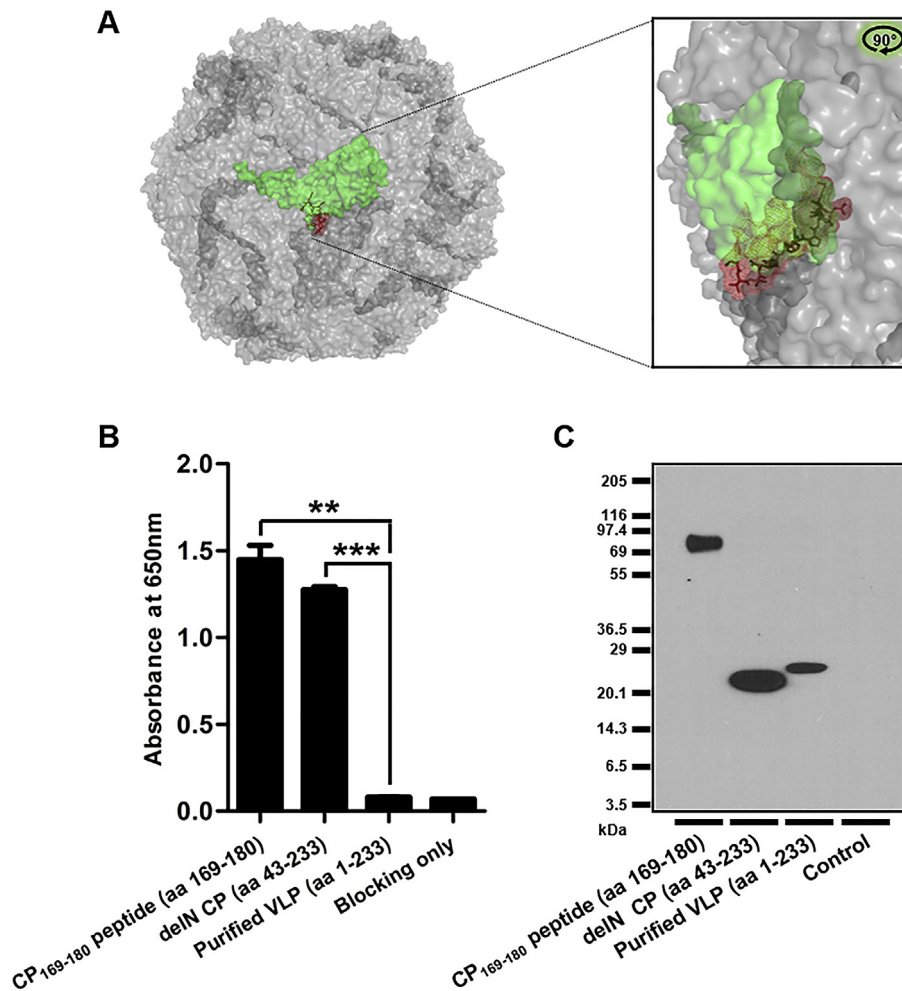
Fig. 1. A novel PCV2 antibody is specific to the CP$_{169-180}$ epitope. (A) The three-dimensional structure of PCV2 (PDB: 3JCI) was visualized by PyMOL 1.3. One capsid protein (CP) and the immunodominant CP$_{169-180}$ decoy epitope are marked in green and red, respectively. (B) Enzyme immunoassay using the anti-CP$_{169-180}$ antibody. The amount of antibody bound to antigens coated on microtiter plate was detected by HRP-conjugated goat anti-human C$_\kappa$ antibody. The results are the means $\pm$ standard deviations from experiments conducted in triplicate; $^{**}p < 0.01$, $^{***}p < 0.001$ by two-tailed unpaired Student's t-test. (C) Immunoblot analysis using the anti-CP$_{169-180}$ antibody. After the gel electrophoresis, antigens were transfected to nitrocellulose membrane. The membrane was probed with anti-CP$_{169-180}$ antibody.

to correlate epitope levels with neutralizing activity after vaccination.

In this study, we developed an antibody specific to the CP$_{169-180}$ epitope, determined the relative amounts of CP$_{169-180}$ epitope in two commercially available vaccines, and analyzed the influence of the epitope level on the induction of neutralizing antibody.

2. Materials and methods

2.1. Preparation of VLP, PCV2 recombinant proteins, and peptide conjugates

PCV2 CP (amino acids [aa] 1–233) was expressed in baculovirus-infected Sf9 insect cells, as previously described [19]. The PCV2 *ORF2* gene (GenBank no. EU747125) was cloned into the pFastBac expression vector (Invitrogen, Carlsbad, CA, USA) and transfected into Sf9 cells. The culture supernatant containing the recombinant baculovirus was harvested and used to infect a separate batch of Sf9 cells. After 3 days, VLPs were purified from culture supernatant by sucrose gradient ultracentrifugation.

Recombinant CP with a deletion of the N-terminal region of the protein (delN CP, aa 44–233) was expressed in *Escherichia coli* and purified, as described previously [12,20,21]. A synthetic CP$_{169-180}$ peptide (STIDYFQPNNKRC) was chemically synthesized using Fmoc solid-phase peptide synthesis (Peptron, Inc., Daejeon, South Korea) and conjugated to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) [10].

2.2. Generation of recombinant chicken anti-CP$_{169-180}$ antibody

White leghorn chickens were immunized with KLH-conjugated CP$_{169-180}$ peptides. A phage-displayed chicken single-chain variable fragment (scFv) library was constructed using total RNA isolated from the bone marrow, spleen, and bursa of Fabricius of immunized chickens, as previously described [22]. A positive clone was enriched by biopanning and screened in a phage enzyme immunoassay, as described previously [23,24].

The gene encoding the selected scFv clone was subcloned into a modified mammalian expression vector encoding the C$_\kappa$ domain of human IgG at the 3′ region [25]. The scFv-C$_\kappa$ fusion protein (anti-CP$_{169-180}$ antibody) was purified from the culture supernatants of transiently transfected HEK293F cells using KappaSelect resin (GE

Healthcare, London, UK), according to the manufacturer's instructions.

2.3. Enzyme immunoassay

The wells of a 96-well microtiter plate (Corning) were coated with BSA-conjugated $CP_{169-180}$ peptides, delN CP, and purified VLP in coating buffer (0.1 M $NaHCO_3$, pH 8.6) and then blocked with 3% (w/v) BSA in phosphate-buffered saline (PBS). After incubation with 1 μg/mL of anti-$CP_{169-180}$ antibody, horseradish peroxidase (HRP)-conjugated goat anti-human C_K antibodies (Chemicon-Millipore, Billerica, MA, USA) were added to each well. After washing with 0.05% (v/v) Tween 20 in PBS (PBST), 3,3′,5,5′-tetramethyl benzidine (TMB) (GenDEPOT, Barker, TX, USA) substrate solution was added and the absorbance was measured at 650 nm with a Multiskan Ascent microplate reader (LabSystems, Helsinki, Finland) [26].

2.4. Immunoblot analysis

Dissolved proteins and BSA-conjugated $CP_{169-180}$ peptides were boiled for 10 min in lithium dodecyl sulfate sample buffer with reducing agents (Invitrogen). The samples were then electrophoretically separated on a NuPage 4—12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane [27]. After blocking with 5% (w/v) skim milk in Tris-buffered saline, the membrane was incubated with 1 μg/mL of anti-$CP_{169-180}$ antibody and probed with HRP-conjugated goat anti-human C_K antibodies (Chemicon-Millipore). The blot was visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, IL, USA).

2.5. Competition enzyme immunoassay using PCV2-infected pig's sera

All animal experiments were performed under the approval of the Institutional Animal Care and Use Committee of the BioPOA (permit no. BP-2015-030-2). After the collection of sera from PCV2-infected pigs and pigs with no infection history, the competition enzyme immunoassay was performed as described previously with the following appropriate modifications [25]. After pre-incubating non-infected and infected porcine sera (n = 3/group) with serially diluted anti-$CP_{169-180}$ antibody, the mixtures were added to microtiter plate coated with BSA-conjugated $CP_{169-180}$ peptide. An irrelevant scFv fused with a C_K domain was used as an isotype-matched control. The amount of bound porcine IgG was determined by HRP-conjugated goat anti-porcine IgG antibodies (Santa Cruz Biotechnology, TX, USA) and TMB was used for the substrate of HRP.

2.6. Enzyme immunoassay using two commercial vaccines

We randomly designated two of out nine commercial vaccines (Ingelvac CircoFlex [Boehringer Ingelheim Vetmedica], Porcilis PCV-one [MSD Animal Health], Circumvent PCV [Merck Animal Health], Fostera PCV [Zoetis], Suvaxyn PCV [Fort Dodge Animal Health], Circovac [Merial], CircoShield [Green Cross Veterinary Products], SuiShot Circo-ONE [ChoongAng Vaccine Laboratory] and DS Circo PigVac [Daesung Microbiological LABS]) as vaccine A or vaccine B. Then vaccine A and B were diluted four-fold in coating buffer. After vigorous vortexing for 10 min, microtiter plates were coated with vaccines at 37 °C for 2 h and blocked with 3% BSA in PBS.

The level of total PCV2 antigen in prepared VLP, delN CP, and the two commercial vaccines was measured by enzyme immunoassay using an anti-PCV2 polyclonal antibody (Veterinary Diagnostic Laboratory, Iowa State University, Iowa, USA) [28] and HRP-conjugated goat anti-rabbit IgG antibodies (Bethyl Laboratories, Montgomery, TX, USA), as described previously.

To monitor the $CP_{169-180}$ epitope levels, we performed the enzyme immunoassay using the anti-$CP_{169-180}$ antibody and HRP-conjugated goat anti-human C_K antibodies (Chemicon-Millipore), as previously described.

2.7. Immunization and the analysis of immune sera

Twenty guinea pigs were randomly divided into four groups (n = 5/group). Each guinea pig was immunized intradermally with 200 μL of VLP, delN CP, or commercial vaccine. Four weeks after immunization, whole blood was collected and sera were prepared.

An indirect fluorescence assay (IFA) was performed using the sera and PCV2-infected PK-15 cells, as previously described [18,29]. To monitor the reactivity of sera to the $CP_{169-180}$ epitope, enzyme immunoassays, using BSA-conjugated $CP_{169-180}$ peptides and HRP-conjugated anti-guinea pig IgG antibodies (Bethyl Laboratories), were performed as described previously. We performed serum virus neutralization assays using PK-15 cells, as described previously [30,31].

2.8. Statistical analysis

Statistical analysis was conducted using GraphPad Prism (v5.0; GraphPad Software Inc., San Diego, CA, USA). Results are expressed as means and standard deviations of the indicated number of independent measurements. Statistical significance was determined using two-tailed unpaired Student's t tests and non-parametric Mann-Whitney's u test. Two-way analysis of variance (ANOVA) was used to analyze the relationship between the two independent factors. For all statistical analyses, p values of <0.05 were considered statistically significant.

3. Results

3.1. Novel PCV2 antibodies are specific to the $CP_{169-180}$ epitope

A phage display library of combinatorial scFv was prepared using chickens immunized with KLH-conjugated $CP_{169-180}$ peptides. The complexity of the library exceeded 1.2×10^9. To enrich for scFv clones with specific reactivity to the $CP_{169-180}$ epitope, six total rounds of biopanning were performed. For each round of biopanning, monomeric delN CP and $CP_{169-180}$ peptides were alternately used as antigens. The N terminus of CP plays a key role in the assembly of the VLP structure. Thus, deletion of the N-terminal region exposes the $CP_{169-180}$ epitope (del CP).

After biopanning, the reactivity of individual clones to $CP_{169-180}$ peptide, delN CP, and PCV2 VLPs was tested by phage enzyme immunoassay. A clone was selected, and its recombinant scFv-C_K fusion protein prepared using a mammalian expression system. The recombinant scFv-C_K fusion protein reacted to $CP_{169-180}$ peptide and delN CP, but not to VLPs (Fig. 1B). In immunoblot analysis, the recombinant scFv-C_K fusion protein was reactive not only to the $CP_{169-180}$ peptide and delN CP, but also to VLPs, because the $CP_{169-180}$ epitope on VLP was exposed by the denaturation that occurs during sample preparation (Fig. 1C).

Although the $CP_{169-180}$ segment is too short to harbor more than one antigenic determinant, we further confirmed a number of antigenic determinants using the polyclonal sera of pigs infected with PCV2. In a competition enzyme immunoassay employing a microtiter plate coated with BSA-conjugated $CP_{169-180}$ peptide and HRP-conjugated anti-porcine IgG antibody, the recombinant anti-$CP_{169-180}$ antibody almost completely inhibited the binding of naturally occurring anti-$CP_{169-180}$ antibody to the peptide in a

PCV2-infected pig's sera (Fig. 2). From these results, we concluded that there is only one antigenic determinant in $CP_{169-180}$ residues.

3.2. Amounts of $CP_{169-180}$ epitope differ in two commercial vaccines

The relative amount of PCV2 antigen in two commercially available vaccines was determined in an enzyme immunoassay, using purified VLP and the recombinant delN CP protein. The amount of coated antigen was then determined by anti-PCV2 polyclonal antibody. Because the quantity of bound antibody was not statistically different between the vaccines, we concluded that they contained similar amounts of the recombinant CP (Fig. 3A). We then determined the relative amount of $CP_{169-180}$ epitope in these two vaccines with an enzyme immunoassay employing anti-$CP_{169-180}$ antibody (Fig. 3B). Because we found a higher amount of anti-$CP_{169-180}$ antibody bound to vaccine A-coated wells than in vaccine B-coated wells, we concluded that vaccine A contained a higher amount of $CP_{169-180}$ epitope.

3.3. Humoral immune responses to two commercial vaccines corresponded to $CP_{169-180}$ epitope levels

To monitor the humoral response, we collected sera from guinea pigs (n = 5/group) that were immunized with vaccines. For control sera, we also immunized with VLPs and the recombinant delN CP in parallel. To confirm successful vaccination, we performed IFA as described previously using PCV2-infected PK-15 cells [18,29]. There was no statistical difference between the antibody titer of sera obtained from animals immunized with the two vaccines. As expected, the sera of animals immunized with delN CP showed a statistically lower antibody titer compared to other groups ($p < 0.05$) (Fig. 3C). In an enzyme immunoassay employing a microtiter plate coated with BSA-conjugated $CP_{169-180}$ peptide, sera from animals immunized with vaccine A had a significantly higher antibody titer than vaccine B-vaccinated animals ($p < 0.01$) (Fig. 3D). Additionally, the sera of delN CP-immunized animals had a significantly higher antibody titer than those of VLP-immunized animals ($p < 0.01$). Then we measured and compared the neutralizing activity of sera in an *in vitro* infection experiment employing PCV2 virus and PK-15 cells. The sera from vaccine B-immunized animals had a significantly higher neutralizing antibody titer than sera from vaccine A-immunized animals ($p < 0.001$) (Fig. 3E). Also, the sera of VLP-immunized animals showed significantly higher neutralizing activity than sera of VLP-immunized animals ($p < 0.01$).

4. Discussion

Since the emergence of PCV2, there has been considerable effort to produce an effective vaccine [32]. For some time, the main hurdle was efficient overexpression of CP [19,33]. Later, it was found that the immunodominant $CP_{169-180}$ epitope of PCV2 is buried during viral capsid assembly and that the antibodies targeting this epitope are non-neutralizing, which is a common viral immune-evasion mechanism [14]. PCV2 infection inevitably provokes exposure to the decoy epitope and therefore is less effective at generating a protective humoral response than immunization of the subunit vaccine with a minimal amount of exposed decoy epitope [18].

Researchers had predicted that incomplete VLPs with an exposed immunodominant $CP_{169-180}$ epitope can be contaminated during the preparation of subunit vaccine and drive the humoral response to produce non-neutralizing antibodies, but could not quantify the exposed immunodominant $CP_{169-180}$ epitope in the subunit vaccines because of the lack of proper analytical tools [33–36]. To rectify this, we generated a monoclonal antibody specific to the immunodominant $CP_{169-180}$ epitope from an antibody library generated using chickens immunized with $CP_{169-180}$ peptide. This recombinant anti-$CP_{169-180}$ antibody preferentially reacted to delN CP with an exposed $CP_{169-180}$ epitope compared to VLP in an enzyme immunoassay (Fig. 1B). Because the length of linear B-cell epitopes can vary widely from 3 to 38 amino acids [37], we confirmed that $CP_{169-180}$ peptides behave as a single epitope in a competition enzyme immunoassay. The recombinant $CP_{169-180}$ antibody almost completely inhibited the binding of PCV2-infected pig sera to $CP_{169-180}$ peptide.

We then used this antibody to quantify the relative amount of $CP_{169-180}$ epitope in two commercially available vaccines and found that vaccine A contained significantly more decoy epitope than vaccine B. After immunization, vaccine A induced a significantly higher antibody response to decoy epitope than vaccine B, which supported the finding that vaccine B induced a higher amount of neutralizing antibody.

In summary, our research demonstrated that the amount of immunodominant decoy epitope present in PCV2 vaccine can be measured by an epitope-specific antibody. From the measured level of decoy epitope, we could predict the efficacy of a vaccine for the induction of neutralizing antibody titer. We believe that anti-$CP_{169-180}$ antibody can be used to monitor the varying quality of the subunit vaccine from batch to batch.
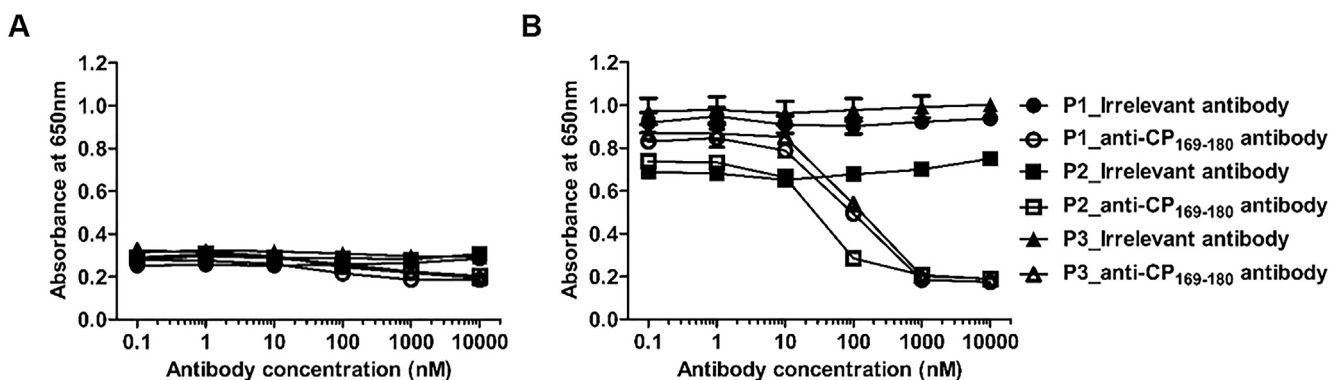
Fig. 2. Recombinant anti-$CP_{169-180}$ antibody inhibited the binding of naturally occurring antibody. After pre-incubation with sera from the non-infected (A) and infected pigs (B) with anti-$CP_{169-180}$ antibody (n = 3/group), the mixtures were added to a $CP_{169-180}$ peptide-coated microtiter plate. The amount of bound porcine antibody was measured by HRP-conjugated anti-porcine IgG antibody. Absorbance was measured at 650 nm. The results are the means ± standard deviations from the experiments, which were conducted in triplicate using the sera from three pigs (P1, P2, and P3 in each group).
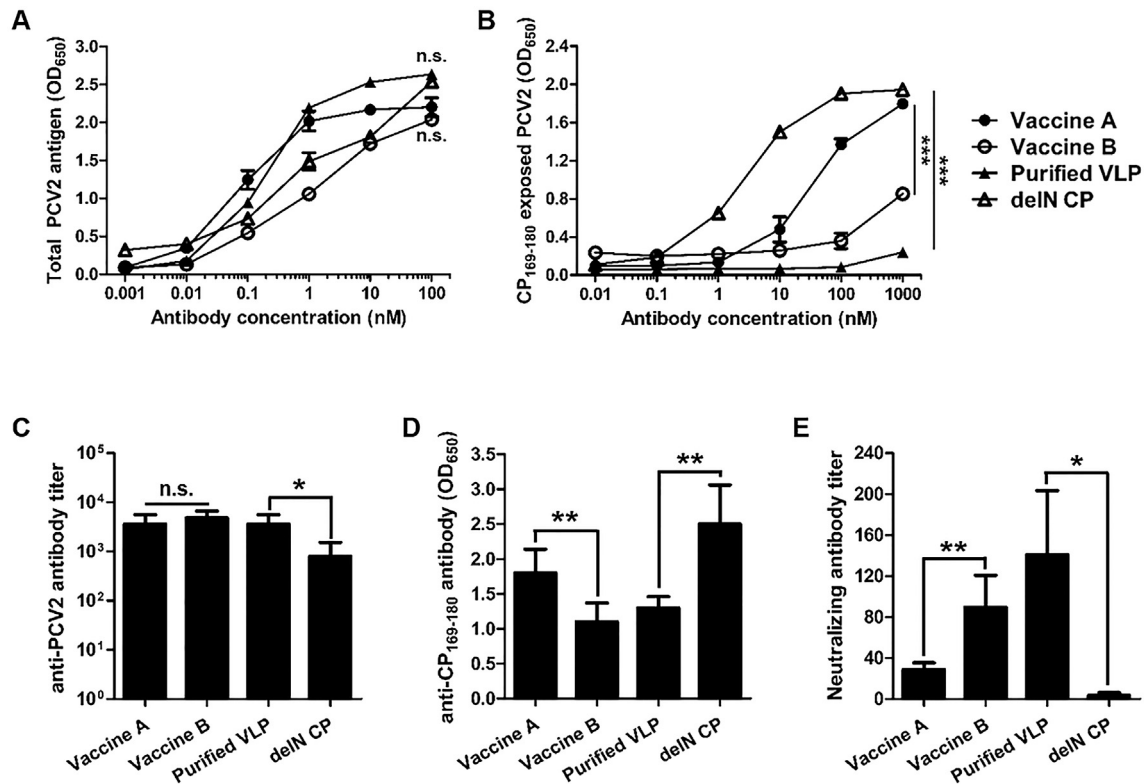
Fig. 3. Levels of $CP_{169-180}$ in vaccines corresponded with humoral responses. (A) PCV2 antigen level was measured in an enzyme immunoassay using anti-PCV2 polyclonal antibody. (B) $CP_{169-180}$ epitope level was measured in an enzyme immunoassay using the anti-$CP_{169-180}$ antibody. (C) Anti-PCV2 antibody in the immunized sera was determined by an indirect fluorescence assay using PCV2-infected PK-15 cells (n = 5/group). (D) The amount of anti-$CP_{169-180}$ antibody in the immunized sera was measured in an enzyme immunoassay using a $CP_{169-180}$ peptide-coated microtiter plate. (E) Neutralizing activity of the immunized sera was measured with a virus neutralization assay. Results are the means ± standard deviations; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by two-tailed non-parametric Mann-Whitney's *u* test.

Conflicts of interest

The authors have declared that there is no conflict of interest.

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Transparency document

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