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Running title: PapGII does not protect against APEC

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Abstract

The aim of this study was to investigate whether immunisation with the sugar binding domain of PapGII (PapGII196) was able to protect chickens against avian pathogenic *Escherichia coli* (APEC). PapGII196 was expressed, purified by Ni-NTA column chromatography and shown to retain its biological activity, as demonstrated by binding to its receptor, globoside. PapGII196 was tested as a vaccine in specific pathogen free broilers and also by vaccinating breeders and assessing protection in their offspring, and using aerosol exposure or air sac inoculation for challenge. Notwithstanding a strong anti-PapGII196 serum IgG response in vaccinated birds in all experiments and inhibition of haemagglutination by serum from PapGII196-vaccinated birds, chickens were not protected against avian pathogenic *E. coli*. These findings suggest that PapGII may not be a useful candidate for inclusion in vaccines against APEC.

Introduction

Escherichia coli infection in avian species (colibacillosis) often initiates an infection of the respiratory tract, but progresses to a general infection of the internal organs, as manifested by pericarditis, perihepatitis, peritonitis and septicemia. APEC infection is often preceded by infection with predisposing agents such as Newcastle disease virus, infectious bronchitis virus or mycoplasmas, or by environmental factors (e.g. ammonia). In broilers, APEC are also associated with the development of cellulitis or necrotic dermatitis. These diseases result in substantial economic losses due to carcass condemnation, reduced growth and death (Gross, 1994; Dho-Moulin and Fairbrother, 1999; Barnes *et al.*, 2003).

Among the many putative virulence factors that have been identified in APEC, fimbriae (or pili) are thought to play an important role in infection. Fimbriae are proteinaceous structures on the outer membrane of *E. coli* mediating attachment to host cell receptors by means of their conserved adhesin. F1A or type 1 fimbriae are believed to be involved in the initial colonization of the respiratory tract due to adhesion of the adhesin FimH to D-mannose residues on host cells (Gross, 1994; Pourbakhsh *et al.*, 1997a, b), although their exact role remains much debated (Marc *et al.*, 1998, Arné *et al.*, 2000, Vandemaele *et al.*, 2005). P fimbriae, on the other hand, are thought to act in the later stages of infection, as they are only expressed *in vivo* in the lower respiratory tract and internal organs (Dozois *et al.*, 1995; Pourbakhsh *et al.*, 1997a).

P fimbriae consist of polymorphic major subunits, PapA (Lund *et al.*, 1988; Johnson *et al.*, 2000), and a conserved PapG adhesin. PapG occurs as three molecular

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variants, encoded by the corresponding *papG* alleles (Johnson *et al.*, 1997) and mediating adhesion (Lund *et al.*, 1987) to different Galα1-4Gal containing glycolipid receptors (Strömberg *et al.*, 1990, 1991; Hansson *et al.*, 1995). The PapG adhesin is composed of an amino-terminal sugar binding domain and a carboxyl-terminal fimbrial domain (Hultgren *et al.*, 1989; Hansson *et al.*, 1995). In a previous study we demonstrated that P fimbriae were present in 29 % of clinical APEC isolates and that 96.6 % of P^+ strains possessed the PapGII adhesin (Vandemaele *et al.*, 2003b).

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It has recently been shown that vaccination with the PapG-PapD adhesin-chaperone complex was able to protect monkeys against pyelonephritis mediated by *E. coli* (Roberts *et al.*, 2004). Furthermore, Kariyawasam *et al.* (2004) showed that experimental transfer of anti-PapG egg antibodies (IgY) protected broilers against subsequent APEC challenge. However, it is not known which PapG variant was used and whether active vaccination with denatured PapG would yield the same result. A vaccine, containing P (F11)-fimbriae and flagellin, is currently available for use in broiler breeders to provide passive protection against APEC to their offspring (Intervet International, Boxmeer, the Netherlands). However, as vaccination with complete fimbriae in general mainly results in antibodies against the polymorphic major subunits, restricting protection to APEC strains with the same major subunit (Dozois *et al.*, 1995; Langermann *et al.*, 1997), we investigated whether a vaccine based on the conserved adhesin PapGII could protect against APEC.

In a previous study (Vandemaele *et al.*, 2005), we demonstrated that the sugar binding domain of FimH, the adhesin of F1A fimbriae, did not protect chickens against APEC infection, indicating a more limited role in infection. However, a major difficulty encountered was the induction of a mucosal immune response in the trachea with a single protein (Vandemaele *et al.*, 2005). Due to the suggested role of PapG in

the systemic phase of APEC infection (Dozois *et al.*, 1995; Pourbakhsh *et al.*, 1997a), the aim of this study was to investigate whether systemic antibodies against the biologically active recombinant PapGII sugar binding domain (i.e. PapGII196) could protect chickens against APEC. To this purpose different vaccination strategies (such as immunisation and challenge of broilers, and immunisation of breeders and challenge of off-spring) and different challenge models (via aerosol **or** intra air sac) were tested. Although the occurrence of PapG is more limited in APEC strains, protection by PapGII could lead to incorporation of this protein into multivalent vaccines, composed of different recombinant virulence factors, possibly broadening protection.

Materials and Methods

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Bacterial strains, plasmids and media. APEC strain CH2 was a virulent O78 *papG*II$^+$ strain (Van den Bosch *et al.*, 1993; Vandemaele *et al.*, 2005). APEC 1 was a virulent O45 *papG*II$^+$ strain (Vandemaele *et al.*, 2003b). Strain CH4 was a virulent O2 *papG*II$^+$ strain (Van den Bosch *et al.*, 1993). All strains were *papG*II$^+$, as verified by PCR and sequencing, and expressed P fimbriae, as shown by mannose-resistant haemagglutination of human blood group A erythrocytes using protocols described by Vandemaele *et al.* (2003a). Plasmid pPCR SCRIPT AMP SK (Stratagene, La Jola, California, USA) was used for direct cloning of blunt-end PCR products and sequencing, and plasmid pET22b (Novagen, Madison, USA) was used as an expression vector. *E. coli* strain BL21 (DE3) was used for expression of the pET22b-*papG*II196 construct. Luria-Bertani broth (DIFCO BD, New Jersey, USA) was used

for growing *E. coli* strain BL21 and strain CH2 for challenge (experiments 1 and 3). Tryptic Soy Broth (TSB, DIFCO BD) was used for growing APEC strain APEC 1 for challenge (Experiment 2). When required, ampicillin (100 µg/ml) was added.

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Newcastle disease virus. Infections with Newcastle disease virus (NDV) lentogenic strains Lasota and Clone30 (Intervet International, Boxmeer, The Netherlands) were used to predispose chickens to colibacillosis, as described by Vandemaele *et al.* (2005). Each chick in Experiment 1 was administered approximately 2 x $10^{6.3}$ median egg infective doses (EID_{50}) of strain Lasota in phosphate buffered saline (PBS) by spray. One-day-old chicks in Experiment 2 received $10^{7.0}$ EID_{50} of strain Clone30 per bird in PBS.

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Plasmid construction. Genomic DNA was extracted (Ausubel *et al.*, 1992) from strain APEC 1 and used as a template to amplify the sugar binding domain of *papG*II, which constitutes the first 196 amino acids of the mature protein (*papG*II196), as previously demonstrated by Dodson *et al.* (2001). The primers used were PapGII196F (5' ATCAACCATGGATATCTGGAATAATATTGTCTTTTAC 3') and PapGII196R **(5' TGATACCATGGTTAATGGTGATGGTGATGGTG TCCGCCGATATTCTTAAATAAG 3')**. Primer PapGII196F contained an *Nco*I cleavage site (CCATGG). A hexahistidine affinity tag was incorporated in the reverse primer, PapGII196R, followed by a stop codon (TAA) and an *Nco*I cleavage site. PCR was performed in 50 µl reactions containing 0.2 µM of each dNTP, *Pfu* buffer, 0.5 µM each of PapGII196F and PapGII196R, 7.5 % DMSO, 2.5 U *Pfu* DNA Polymerase (Stratagene) and 0.2 µg genomic DNA. The reaction was incubated at 95°C for 1 min, then through 30 cycles of incubation at 95°C for 1 min, 56°C for 1

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min and 72°C for 1.5 min, followed by a final incubation at 72°C for 5 min. The PCR product was extracted from a 2 % agarose gel (Qiaquick Gel Extraction Kit, Qiagen, Valencia, USA), and the Stratagene pPCR SCRIPT AMP SK cloning kit (Stratagene) was used to ligate the PCR product into pPCR SCRIPT AMP SK and this used to transform to *E. coli* XL10. The sequence of the cloned product was confirmed by sequencing using the ABI PRISM Bigdye™ Terminator Cycle Sequencing Ready Reaction Kit (ABI, Foster City, USA), following the manufacturer's instructions. Using *Nco*I, *papG*II196 was cleaved from this plasmid, ligated to vector pET22b (subsequently referred to as pET22b-*papG*II196) and used to transform to strain BL21 (DE3) by electroporation. The predicted amino acid sequence of the sugar binding domain of PapGII of virulent strain CH2 was identical to that of strain APEC 1, from which the construct was derived.

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Expression and purification. Starter cultures of BL21 (DE3) in LB broth containing ampicillin were incubated at 37°C with shaking at 250 rpm until turbid and then stored at 4°C. The following day, 300 ml LB containing 100 µg ampicillin / ml and 1 % glucose was inoculated with 12 ml of the starter cultures and incubated at 28°C with shaking at 250 rpm to an OD_{600} of 0.6. Expression was then induced by adding IPTG to a final concentration of 0.1 mM. After 4 hours, cells were harvested by centrifugation at 6500 x g for 15 min and the bacterial pellet was stored at -20°C until purification. As some PapGII196 is present in the soluble fraction, the protein was purified by Ni-NTA affinity chromatography under non-denaturing conditions. The bacterial pellet was resuspended in 2 volumes of Ni-NTA binding buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole). Lysozyme was added to a final concentration of 1 mg/ml and the mixture incubated for 30 min on ice. The lysates

were then sonicated on ice and centrifuged twice at 10,000 x g for 30 min. The cleared supernatant was mixed with 0.33 volume of Ni-NTA slurry (50 %, Novagen) and incubated at 4°C with shaking for 90 min. The mixture was then applied to a chromatography column and the column washed once with a volume of washing buffer 1 (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole) equal to the gel volume. The column was subsequently washed twice with 8 ml washing buffer 2 (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 35 mM imidazole) and once with a volume of washing buffer 3 (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 50 mM imidazole) equal to the gel volume. Finally, the protein was eluted 4 times with elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 250 mM imidazole; 0.5 volume of the Ni-NTA slurry) into tubes already containing half a volume of elution buffer (to prevent possible aggregation due to high concentration). Elution fractions were dialysed extensively against 0.02 M Tris-HCl (pH 8.5) using a 15 kDa dialysis membrane (Spectropore, Spectrum Laboratories Inc., California, USA) to remove imidazole. The presence and purity of PapGII196 was checked by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (12.5 %) and Coomassie blue staining. Western blotting was performed using an anti-His monoclonal antibody (Novagen) to confirm the presence of the His-tagged protein. Protein concentrations were determined using the BCA method (Pierce, Rockford, USA).

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PapGII196 activity assay. The biological activity of the recombinant PapGII196 was confirmed by ELISA. ELISA plates were coated with 150 µl of globoside (GbO4, Sigma, St. Louis, MO, USA) per well at three different concentrations (30, 15 and 7.5 µg/ml) in methanol. Methanol was allowed to evaporate at 37°C for 45 min and plates were blocked using 5 % bovine serum albumin (BSA) in PBST (0.01 % (v/v)

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Tween 20 in PBS) for 1 hour at 37°C. After washing three times with PBST, the recombinant PapGII196 (50 µg/ml in PBST) was added at 150 µl/well and plates were incubated for 2 hours at room temperature. As negative controls, 150 µl FimH156 (50 µg/ml in PBST), the sugar binding domain of the F1A fimbrial adhesin FimH (Vandemaele *et al.*, 2005) or 150 µl PBS were added to the globoside. Plates were washed and incubated with an anti-His monoclonal antibody (Novagen, 1/2000 in 0.5 % bovine serum albumin – PBST (BSA-PBST)) for 1 hour at room temperature. After washing, a secondary goat anti-mouse horseradish peroxidase (HRP) labelled antibody (DAKO Cytomation, Glostrup, Denmark, 1/5000 in 0.5 % BSA-PBST) was added and the plates incubated for 1 hour at room temperature. Bound HRP was detected using 2,2'-azino-di(3-ethylbenzythiazolin-6-sulfonic acid) (ABTS; KPL, Gaithersburg, Maryland, USA) and absorbances at 405 nm were measured using a Titertek microplate reader (MCC/340 Multiskan, Titertek, Alabama, USA).

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Active immunization and aerosol challenge (Experiment 1). Forty-two specific pathogen free (SPF) broiler chicks (Intervet) were randomly divided in 4 groups (12 chicks/group for groups 1-3 and 6 chicks for group 4) and housed in isolators. All chicks were free of infectious bronchitis virus, Newcastle disease virus, *Mycoplasma gallisepticum* and *M. synoviae*. Groups 1 and 2 were mixed and distributed in equal proportions in two isolators to exclude isolator effects. Feed and water were supplied *ad libitum* during the experiment. Group 1 was vaccinated intramuscularly with PapGII196 and challenged (V/C), group 2 was vaccinated intramuscularly with a placebo and challenged (P/C), group 3 was vaccinated intramuscularly with PapGII196 but was not challenged (V/-) and group 4 was not vaccinated or challenged (-/-). At 10 days of age (0 days post vaccination, dpv) chicks in groups 1

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($1^{V/C}$) and 4 ($4^{V/-}$) were vaccinated intramuscularly with 85.5 µg PapGII196 in a water-in-oil (w/o) emulsion (45/55, paraffin based, Intervet). Group 2 ($2^{P/C}$) received an intramuscular injection of 0.02 M Tris-HCl, pH 8.5, in the same w/o emulsion, while group 3 ($3^{-/-}$) was not vaccinated (Table 1). Three weeks later (21 dpv), all groups were spray-infected with NDV strain Lasota. Three days later (24 dpv), an APEC challenge was administered by nebulization of a 10 ml culture of strain CH2 (2.7 x 10^{10} cfu/ml in LB) into each isolator. Birds in groups $3^{-/-}$ and $4^{V/-}$ were not challenged. Seven days later (31 dpv), all birds were euthanised by cervical dislocation and autopsied. Scores were determined for macroscopic lesions in the thoracic (0 – normal, 1 – one air sac very cloudy and presence of fibrinous exudate or limited pinhead-sized foci of fibrinous exudate in both air sacs, 2 - both air sacs very cloudy and presence of fibrinous exudate) and abdominal air sacs (0 - normal, 1 – mild cloudiness and / or pinhead-sized foci of fibrinous exudate, 2 - very cloudy with fibrinous exudate), lungs (0 - normal, 1 – unilateral pneumonia with discoloration and consolidation, 2 - bilateral pneumonia), pericardium (0 - normal; 1 - some cloudy exudate in the pericardium, 2 - pericardium filled with large amounts of purulent exudate) and liver (0 - normal; 1 - some multifocal exudate adherent to surface, 2 - extensive layers of exudate or fibrin adherent to the surface). Birds that died during the challenge period were awarded the maximal score of 10 (2 per organ). Birds that died before APEC challenge and did not show clinical signs of APEC infection were excluded from the results. Total lesion scores were calculated for each chicken and medians and lower and upper quartiles were determined for each group. The medians for the total lesion score and the medians for the lesion scores per organ were analyzed to determine whether there were significant differences between groups.

Blood was collected on days 10 (0 dpv), 17 (7 dpv), 24 (14 dpv) and 31 (21 dpv), and at autopsy (31 dpv). Serum was collected and stored at -20°C for ELISA.

Passive immunization and aerosol challenge (Experiment 2). Twelve SPF broiler breeders (Intervet) were housed in isolators with feed and water *ad libitum*. At 12 weeks of age, six of the breeders were vaccinated with 50 µg of PapGII196 in a water-in-oil (w/o) emulsion (45/55, paraffin based, Intervet), while the others remained unvaccinated. At 20 weeks of age, the six vaccinated breeders were revaccinated in the same way. From 25 weeks of age onwards, eggs were collected from all birds and stored at 4°C. Eggs were incubated and the one-day-old chicks were divided into 3 groups (Table 2, groups $1^{V/C}$, $2^{-/C}$, $3^{-/-}$). Group $1^{V/C}$ contained 40 chicks from the PapGII196-vaccinated breeders. Group $2^{-/C}$ and $3^{-/-}$ contained 40 and 20 chicks, respectively, from the unvaccinated breeders. At one day of age, all chicks were infected by spray with NDV strain Clone30, and subsequently the one-day-old chicks of groups 1 and 2 were challenged by spray with strain APEC 1 in TSB (100 ml at a concentration of 1.8 x 10^9 cfu/ml). Chicks in group $3^{-/-}$ were not challenged. Birds were euthanized one week later and autopsied. Lesion scores were determined as described above (Experiment 1). Blood was taken from breeders at 12 weeks (before vaccination), 20 weeks (before booster), 25 weeks (egg collection) and 29 weeks. Egg yolk was collected from eggs and blood was also taken from 1-day-old chicks.

Active immunization and air sac challenge (Experiment 3). Sixty SPF broiler chicks (Intervet) were randomly divided in 5 groups (12 chicks/group) and housed in a disinfected stable with feed and water *ad libitum*. The 12 birds in group 5 were

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equally distributed over the other groups to act as sentinels. At 10 days of age (0 dpv) chicks in groups 1 ($1^{V/C}$) and 3 ($3^{V/C}$) were vaccinated intramuscularly with 26.1 µg of PapGII196 in a water-in-oil (w/o) emulsion (45/55, paraffin based, Intervet). Groups 2 ($2^{P/C}$) and 4 ($4^{P/C}$) received 0.02 M Tris-HCl, pH 8.5, in the same w/o emulsion, while group 5 ($5^{-/-}$) was not vaccinated (Table 3). Group superscripts are as described for experiment 1. Three weeks later (21 dpv), birds in groups $1^{V/C}$ and $2^{P/C}$ were challenged by air sac inoculation with 10^4 cfu of strain CH2 in LB (inoculation of 200 µl into the right thoracic air sac) and birds in groups $3^{V/C}$ and $4^{P/C}$ were challenged by air sac inoculation of 10^5 cfu of strain CH2 in LB. Group $5^{-/-}$ was not challenged. Seven days later (28 dpv), all birds were euthanised by cervical dislocation and autopsied. Scores were determined as described above, but with some modifications. Left and right thoracic air sacs were scored separately (0 – normal, 1 – presence of fibrinous exudate or limited pinhead-sized foci of fibrinous exudate, 2- air sac very cloudy and presence of fibrinous exudate). The left and right lungs were also scored separately (0 - normal, 1 – pneumonia with discoloration and consolidation, 2 - pneumonia and presence of fibrinous exudate). Blood was collected on days 10 (0 dpv), 17 (7 dpv), 24 (14 dpv) and 31 (21 dpv), and at autopsy (28 dpv). Serum was collected and stored at -20°C for ELISA.

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ELISA for anti-PapGII IgG using recombinant papGII196. ELISA plates were incubated overnight at 4°C with 150 µl purified PapGII196 (10 µg/ml in bicarbonate buffer, pH 9.6) per well. Plates were washed three times with PBS-Tween (PBST, 0.01 % (v/v) Tween 20 in PBS) and blocked with 0.1 % casein in PBS for 1 hour at 37°C. Plates were washed and incubated with sera from experimental birds (for experiments 1 and 3, at dilutions of 1/400 and 1/200, respectively, in 0.5 % bovine

serum albumin – PBST (BSA-PBST)) for 1 hour at room temperature. For experiment 2 a series of twofold dilutions of serum and egg yolk in 0.5 % BSA-PBST, ranging from 2^{-10} to 2^{-17}, were tested to determine the titre. Subsequently plates were washed three times with PBST and incubated with a HRP-labelled goat anti-chicken IgG antibody (Bethyl, Montgomery, Texas, USA, 1/5000 in 0.5 % BSA-PBST) for 1 hour at room temperature. After washing, bound HRP was detected using 150 µl ABTS per well and absorbance determined at 405 nm. Negative controls (no chicken serum, 0.5 % BSA-PBST) were incorporated on each plate. The mean of duplicate wells was determined and the value obtained from the negative control wells was subtracted from this. For determination of titres in experiment 2, the cut-off was defined as two standard deviations above the mean absorbance of the pre-immune sera. When the most dilute sample (2^{-17}) was still above the cut-off, the titre was recorded as 18. When the initial dilution was below the cutoff, of the titre was recorded as 9.

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ELISA for anti-PapGII IgG using bacterial lysate. To evaluate whether antibodies against the recombinant PapGII196 also recognized PapGII in its native context, sera from experiment 1 were analysed by ELISA using a lysate of the PapGII-expressing strain CH2. Strain CH2 was inoculated onto LB agar to favour expression of P fimbriae (Dozois *et al.*, 1995) and allowed to grow overnight at 37°C. The following day, bacteria were harvested by washing each plate with 2 ml sterile PBS. This procedure was repeated and the harvested bacteria were centrifuged for 10 min at 4000 ***g*** at 4°C and resuspended in ELISA coating buffer (bicarbonate buffer, pH 9.6). The optical density (OD) was determined at 600 nm and bacterial suspensions were diluted to a concentration of 10^9 bacteria/ml (OD_{600} of 1 equalled 1 x 10^9 bacteria/ml). The suspension was incubated in lysozyme at 100 µg/ml for 15 min at 30°C on a

shaking platform at 250 rpm. Thereafter, bacterial suspensions were sonicated on ice for 3 minutes (5 sec pulse, 5 sec rest) and stored at 4°C. ELISA plates were coated with 100 µl per well of the bacterial lysate and incubated overnight at 4°C. Blocking, primary antibodies, secondary antibodies and development were as described above for the PapGII196 ELISA.

Inhibition of haemagglutination. In order to evaluate whether sera from PapGII196-vaccinated birds could inhibit binding of a heterologous P^+ / $PapGII^+$ challenge strain to the globoside receptor on human erythrocytes, inhibition of haemagglutination was investigated. Strain CH4 was grown as described above to induce expression of P fimbriae, except that bacteria were harvested in PBS containing 3 % mannose (PBS-Man) to inhibit any potential haemagglutination by F1A fimbriae. Twenty-five microlitres of the bacterial suspension was mixed with 25 µl of chicken serum or 25 µl of PBS-Man (as a control) and the mixture incubated for 1 hour at 37 °C with gentle rotation at 125 rpm. The following sera were analyzed: serum from PapGII196-vaccinated chickens at 21 dpv (pooled serum from birds in group $1^{V/C}$, Experiment 1), serum from a placebo-vaccinated bird at 21 dpv (group $2^{P/C}$, Experiment 1) and serum from an untreated control bird at 21 dpv (group $3^{-/-}$, Experiment 1). All sera were first heat-inactivated for 25 min at 56°C. Following incubation, the bacterial suspensions were transferred to microscope slides and 25 µl of a 3 % human erythrocyte solution (blood group A, donor F. Vandemaele) in PBS was added. Slides were agitated gently to mix and examined microscopically (magnification 200 x).

Statistical analysis. All statistical analyses were performed using SAS software, version 8.2 (the SAS Institute, Cary, NC, USA). Lesion scores were compared using

Kruskal-Wallis analysis to detect significant differences between multiple groups and the Wilcoxon Rank Sum Test was used to compare differences between each pair of groups. IgG concentrations in serum and egg yolk were analyzed using Proc GLM (multiple two-tailed t-test) or the Wilcoxon Rank Sum Test. Fisher's exact test or the Wilcoxon Rank Sum Test were used to compare the morbidity and mortality rates. A $P \leq 0.05$ was considered significant.

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Results

Expression and purification of PapGII196. The amino-terminal sugar binding domain of PapG, PapGII196, was successfully expressed using pET22b in *E. coli* strain BL21 (DE3). SDS-PAGE and Western blotting demonstrated that most of the PapGII196 was present in inclusion bodies after expression (data not shown). However, induction of expression with a low IPTG concentration (0.1 mM) at 28°C yielded sufficient PapGII196 in the soluble fraction to enable purification by Ni-NTA affinity chromatography under non-denaturing conditions. One major band corresponding to the size of the recombinant protein (22.4 kDa) was observed in eluted fractions (Figure 1A). Some higher molecular weight bands between 60 and 80 kDa were also seen. Approximately 1.4 mg of purified PapGII196 was obtained per litre of *E. coli* culture. Western blotting with an anti-His monoclonal antibody confirmed that the major band was PapGII196 (data not shown). In an activity assay (Figure 2), PapGII196 was shown to bind specifically to GbO4-coated ELISA plates in a GbO4-concentration-dependent manner, while no binding was detected to the wells in the absence of GbO4 or when recombinant FimH156 (sugar binding domain of the F1A fimbrial adhesin FimH, Vandemaele *et al.*, 2005) or PBS were used

instead of PapGII196. When a higher PapGII196 concentration (100 µg/ml) was used in combination with 30 µg/ml globoside, no increase in absorbance was observed, indicating maximal occupancy of the globoside (data not shown).

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Active immunization and aerosol challenge (Experiment 1). *Lesions.* Mortality rates and macroscopic lesions are shown in Table 1. There was no significant difference in mortality rates between different treatments. Groups $3^{-/-}$ and $4^{V/-}$ had some slightly affected birds, even though they had not been experimentally infected. However, the total lesion scores of each of the birds in these groups was less than 1 (except for one bird in group $3^{-/-}$, which had a lesion score of 6). The birds in groups $1^{V/C}$ and $2^{P/C}$ had higher median total lesion scores ($1^{V/C}$ vs. $3^{-/-}$, $P = 1.3 \times 10^{-4}$; $1^{V/C}$ vs. $4^{V/-}$, $P = 2.4 \times 10^{-4}$; $2^{P/C}$ vs. $3^{-/-}$, $P = 4.3 \times 10^{-4}$ and $2^{P/C}$ vs. $4^{V/-}$, $P = 0.001$) than birds in the unchallenged groups. No significant differences were seen between the medians of the total lesion scores of birds in the APEC challenged groups ($1^{V/C}$ vs. $2^{P/C}$, $P = 0.4$), nor between the unchallenged control groups ($3^{-/-}$ and $4^{V/-}$, $P = 1$). The same differences in median lesion scores were seen for the thoracic air sacs, abdominal air sacs and lungs, but not the liver or pericardium (Table 1).

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Analysis of the immune response. PapGII196-specific serum IgG responses were analysed by ELISA using recombinant PapGII (Figure 3A) or whole cell lysate (Figure 3B) as the coating antigen. Both analyses showed a significantly higher antibody response in the PapGII196-vaccinated groups ($1^{V/C}$ and $4^{V/-}$) than in the unvaccinated groups from day 14 onwards. Until challenge, no differences were observed between the PapGII196-vaccinated groups ($1^{V/C}$ and $4^{V/-}$), nor between the placebo-vaccinated group ($2^{P/C}$) and the control group ($3^{-/-}$), except for a slight

difference at day 14 in the PapGII196-ELISA and at day 21 in the whole cell lysate ELISA. After challenge, the PapGII196-vaccinated groups ($1^{V/C}$ and $4^{V/-}$) still had significantly higher antibody responses than the placebo-vaccinated group ($2^{P/C}$) and control group ($3^{-/-}$) in the PapGII196-ELISA ($P < 0.0001$), but were not different from the placebo-vaccinated group ($2^{P/C}$) in the whole cell lysate ELISA ($1^{V/C}$ vs. $2^{P/C}$, $P = 0.06$; $4^{V/-}$ vs. $2^{P/C}$, $P = 0.3$), as the latter ELISA detects all anti-*E. coli* antibodies and APEC challenge mainly results in induction of antibodies other than anti-PapG antibodies (as there is only a limited presence of PapG on *E. coli*). At the time of autopsy, an increase in the anti-PapGII196 IgG response was observed in the unchallenged control group (group $3^{-/-}$, time of autopsy versus all other time points, $P = 5.6 \times 10^{-6}$). Sera from vaccinated birds recognised purified PapGII196 in Western blots (Figure 1B) as well as some co-purified higher molecular weight proteins. However, the same sera could not differentiate PapGII in the lysate of strain CH2, which expressed PapGII, as demonstrated by mannose-resistant haemagglutination of human erythrocytes. At 0 dpv, anti-PapGII196 IgG was present in all groups as detected by ELISA (both using recombinant protein and the whole cell lysate), but decreased over time in the unvaccinated groups until challenge. Western blotting confirmed that the pre-immune sera could detect PapGII196 (data not shown).

Passive immunization and aerosol challenge (Experiment 2). *Lesions.* Mortality rates and lesion scores in the offspring of PapGII196-vaccinated and unvaccinated breeders are shown in Table 2. Although fewer birds died in group $1^{V/C}$ than in group $2^{-/C}$, this difference was not significant. However, there were significantly more deaths in both challenged groups ($1^{V/C}$ and $2^{-/C}$) than in the unchallenged control group ($3^{-/-}$). Total lesion scores were higher in the challenged groups (significantly different for

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group 1$^{V/C}$, P = 0.04, but not for group 2$^{-/C}$, P = 0.06) than in the unchallenged control group (3$^{-/-}$), in which some birds had lesions, possibly indicating infection by resident *E. coli*. The lesion scores for the individual organs did not differ between groups 1$^{V/C}$ and 2$^{-/C}$. While lesion scores for the respiratory tract (thoracic and abdominal air sacs and lungs) were not different between the unchallenged control group (3$^{-/-}$) and either of the challenged groups, lesion scores for the liver and pericardium were significantly higher in the challenged groups. *E. coli* could be isolated from lesions in all the chickens in the challenged groups and from chickens with lesions in the unchallenged control group.

Analysis of the immune response. PapGII196-specific IgG titres were determined for serum from the breeders, egg yolk and serum from 1-day-old chicks (Figure 4). Four weeks after primary vaccination, a significantly higher serum antibody titre was detected in vaccinated breeders than in unvaccinated breeders (P = 2.3 x 10^{-4}). Although the titre decreased until after the secondary vaccination, it remained at a high level from then onwards (no difference between 4 and 9 weeks post secondary vaccination, P = 0.5). Anti-PapGII196 antibodies were also detected in egg yolk and in sera from 1-day-old chicks. The anti-PapGII196 serum IgG titres of 1-day-old chicks were similar to those of breeders 4 weeks after secondary vaccination, when they started laying eggs (P = 0.16). The IgG titre in egg yolk from vaccinated breeders was about 2.7 times higher than in the serum of the breeders. The anti-PapGII196 titre in the unvaccinated breeders and their 1-day-old chicks was below the cut-off. Only in the egg yolk of the unvaccinated breeders was the titre slightly above the cut-off.

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Active immunization and air sac challenge (Experiment 3). *Lesions.* Mortality rates and lesion scores are shown in Table 3. There were significantly more deaths in the placebo-vaccinated and challenged group ($2^{P/C}$) than in the unchallenged control group ($5^{-/-}$) (P = 0.01). All challenged groups were significantly different from the unchallenged control group ($5^{-/-}$), in both the median total lesion score and the median lesion scores for each individual organ, except the left lung ($1^{V/C}$ vs $5^{-/-}$, P = 0.09; $3^{V/C}$ vs $5^{-/-}$, P = 0.09). There were no significant differences between the median total lesion scores of the PapGII-vaccinated and the placebo-vaccinated birds at either challenge dose ($1^{V/C}$ vs $2^{P/C}$, P = 0.1; $3^{V/C}$ vs $4^{P/C}$, P = 0.4), and nor were there differences between the median lesion scores for each individual organ. There was no effect of challenge dose on the median total lesion score in the PapGII196-vaccinated birds ($1^{V/C}$ vs $3^{V/C}$, P = 0.6), nor in the placebo-vaccinated birds ($2^{P/C}$ vs $4^{P/C}$, P = 0.1) and there were no differences in the median lesion scores for each individual organ.

Analysis of the immune response. A significant increase in serum anti-PapGII196 IgG was detected from day 14 onwards in the two vaccinated groups ($1^{V/C}$ and $3^{V/C}$) compared to the two placebo-vaccinated groups ($2^{P/C}$ and $4^{P/C}$) and the control group ($5^{-/-}$) (Figure 5). No differences were observed between the two vaccinated groups ($1^{V/C}$ and $3^{V/C}$), nor between the two placebo-vaccinated groups ($2^{P/C}$ and $4^{P/C}$) before challenge. After challenge, all challenged groups ($1^{V/C}$ to $4^{P/C}$) had significantly higher anti-PapGII196 IgG than the unchallenged control group ($5^{-/-}$) (P < 0.0001). At 0 dpv anti-PapGII196 IgG was detected, but the titres dropped over time in all groups (at 7 dpv), staying low in the placebo-vaccinated and control birds and increasing in the vaccinated birds. A significant increase in serum IgG was observed in the unchallenged control group ($5^{-/-}$) at time of autopsy (time of autopsy versus all other

time points, P = 0.007). The ELISA was repeated using whole cell lysates of the P^+ challenge strain CH2 as coating antigen and a similar immune response was detected (data not shown).

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Comparison of challenge by aerosol and by air sac inoculation (Experiment 1 versus Experiment 3). The aim of the air sac inoculation challenge was to induce more severe systemic lesions than exposure to aerosols of NDV and *E. coli*. In the PapGII196-vaccinated groups (Experiment 3: group $3^{V/C}$ but not group $1^{V/C}$) the median total lesion score and the median lesion scores for pericardium and liver were significantly higher after air sac inoculation than after aerosol exposure (Experiment 1: group $1^{V/C}$) (P = 0.03 for total lesion scores; P = 0.008 for pericardium and P = 0.03 for liver). This was also seen in the placebo-vaccinated birds when comparing birds challenged by air sac inoculation (Experiment 3: group $2^{P/C}$, but not group $4^{P/C}$, except for the pericardial lesion scores) to those challenged by aerosol (Experiment 1: group $2^{P/C}$) (P = 0.009 for total lesion scores; P = 0.003 for pericardial lesion scores and P = 0.02 for hepatic lesion scores). As expected there was no difference between the the unchallenged control groups (Experiment 1: group $3^{-/-}$ vs Experiment 3: group $5^{-/-}$) in the median total lesion score (P = 0.6) or the median lesion scores for the pericardium (P = 1) or the liver (P = 0.3).

The serum anti-PapGII196 IgG responses in the vaccinated groups in Experiment 3 (dilution 1/200; absorbance of 1.1 +/- 0.3 for $1^{V/C}$ and 1.0 +/- 0.6 for $3^{V/C}$) were significantly lower than in the vaccinated group ($1^{V/C}$) from Experiment 1, which was re-analyzed in ELISA at a dilution of 1/200 (2.1 +/- 0.2).

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Inhibition of haemagglutination.

The results from the haemagglutination assay are shown in Figure 6. When bacteria were pre-incubated with PBS-Man (as a control), clear haemagglutination of human erythrocytes was observed in the presence of D-mannose (Figure 6A), indicating that P fimbriae were expressed on APEC strain CH4. Pre-incubation of CH4 with serum from PapGII196-vaccinated chickens completely inhibited haemagglutination (Figure 6B), while sera from placebo-vaccinated or untreated control chickens did not (Figure 6C and D, respectively). When erythrocytes were added to wells containing bacterial suspensions, mixed and then transferred to microscope slides, identical results were obtained.

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Discussion

The aim of this study was to produce biologically active PapGII196 and to investigate whether immunisation with this protein was able to protect chickens against APEC infection using different approaches to vaccination and different challenge methods. Sung *et al.* (2001a, b) and Dodson *et al.* (2001) previously expressed the sugar binding domain of PapGII (from *E. coli* of human origin) in order to determine the three-dimensional protein structure of the adhesin. Similarly, we engineered a construct comprising the first 196 amino acids of the mature protein (PapGII196) and expressed it in an *E. coli* BL21 (DE3) strain at low temperature (28°C) and, using a low concentration of inducer (0.1 mM IPTG), obtained low yields of PapGII196 in the soluble fraction. The recombinant protein was able to bind to globoside (GbO4), the preferred receptor for PapGII, demonstrating its biological activity.

A strong anti-PapGII196 serum IgG response was detected after a single intramuscular vaccination of chickens. Mast and Goddeeris (1999) and Vandemaele *et al.* (2005) have shown that it is possible to induce an immune response in chickens at that early age. A strong responses was also detected by ELISAs using whole cell lysates of the P fimbriae-expressing challenge strain CH2, suggesting that the serum antibodies recognised PapGII in its native context. Challenge strain CH2 expressed P fimbriae and PapGII, as demonstrated by mannose-resistant haemagglutination of human erythrocytes, but PapGII could not be detected in Western blots of whole cell lysates. Similarly we were unable to detect PapGII in Western blots of whole cell lysates of strain CH4 (data not shown). This could be due to the very low concentration of PapG molecules in wild type P^+ strains (only 1 PapG subunit per P pilus). Additional proteins were detected in the whole cell lysate, probably by antibodies induced by resident *E. coli*. Furthermore, serum from PapGII-vaccinated birds was able to completely inhibit agglutination of human erythrocytes by the heterologous P^+ / $PapGII^+$ APEC strain CH4, whereas sera from placebo-vaccinated or untreated control birds could not, indicating that adhesion-inhibiting anti-PapGII196 antibodies had been induced. Despite the strong IgG response, no protective effects were observed.

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In the passive immunization experiment, a strong anti-PapGII196 IgG response was detected in the breeders, egg yolk and 1-day-old chicks. The anti-PapGII196 IgG titre in the 1-day-old chicks was similar to that in breeders at 4 weeks after secondary vaccination (when they started laying eggs), indicating successful transfer of anti-PapGII196 antibodies from breeder to chicks. However, no protective effects could be detected against strain APEC 1. Although a 33.3 % reduction in mortality was seen in the vaccinated group compared to the unvaccinated group, this

decrease was not significant. It is possible that larger groups of birds (e.g. 10000 birds in field conditions) would yield a significant reduction in mortalities. Some lesions were seen in unchallenged birds and *E. coli* were isolated from these lesions. Administration of NDV probably sensitized the unchallenged control birds to intestinal APEC excreted by the birds, as the isolators used to house the birds would be expected to exclude contamination with environmental *E. coli*.

In order to produce a more severe systemic infection, air sac inoculation was used for challenge, as preliminary studies with strain CH2 resulted in typical APEC lesions in multiple organs (F. Vandemaele, unpublished results). Significantly more severe and more reproducible total lesion scores were obtained using air sac inoculation and lesion were also seen in the pericardium and liver, indicating that although this route of inoculation bypassed the natural route of infection via the trachea, it may be more suitable for studies of immunization against APEC and for studies of virulence factors involved in the systemic stages of infection. Despite a good IgG response, there was no evidence of a protective effect of PapGII196 vaccination at any of the challenge doses tested. The serum IgG response was weaker than in Experiment 1, as might be expected given the lower PapGII196 concentration in the vaccine. A significant increase in anti-PapGII196 IgG titre was seen in the control birds, which had been distributed among the challenged groups, after challenge. However, as no lesions were observed in control birds, the transmission of APEC from challenged to unchallenged birds in the absence of predisposing NDV infection appeared to be insufficient to generate in clinical disease.

Our results contrast with those of Kariyawasam *et al.* (2002, 2004), who found that experimental transfer of IgY against recombinant full-length denatured PapG protected broilers against *E. coli* challenge. It is unlikely that antibodies to the

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carboxyl-terminal domain (absent in PapGII196) would contribute to protection, as Hansson *et al.* (1995) have demonstrated that carbohydrate binding and specificity only reside in the amino-terminal part of the PapG adhesin. Although a strong anti-PapGII196 immune response was obtained, we cannot exclude the possibility that the full-size protein might be more immunogenic and hence might induce a stronger immune response. However, we demonstrated that the serum from PapGII196-vaccinated birds was able to inhibit binding of APEC to human erythrocytes and hence haemagglutination. The difference might be explained by the different challenge strains used. APEC infection is a multi-factorial event, involving different virulence factors. Although Mellata *et al.* (2003) showed that a PapG deletion mutant was less virulent than the wild type APEC strain, Stordeur *et al.* (2004) demonstrated that *pap*-negative APEC strains isolated from lesions in birds with septicaemia were virulent, indicating the importance of other virulence factors. Similarly, several other studies have demonstrated the virulence of *pap*-negative APEC strains (Dozois *et al.*, 1992; Li *et al.*, 2005). Blockage of the PapG adhesin by antibodies might have a differential effect depending on the presence of additional virulence factors (such as Tsh and IutA.). Our results and the limited proportion of APEC strains that are pap^+ (Vandemaele *et al.*, 2003b) indicate that, notwithstanding their contribution to virulence, P fimbriae are by no means indispensable for APEC infection.

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Several studies have questioned the importance of the role of type 1 and P fimbriae and their respective adhesins FimH and PapG, in APEC infections (Marc *et al.*, 1998; Arné *et al.*, 2000; Stordeur *et al.*, 2004). In contrast, these adhesins have been shown to play a major role in human urinary tract infection (Roberts *et al.*, 1994; Connell *et al.*, 1996), and FimH-FimC and PapG-PapD adhesin-chaperone vaccines have been able to protect cynomolgous monkeys against infection with uropathogenic

E. coli (Langermann *et al.*, 2000; Roberts *et al.*, 2004). Targeting a single virulence factor of APEC might not be an ideal vaccination strategy. Multivalent vaccines containing multiple recombinant virulence factors might offer a solution. However, determining which virulence factors should be incorporated in such vaccines requires further research. Our results suggest that PapG may not be a candidate for inclusion such a vaccine. The alternative is to rely on the use of live attenuated or inactivated whole cell vaccines (Rosenberger *et al.*, 1985; Gyimah *et al.*, 1985). Our findings indicate that biologically active PapGII196 was unable to protect chickens against APEC, notwithstanding a strong immune response. This underlines the fact that APEC infection is a multi-factorial event and that the PapG adhesin may play a limited role in infection, particularly in the strains that were used for challenge in our study.

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Figure legends

Figure 1 *(A) Sodium dodecylsulphate-polyacrylamide gel electrophoresis of the purified PapGII196. (B) Western blot with immune serum from vaccinated birds. TCP0: total cell protein (soluble + insoluble fraction) (TCP) before induction of expression, TCP4: TCP 4 hours after induction of expression, MW: molecular mass (kDa), E1/2: combined elution fractions 1 and 2 after Ni-NTA purification. This fraction was used for the final vaccine. E3: third elution fraction, TCP CH2: total cell protein of challenge strain CH2. The SDS-PAGE gel was stained with Coomassie blue. Arrow: PapGII196.*

Figure 2 *Biological activity assay of PapGII196. Globoside was coated at three different concentrations. The adhesins PapGII196, FimH156 or PBS were allowed to react with globoside and subsequently detected with an anti-His monoclonal antibody. BSA – not coated with GbO4 .*

Figure 3 *Serum IgG response (dilution 1/400) to PapGII196 (A) or to CH2 bacterial lysate (B) in Experiment 1. The bracketed numbers indicate groups described in Table 1. Arrow: APEC infection at 25 dpv. Significant differences between treatments at each time point are indicated by different lowercase letters. OD was measured at 405 nm. PapG: vaccinated intramuscularly with PapGII196 and challenged, PapG-control: vaccinated intramuscularly with PapGII196 and not challenged (NDV only), Placebo: vaccinated intramuscularly with Tris-HCl buffer and challenged, NDV: unchallenged control group (NDV only).*

Figure 4 *Serum IgG titres to PapGII196 in Experiment 2. The lower dashed line represents the lower limit (a titre of 10, corresponding to the lowest dilution 1/1024) and the upper dashed line represents the upper limit (a titre of 17, corresponding to the highest dilution 1/131072. If the initial dilution was already below the cut-off, a titre of 9 was recorded. If the highest dilution was still not below the cut-off, a titre of 18 was recorded. PapG: vaccinated twice intramuscularly with PapGII196, placebo: unvaccinated. w ppv: weeks post primary vaccination (for the breeders), w psv: weeks post secondary vaccination (for the breeders), yolk: egg yolk from the collected eggs, chick: serum from 1-day-old chicks (day of infection). Significant differences between treatments at each time point are indicated by different lowercase letters. OD was*

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*measured at 405 nm. *: primary vaccination, **: booster vaccination. The vertical dashed lines separate the breeders, egg yolk and 1-day-old chicks.*

Figure 5 *Serum IgG response (dilution 1/200) to PapGII196 in Experiment 3. The bracketed numbers indicate groups described in Table 3. Arrow: APEC infection at 21 dpv. Significant differences between treatments at each time point are indicated by different lowercase letters. OD was measured at 405 nm. PapG: intramuscularly vaccinated with papGII196 and challenged (10^4 cfu/bird for group $1^{V/C}$ and 10^5 cfu/bird for group $3^{V/C}$), placebo: intramuscularly vaccinated with Tris-HCl buffer and challenged (10^4 cfu/bird for group $2^{P/C}$ and 10^5 cfu/bird for group $4^{P/C}$), control: unvaccinated and notchallenged.*

Figure 6 *Inhibition assay. APEC strain CH4 was pre-incubated with PBS-Man (A), serum from PapGII196-vaccinated birds (B), serum from a placebo-vaccinated bird (C) and serum from an untreated control bird (D). Following incubation, human erythrocytes were added. Haemagglutination was examined by microscope (200 x magnification). Only serum from PapGII196-vaccinated birds was able to inhibit haemagglutination. All sera were taken 21 dpv from birds in Experiment 1.*

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Table 1 *Mortality and lesion scores of chickens after direct immunization and aerosol challenge (experiment 1).*

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Group[a]	Vaccine[b]	Challenge[c]	Death[d]	Total[e]	Median of lesion scores[f] (lower quartile - upper quartile)					
					TAS	AAS	Liver	PRD	Lung	Total[g]
$1^{V/C}$	PapGII	CH2	1	10	1^{A} (1-1)	1^{A} (1-1)	0^{A} (0-1)	0^{A} (0-1)	1^{A} (0-1)	4^{A} (3-5)
$2^{P/C}$	Tris-HCl	CH2	1	11	1^{A} (1-1.5)	1^{A} (1-1.5)	0^{A} (0-1)	0^{A} (0-0.5)	0^{AB} (0-1)	3^{A} (2-4.5)
$3^{-/-}$	---	---	0	12	0^{B} (0-0)	0^{B} (0-0)	0^{A} (0-0)	0^{A} (0-0)	0^{B} (0-0)	0^{B} (0-0)
$4^{V/-}$	PapGII	---	0	6	0^{B} (0-0)	0^{B} (0-0)	0^{A} (0-0)	0^{A} (0-0)	0^{B} (0-0)	0^{B} (0-0)

[a]: V/C: intramuscularly vaccinated and challenged, P/C: intramuscularly placebo-vaccinated and challenged, -/-: unvaccinated and unchallenged, V/-: intramuscularly vaccinated and unchallenged.
[b] PapGII: PapGII196 (intramuscular), Tris-HCl buffer: placebo (intramuscular). Vaccination was performed at day 10.
[c] All groups received NDV at day 31 by spray. Infection with APEC strain CH2 was performed at day 35 by nebulization in isolators.
[d, e] Some chickens died (or were euthanized when diseased) before APEC challenge, but showed no clinical signs of APEC infection, so they were not recorded as deaths. Therefore, the total number of birds is 10 or 11 per group, instead of the initial 12 birds per group. Group $4^{V/-}$ consisted of 6 birds. Birds that died after APEC challenge were awarded a total lesion score of 10.
[f] TAS: thoracic air sacs, AAS: abdominal air sacs, PRD: pericardium, Tot: total lesion score. Values within a column with different uppercase superscript letters differ significantly ($P \leq 0.05$).
[g] Some minor lesions were present in a few birds in the unchallenged control groups $3^{-/-}$ and $4^{V/-}$but these were not sufficiently severe to warrant a lesion score.

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Table 2 *Mortality and lesion scores of one-day old chicks after passive immunization and aerosol challenge (experiment 2).*

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Group[a]	Vaccine[b]	Challenge[c]	Death[d]	Total	Median of lesion scores[e] (lower quartile - upper quartile)					
					TAS	AAS	Liver	PRD	Lung	Total
$1^{V/C}$	PapGII	APEC 1	8^{A}	40	2^{AB} (1.5-2)	1^{AB} (0-2)	0^{A} (0-1)	0^{A} (0-1.5)	1^{A} (0-2)	4^{A} (2-6.5)
$2^{-/C}$	---	APEC 1	12^{A}	40	2^{AB} (1.5-2)	1^{AB} (0-2)	0^{A} (0-2)	0^{A} (0-2)	1^{A} (0-2)	4^{AB} (2-10)
$3^{-/-}$	---	---	0^{B}	20	2^{B} (1-2)	1^{B} (0-1)	0^{B} (0-0)	0^{B} (0-0)	0^{A} (0-1)	3^{B} (2-3.5)

[a]: V/C: offspring from vaccinated breeders and challenged, -/C: offspring from unvaccinated breeders and challenged, -/-: offspring from unvaccinated breeders and unchallenged.
[b] Vaccination of SPF parent breeder chickens :PapGII: PapGII196 (intramuscular), --- : unvaccinated.
[c] NDV was administered to all groups by spray at day 1 in combination with APEC 1 for groups 1 and 2.
[d] Birds that died after APEC challenge were allocated a total lesion score of 10.
[e] TAS: thoracic air sacs, AAS: abdominal air sacs, PRD: pericardium, Tot: total lesion score. Values within a column with different uppercase superscript letters differ significantly ($P \leq 0.05$).

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Table 3 *Mortality and lesion scores of chickens after direct immunization and challenge by air sac inoculation.*

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Group[a]	Vaccine[b]	Challenge[c] cfu/bird	Death[d]	Total	Median of lesion scores[e] (lower quartile - upper quartile)							
					TASR	TASL	AAS	PRD	Liver	Lu-R	Lu-L	Tot
$1^{V/C}$	PapGII	CH2 10^4	2^{AB}	12	1.5^{A} (0.5-2)	1^{A} (0-2)	1^{A} (0-2)	1^{A} (0-2)	0.5^{A} (0-2)	1^{A} (0-2)	0^{AB} (0-1.5)	6.5^{A} (1-12.5)
$2^{P/C}$	Tris-HCl	CH2 10^4	6^{A}	12	2^{A} (1-2)	2^{A} (1-2)	2^{A} (1-2)	2^{A} (1-2)	1.5^{A} (1-2)	2^{A} (1.5-2)	2^{A} (0-2)	13.5^{A} (6-14)
$3^{V/C}$	PapGII	CH2 10^5	2^{AB}	12	2^{A} (1-2)	2^{A} (1-2)	1^{A} (1-2)	2^{A} (2-2)	1^{A} (1-1.5)	1^{A} (0-2)	0^{AB} (0-1)	8.5^{A} (7.5-11.5)
$4^{P/C}$	Tris-HCl	CH2 10^5	2^{AB}	12	1.5^{A} (0-2)	1^{A} (0-2)	1^{A} (0-2)	1^{A} (0.5-2)	1^{A} (0.5-2)	0.5^{A} (0-2)	0^{A} (0-1)	6.5^{A} (1.5-11.5)
$5^{-/-}$	---	---	0^{B}	12	0^{B} (0-0)	0^{B} (0-0)	0^{B} (0-0.5)	0^{B} (0-0)	0^{B} (0-0)	0^{B} (0-0)	0^{B} (0-0)	0^{B} (0-0)

[a]: V/C: intramuscularly vaccinated and challenged, P/C: intramuscularly placebo-vaccinated and challenged, -/-: unvaccinated and unchallenged.

[b] PapGII: PapGII196 (intramuscular), Tris-HCl buffer: placebo (intramuscular). Vaccination was performed at day 10.

[c] Challenge consisted of inoculation of 200 µl bacteria of strain CH2 in the right thoracic air sac at a dose of 10^4 cfu/bird or 10^5 cfu/bird. The control group was not inoculated.

[d] Birds which died after challenge all showed signs of infection and were awarded a maximum score of 14

[e] TASR: right thoracic air sac, TASL: left thoracic air sac, AAS: abdominal air sacs, PRD: pericardium, Lu-R: right lung, Lu-L: left lung. Values within a column with different uppercase superscript letters differ significantly ($P \leq 0.05$). The combinations group 1 - group 4 and group 2 - group 3 were not analyzed statistically.

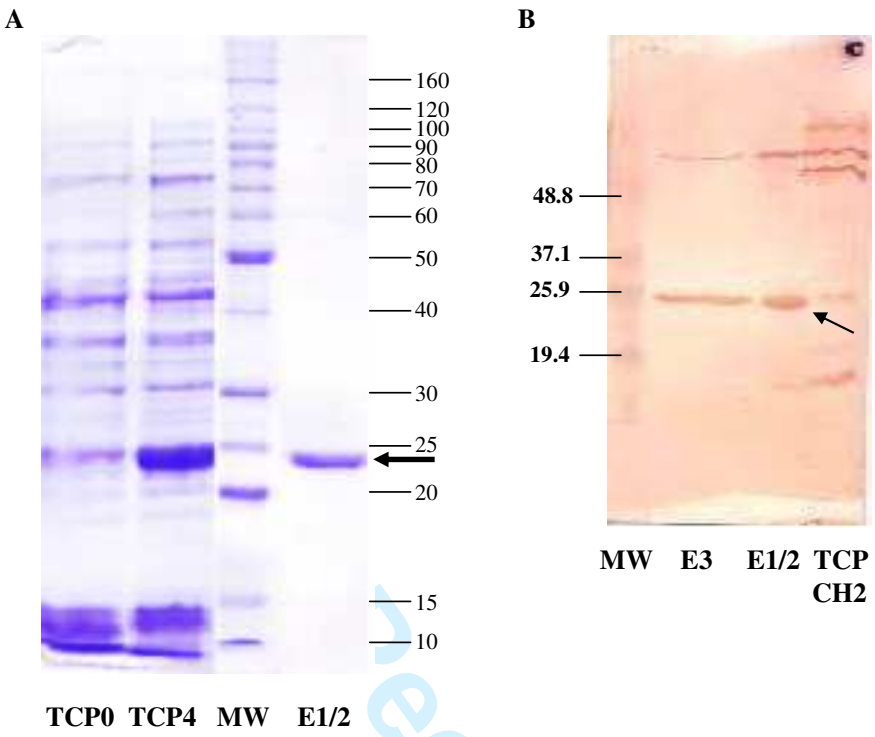

Figure 1 *(A) Sodium dodecylsulphate-polyacrylamide gel electrophoresis of the purified PapGII196. (B) Western blot with immune serum from vaccinated birds.*

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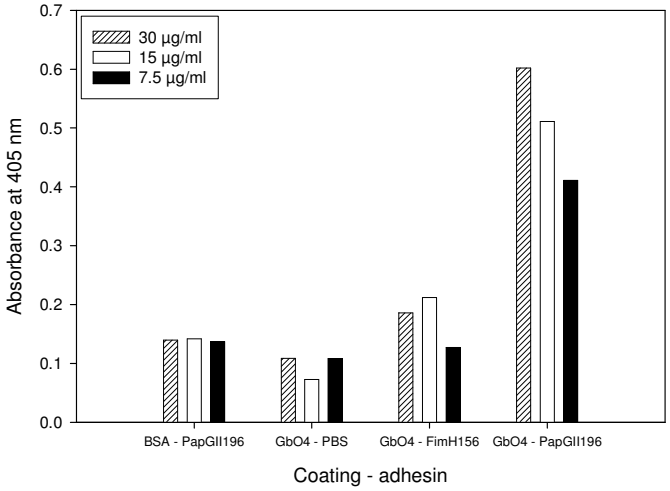

Figure 2 *Biologically activity assay of PapGII196.*

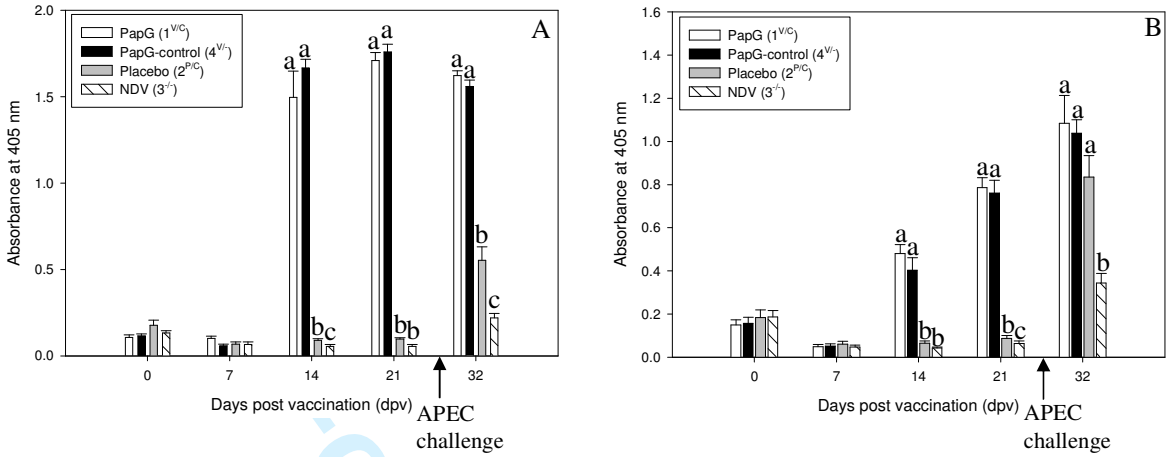

Figure 3 *Serum IgG response (dilution 1/400) to PapGII196 (A) or to CH2 bacterial lysate (B) in Experiment 1.*

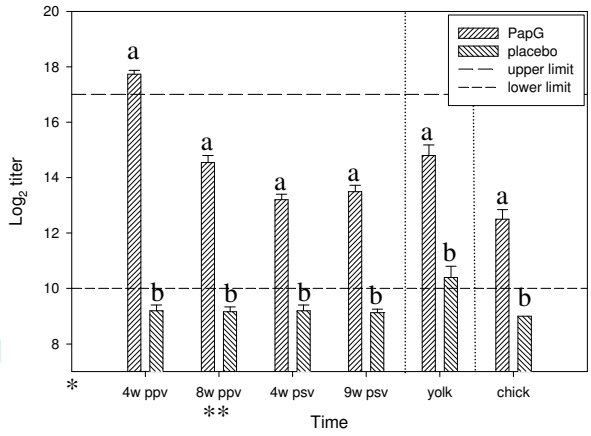

Figure 4 *Serum IgG titres to PapGII196 in Experiment 2.*

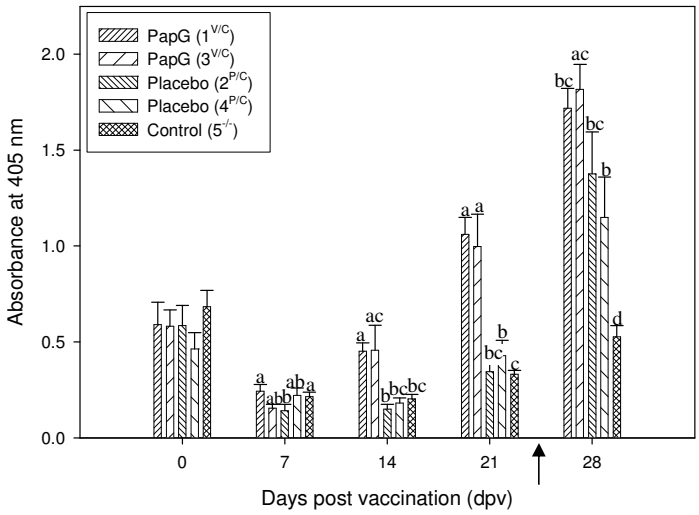

Figure 5 *Serum IgG response (dilution 1/200) to PapGII196 in Experiment 3.*

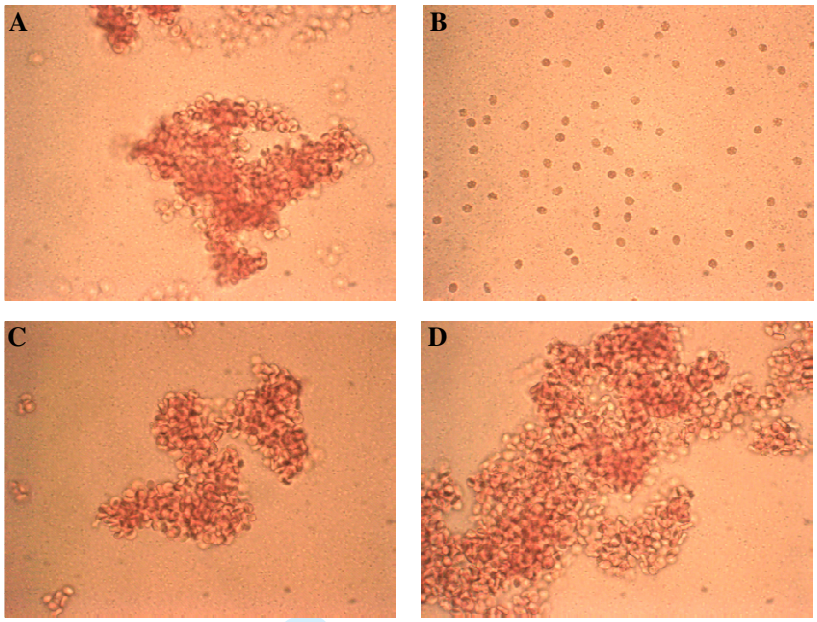

Figure 6 *Inhibition assay.*

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