Glycoengineered Outer Membrane Vesicles: A Novel Platform for Bacterial Vaccines

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The World Health Organization has indicated that we are entering into a post-antibiotic era in which infections that were routinely and successfully treated with antibiotics can now be lethal due to the global dissemination of multidrug resistant strains. Conjugate vaccines are an effective way to create a long-lasting immune response against bacteria. However, these vaccines present many drawbacks such as slow development, high price, and batch-to-batch inconsistencies. Alternate approaches for vaccine development are urgently needed. Here we present a new vaccine consisting of glycoengineered outer membrane vesicles (geOMVs). This platform exploits the fact that the initial steps in the biosynthesis of most bacterial glycans are similar. Therefore, it is possible to easily engineer non-pathogenic Escherichia coli lab strains to produce geOMVs displaying the glycan of the pathogen of interest. In this work we demonstrate the versatility of this platform by showing the efficacy of geOMVs as vaccines against Streptococcus pneumoniae in mice, and against Campylobacter jejuni in chicken. This cost-effective platform could be employed to generate vaccines to prevent infections caused by a wide variety of microbial agents in human and animals.

Most successful current antibacterial vaccines are glycoconjugates, composed of cell surface carbohydrates chemically attached to an appropriate carrier protein. These are effective means to generate protective immune responses to prevent a wide range of diseases. One of the best examples is the conjugate vaccine against Haemophilus influenzae type b, which practically eliminated the disease caused by this bacterium in vast parts of the world. Other examples are the vaccines against Streptococcus pneumoniae and Neisseria meningitidis, both based on capsular polysaccharides. However, the current technology to produce conjugate vaccines presents some drawbacks. These require complex synthetic chemistry for obtaining, activating, and attaching the polysaccharides to protein carriers. The carbohydrates employed for the formulation of these vaccines are usually obtained from pathogenic organisms, which may constitute a hazard and may require higher levels of biosafety for production. Some of the glycans containing acid-labile sugars do not resist the chemical treatment required for their purification and crosslinking to proteins. Furthermore, the chemical crosslinking is often not reproducible, resulting in unreliable products with batch to batch variability. Novel approaches are needed to effectively prevent bacterial infections, especially in the context of the alarming increase of MDR bacterial strains.

Gram-negative bacteria are able to produce outer membrane vesicles (OMVs). OMVs are mainly composed of LPS, outer membrane and periplasmic proteins, and phospholipids. OMVs are formed by blebbing of the outer membrane, although their biogenesis is poorly understood. Due to their immunogenic properties, self-adjuvanticity, ability to be taken up by mammalian cells, and capacity for enhancement by recombinant engineering, OMVs are attractive candidates for vaccine delivery platform. Within the last 25 years, vesicle based meningococcal vaccines (i.e., MenBvac, MenZB, and BexSero) have been successfully developed and employed in various countries. Due to their success, other OMV vaccine candidates for various pathogenic bacteria including Vibrio cholerae, Bordetella pertussis, Burkholderia pseudomallei, Acinetobacter baumannii and even Gram-positive bacteria, such as Bacillus anthracis, have been tested. Such OMV based vaccines involve direct manipulations of large volumes of pathogenic bacteria and may pack unwanted bacterial toxins.
Pneumococcal disease kills more patients worldwide than any other vaccine-preventable disease. Annual worldwide statistics show that 1.6 million people die of pneumococcal disease\(^2\). The current vaccine (Prevnar 13\(^(\text{®})\)) protects against the 13 predominant serotypes in the USA, but serotypes that are important in other countries are not included in the vaccine. Furthermore, serotypes with lower prevalence often take over the niche left by the serotypes included in the vaccines\(^25\). Therefore, effective vaccines for the remaining \(S. \text{pneumoniae}\) capsule serotypes are needed.

\(C. \text{jejuni}\) is a natural commensal in all birds, including chickens. Humans are frequently infected with \(C. \text{jejuni}\) through the consumption of improperly cooked poultry. In most cases, infection only causes diarrhea, fever and abdominal pain. However, approximately, one in one thousand patients will develop a severe polyneuropathy known as Guillain-Barré syndrome (GBS)\(^26\). One way to control human infection by \(C. \text{jejuni}\) would be to reduce the load of the bacterium in chickens. Here we present an alternative platform for vaccines consisting of glycoengineered OMVs (geOMVs) derived from non-pathogenic engineered \(E. \text{coli}\) strains expressing bacterial surface glycans encoded by the bacterial pathogenic organisms. As a proof of principle, we demonstrate the efficacy of geOMVs as vaccines for \(S. \text{pneumoniae}\) serotype 14, and the common foodborne pathogen \(C. \text{jejuni}\).

**Results**

**Generation of geOMV displaying \(S. \text{pneumoniae}\) serotype 14 capsule (CPS14).** The general strategy for the production of geOMV is represented in Fig. 1. In principle, our platform can be applied to any surface glycan, such as O antigens, capsules, exopolysaccharides, or glycans present in \(N\)- and \(O\)-glycoproteins. The initial steps in the biosynthesis of these glycans are common. These include the assembly of the glycan onto the undecaprenylpyrophosphate carrier and the flipping of the lipid-linked sugars across the inner membrane of Gram-negative or the cell membrane in Gram-positive bacteria. If these pathways are reconstituted in \(E. \text{coli}\), the WaaL ligase transfers the glycan from the carrier to the lipid A, which is synthesized independently, and exported to the bacterial surface. OMVs displaying such antigens are then naturally produced. The biosynthetic pathways of type I capsular polysaccharides and O antigens only diverge once the polysaccharide has been translocated across the inner membrane (Supplementary Data, Fig. S1a). WaaL is not glycan-specific and can transfer a variety of sugars onto the lipid A core\(^27\). We predicted that expressing the \(S. \text{pneumoniae}\) capsule in an \(E. \text{coli}\) strain lacking its own O antigen would result in an LPS consisting of capsular polysaccharide attached to the lipid A core. To obtain such a strain, we constructed an inducible plasmid (pNLP80) which expresses the capsule synthesis \(cps\) locus and includes all essential glycosyltransferases, the flipase (Wzx), and the polymerase (Wzy) required for the synthesis of the \(S. \text{pneumoniae}\) serotype 14 capsule (CPS14). The expression of CPS14 was evaluated in two different \(E. \text{coli}\) mutant strains, CLM37\(^28\) and CLM24\(^29\) (Fig. 2a). CLM37 harboures a mutation which interrupts the initiator glycosyltransferase (\(wecA\)) for O antigen and Enterobacterial common antigen (ECA) synthesis\(^28\). In \(S. \text{pneumoniae}\) capsule synthesis the initiating glycosyltransferase is WchA, which transfers a glucose residue to the Und-PP carrier. WecA and WchA attach different sugars to the lipid and therefore would compete for the carrier. We reasoned that a \(wecA\) mutant strain should produce more CPS14 than the wild-type strain. To demonstrate the attachment of the CPS14 to lipid A, we employed strain CLM24 (\(waaL\) mutant)\(^32\). In this strain, the signal detected in the WaaL mutant strain corresponds to UndPP-linked CPS14. The geOMVs were visualized by transmission electron microscopy (TEM) (Fig. 2b).
geOMV displaying *S. pneumoniae* CPS14 raise specific antibodies and are effective in OPA assays. To determine whether geOMVs from CLM37 strain displaying the CPS14 would generate an immunogenic response in a murine model, we injected 2 μg of geOMVs per mouse (n = 10). Control groups immunized with either geOMVs lacking the capsule (empty geOMVs) or the commercially available Prevnar 13®, were included for comparison. Booster doses of geOMVs and Prevnar® were administered on Days 14 and 28 and sera was collected weekly over a 42 day period. Collected sera of the immunized mice were assayed via Western blot analysis (Fig. 3a). Initial immunization with geOMVs did not elicit an IgG immunogenic response. However one week after the first booster dose (Day 21), high IgG titers in response to CPS14 were generated (Fig. 3a). The IgG response continued to be observed over the remaining course of the immunization schedule. To determine whether the immunogenic response was specific for serotype 14, we performed ELISAs with whole pneumococcal cells of serotypes 14 and 9 V (Supplementary Data, Fig. S1b,c). Sera from mice injected with geOMVs with CPS14 reacted only with *S. pneumoniae* serotype 14, thus demonstrating that the immunogenic response is specific for the glycoengineered serotype. Furthermore, at day 42, the levels of the IgG response of mice injected with pneumococcal geOMVs were comparable to the immunogenic response of mice injected with the commercially marketed pneumococcal vaccine Prevnar 13® (Fig. 3b) which suggested that the level of the protective effect could also be similar.
Opsonophagocytosis assays (OPA) are accepted as one the most reliable ways to evaluate the efficacy of pneumococcal vaccines. OPA were performed to evaluate the efficacy of geOMVs against an S. pneumoniae serotype 14 infection. Day 42 sera obtained from the three groups of immunized mice (i.e., placebo geOMVs, pneumococcal geOMVs, and Prevnar 13®) were employed. OPA showed a significantly increased killing effect using pneumococcal geOMVs compared to placebo geOMVs (Fig. 4a), which suggests that isolated pneumococcal geOMVs elicit a protective effect in vitro. In addition, varying mouse serum concentrations (5% and 20%) showed a similar killing effect between pneumococcal geOMVs and the Prevnar 13 pneumococcal vaccine (Fig. 4b), thus demonstrating that geOMVs have a similar protective effect when compared to the commercially available Prevnar 13® vaccine.

**geOMV reduce chicken colonization by C. jejuni.** We next evaluated the use of geOMVs to vaccinate chickens against C. jejuni. We introduced a plasmid (pACYCpplBmut) that expresses the C. jejuni heptasaccharide N-glycan into E. coli EVV11. This strain carries a mutation in Wzy and therefore does not polymerize the C. jejuni glycan as the wild-type strain does. We isolated geOMVs from this strain and analyzed them by Western-blot employing an N-glycan specific antibody. An immunoreactive signal migrating around 15 kDa that was absent in control OMVs clearly indicates that the C. jejuni N-glycan was present in geOMVs (Fig. 5a). To analyze the efficacy of the geOMVs containing the C. jejuni N-glycan, we tested the protective effect of these geOMVs in a C. jejuni chicken challenge model. Four groups of chickens (n = 8 for the positive control, n = 6 for the negative and experimental groups) were vaccinated orally with PBS, placebo geOMVs or Campylobacter geOMVs, and challenged with oral doses of PBS (negative control) or C. jejuni strain 81–176. Birds were monitored for 7 days before being euthanized and the amount of C. jejuni isolated from the cecum was determined for each bird. Birds that received PBS (Fig. 5b, group 2) and placebo geOMVs (Fig. 5b, group 3) showed similar levels of C. jejuni colonization after challenge. Chickens vaccinated with the N-glycan-containing geOMVs exhibited an almost 104-fold reduction in C. jejuni colonization after challenge when compared to the naïve and placebo groups (Fig. 5b, group 4). In agreement with this data, the IgY levels of chicken that received the geOMV containing the glycan were higher than the ones that received the empty OMV (Fig. S1d).

**Discussion**
Conjugate vaccines are an effective way to create a long-lasting IgG immune response. However, besides being expensive to produce, these vaccines present a series of drawbacks. Large volumes of pathogenic cells need to be cultured to obtain the polysaccharides and the chemical crosslinking of the sugars to the protein is complex, with large batch to batch variability. The development of novel conjugate vaccines is very slow. Diverse serotypes not included in Prevnar 13® are prevalently being isolated in different geographical locations, and therefore novel technologies that can accelerate the development of multivalent vaccines are required. The generation of conjugate vaccines through exploitation of bacterial protein glycosylation systems is a very promising alternative, but not all the glycan chains are efficiently attached to proteins by the oligosaccharyltransferase PglB, which requires a HexNAc residue at the reducing end of the sugar chain. PglL, the N. meningitidis O-oligosaccharyltransferase, can recognize a galactose but not glucose residue at the reducing end (Feldman, manuscript in preparation). Interestingly, about 90% of the S. pneumoniae strains contain glucose at the reducing end. Here we demonstrated that OMVs produced by glycoengineered E. coli expressing a glycan from unrelated bacterial pathogens, such as the CPS14 capsule from S. pneumoniae or the heptasaccharide derived from N-linked glycans from C. jejuni, can be effective in producing a significant immune response.

![Figure 4. Bactericidal activity of sera from vaccinated mice against S. pneumoniae serotype 14.](image-url)

(a) OPA evaluation of the effect of the incubation time (2 h and 4 h) on S. pneumoniae type 14 killing in presence of either 20% (v/v) placebo or vaccinated mice sera. Statistically significant differences were found at 2 hours and at 4 hours between the placebo and the vaccinated mice sera (*p-value < 0.0001; **p-value < 0.0001). Plotted data are means of percentages and SD as error bars. Kruskal-Wallis (non-parametric) test was used to calculate p-values. (b) OPA evaluation of the effect of the serum concentration (5% and 20%) on S. pneumoniae type 14 serum was used as positive control to validate OPA methodology. No statistically significant differences could be observed between different serum concentrations (NS1 p-value = 0.15; NS2 p-value = 0.21). P-values were calculated using Kruskal-Wallis test. No bacteria were found when using 20% serum concentration.
In the case of *S. pneumoniae*, geOMVs induced an immune response, as measured by serum IgG levels and efficacy in OPA tests, which was similar to the one generated by the most widely used commercial conjugate vaccine. In the future, a multivalent vaccine could be generated by mixing geOMVs carrying capsules from different serotypes. Furthermore, geOMVs could complement the current conjugate vaccines, especially for serotypes corresponding to glycan structures for which the conjugation has not been solved. Although the Western blots together with the specific antibody response to CPS14 and the OPA assays suggest that the right structure of the CPS14 has been displayed in the geOMVs, future work will confirm the exact structure attached to lipid A. In the case of the *C. jejuni* geOMV vaccine candidate, the 4 log reduction in chicken colonization is, to our knowledge, unprecedented for this microorganism. However, the power of the vaccine could be increased if, in addition to engineering *E. coli* to produce pathogenic bacterial surface glycans, the geOMVs were modified to express antigenic membrane proteins to be directed to the vesicles. Previous studies have already explored incorporating antigenic proteins from pathogenic species (*Neisseria*, *Streptococcus*, *Leishmania*, *Vibrio*, and *Yersinia*) into vesicles derived from laboratory *E. coli* and *Salmonella* strains17,32–35. Furthermore, OMV produced in *Salmonella* carrying pneumococcal protein antigens showed promise in murine models36. The geOMV platform would enable the glycans and proteins to synergistically increase the vaccine immunogenicity capacity. This can also be important for cases like in *S. pneumoniae*, in which a protein antigen could expand the protection to serotypes not included in the vaccines.

One of the main concerns with vesicle based vaccines is the safety issues as OMVs contain endotoxic LPS37. LPS lipid A has been shown to provoke severe/lethal inflammatory responses in the host38–40. The OMV vaccines employed in humans were derived from *Neisseria meningitidis*. Several studies have analyzed the effect of modifying the lipid A to abrogate its interactions to obtain OMVs with LPS preparations tailored for human vaccine development41–44. For example, *N. meningitidis* strains lacking LpxM or LpxL also render a lipid A with minimal toxicity41,44. It has been shown that OMVs produced in *E. coli* can also be detoxified through modifications of the lipid A37. These modifications can be carried out through the action of lipid A deacylases, such as PagL45. The overexpression of *B. pertussis* PagL resulted in OMVs with lower endotoxic activity compared to wild type *B. pertussis* OMVs. Monophosphorylated lipid A species recently became the first new Food and Drug Administration-approved adjuvant in several decades46. Therefore, it might be possible to generate geOMVs with a perfect balance between reduced toxicity and optimal adjuvanticity by generating strains containing modifications in the levels of lipid A acylation, phosphorylation, and/or other modifications.
Material and Methods

Animal ethics statement. All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Welfare Committee of the University of Montreal (protocol #RECH-1523). Studies involving chickens were carried out in accordance with the protocol approved by the Animal Care and Use Committee at the University of Alberta using a 35 day challenge protocol.

Construction of pneumococcal glycan expression plasmid. The cps gene cluster responsible for the synthesis of S. pneumoniae capsule serotype 14, CPS14, (excluding the regulatory genes) was cloned via a 3-way ligation method. Two separate fragments of the CPS14 locus were amplified using a high fidelity polymerase (iProof™, BioRad). Fragment 1 amplified the wchA-wchM region using the forward primer (5′-ATAGACGTCTAGGATAAAAAAGGATTGGAAT-3′) and reverse primer (5′-TTAAGAAAAATCCCTGTTACAAA-3′). Fragment 2 amplified the wchM-wciY region using the forward primer (5′-CTGGTCAACAAATATAGAAAA-3′) and reverse primer (5′-ATACCTGAGATTCTTTGTAACCTCAGAA-3′). The underlined sequences denote SacI and Xhol restriction enzyme sites, respectively. The PCR fragments 1 and 2 were both designed to include the native HindIII site within the wchM gene, thus, after a HindIII RE digest, the two fragments were compatible and reconstituted the wchM gene after ligation. Both fragments were successfully amplified and digested with SacI/HindIII (fragment 1) or HindIII/Xhol (fragment 2) and cloned into the plasmid vector pBRR1MCS-2, which was double digested with SacI and Xhol, generating pBRR1MCS-CPS14. The cloned CPS14 locus was confirmed by restriction enzyme digests and sequencing. For proper expression of the CPS14 locus in E. coli, the CPS14 locus was subcloned into pWSK129. The wchA-wciY CPS14 fragment was cleaved from pBRR1MCS-CPS14 using SacI and Xhol restriction enzymes. pWSK129 was digested with SacI and Xhol to reconstitute restriction enzymes. As Xhol and XbaI generate compatible sticky ends, the wchA-wciY fragment was ligated into pWSK129. The resulting plasmid, named pNLP80 was introduced in E. coli strains.

Isolation of geOMVs from E. coli expressing bacterial glycans. geOMVs were isolated following the protocol described by Haurat et al.47. Briefly, E. coli strains harbouring plasmids expressing genes involved in glycan synthesis (i.e., pNLP80, pEQ3, pACYCpIgBmut) and the respective vector control plasmids were grown at 37 °C overnight with shaking in 50 mL of Luria-Bertani (LB) broth plus appropriate antibiotics. The following day, each culture was subcultured (1:100 dilution) into 1 L LB or Terrific Broth (TB) plus antibiotics and grown at 37 °C for 3 min. Excess geOMVs were blotted away from membrane grid and samples were negatively stained with 2% ammonium molybdate. Transmission electron microscopy. Isolated geOMVs were absorbed onto carbon-coated copper membrane grids for 3 min. Excess geOMVs were blotted away from membrane grid and samples were negatively stained with 2% (w/v) uranyl acetate (3 min). The grids were analyzed for presence of geOMVs using a Morgagni (FEI) transmission electron microscope (Biological Sciences Microscopy Facility, University of Alberta).

Murine studies with Pneumococcal geOMVs. Three groups of BALB/c mice (n = 10 per group, female, 4–6 weeks old, obtained from Charles River, WA) were immunized intraperitoneally. The test group was injected with 2 μg of geOMV’s isolated from E. coli CLM37 strain expressing CPS14 (pNLP80). A placebo control group received geOMVs isolated from E. coli CLM37 strain not expressing CPS14. The third group was injected with commercially available dose of Prevnar 13 (500 μL as supplied). Sera were collected weekly via tail bleeds over a 42 day period and booster doses were administered on Days 14 and 28. Final bleed (Day 42) was via cardiac puncture. The presence of antibodies against CPS14 was analyzed by Western-blot using Odyssey imaging systems (LI-COR Biosciences, USA). geOMVs preparations digested with proteinase K were analyzed using 1:500 dilutions of the mouse sera.
the wells were washed again three times with PBS and 100 μL of p-nitrophenyl phosphate was added to each well and the plates were incubated at 37 °C for 1 h followed by reading the absorbance at 405 nm on a BioTek plate reader.

**Opsonophagocytosis Assay (OPA).** Blood collection. Blood was collected by intracardiac puncture from naïve female mice (Charles River, Wilmington, MA), treated with sodium heparin, then diluted to obtain 6.25 × 10⁶ leukocytes/mL in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. All reagents were from Gibco (Invitrogen, Burlington, ON, Canada).

**Bacterial suspension preparation.** Isolated colonies on sheep blood agar plates of *S. pneumoniae* serotype 14 (Statens Serum Institut, Denmark) were inoculated in 5 mL of Todd-Hewitt Broth (THB) (Becton Dickinson, Mississauga, ON, Canada) and incubated for 16 h at 37 °C with 5% CO₂. Working cultures were prepared by transferring 0.1 mL of 16 h-cultures into 10 mL of THB which was incubated for 5 h. Bacteria were washed 3 times and resuspended in PBS to obtain an OD₆₅₀ value of 0.6, which corresponds to 2 × 10⁸ colony forming units (CFU)/mL. Final bacterial suspension was prepared in complete cell culture medium to obtain a concentration of 6.25 × 10⁶ CFU/mL. The number of CFU/mL in the final suspension was determined by plating samples onto Todd-Hewitt Agar (THA) using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA).

Assay. Diluted whole blood (5 × 10⁴ total leukocytes) was mixed with 5 × 10³ CFU of *S. pneumoniae* (MOI of 0.01) and either 5% or 20% (v/v) of serum from control (placebo) or vaccinated mice in a microtube to a final volume of 0.2 mL. Microtubes were incubated for 2 h or 4 h at 37 °C with 5% CO₂, with shaking. After incubation, viable bacterial counts were performed on THA using an Autoplate 4000 Automated Spiral Plater. Tubes with addition of naive mouse sera (5% or 20% v/v) or of commercial rabbit anti-*S. pneumoniae* type 14 serum (20% v/v) (Statens Serum Institut, Denmark), were used as negative and positive controls, respectively. The % of bacteria killed was determined using the following formula: % Bacteria killed = [1 – (bacteria recovered from sample tubes/ bacteria recovered from negative control tubes with naïve sera)] × 100. Final OPA conditions were selected based in several pre-trials using different incubation times and MOIs (data not shown). A more detailed procedure can be found in ⁴⁹.

**Chicken vaccination and challenge.** In general each group contained up to 8 leghorn birds (Poultry Research Facility, University of Alberta) that were randomly tested for the presence of *Campylobacter* on the day of hatch (Day 1) by plating cloacal swabs onto selective Karmali agar. In all cases no *Campylobacter* colonies were observed after 48 h of incubation under microaerobic conditions at 37 °C. Vaccination was performed by orally gavaging with 300 μL of PBS containing 500 ng of geOMVs on Days 7 and 21. Control groups were gavaged with 300 μL of PBS only.

Birds were challenged on day 28 by oral gavaging with either PBS (negative control) or with 300 μL PBS containing 10⁵ C. jejuni 81–176 cells. The challenge strain was prepared as follows: C. jejuni 81–176 was grown for 18 h on Mueller-Hinton (MH) agar and harvested with cold MH broth. Cells were washed twice with cold PBS and adjusted to an OD₆₅₀ of 1.2 (OD₆₅₀ of 1.2 equals 3 × 10⁶ cell/mL). Serial dilutions in PBS were performed dependent on the final amount of cells that were administered. For example: 3 × 10⁵ cells/mL (1 × 10³ cells per 300 μL (=1 dose)). Cells were harvested on ice until usage. Birds were maintained on an additional 7 days after challenge and then euthanized. Ceca were removed and the contents were adjusted to 1 mg cecal content per 1 mL with sterile PBS. Aliquots of 10-fold serial dilutions (in PBS) of the cecal contents were plated onto selective Karmali agar. CFU were determined after incubation of the plates for 48 h under microaerobic conditions.

**ELISA for C. jejuni N-glycan-specific antibodies.** Blood samples were collected on Day 28 (vaccine response prior to challenge) and kept at 37 °C until a firm blood clot was formed. Samples were centrifuged (5 min, 18,000 × g, 4 °C) and the supernatants (sera) were transferred to fresh tubes. After addition of glycerol to a final concentration of 10%, sera were stored at −20 °C until further use. For ELISA coating, Campylobacter N-glycan compounds (Cj-N-glycan) and their chemical conjugation to the protein carrier bovine serum albumin (BSA) was performed as described ⁵⁰, ⁵¹. Maxisorb plates (Thermo Fisher) were coated with 500 ng of BSA-Cj-N-glycan conjugate per well for 18 h at 4 °C. After removal of unbound antigen the plate was blocked for 1 h at room temperature with PBS-Tween, 5% skim milk. After discarding the blocking solution 100 μL of chicken sera diluted 1:10 in PBS-Tween, 1% skim milk was added to each well. Plates were incubated for 1 h at room temperature and then washed 3 times with 5 min PBS-Tween. After addition of 100 μL of 2nd antibody solution (anti chicken IgY, diluted 1:500 in PBS-Tween, 1% skim milk) and incubation for 1 h at room temperature wells were washed 4-times for 5 min with 100 μL of PBS-Tween and developed using the 1-Step p-Nitrophenyl Phosphate (PNPP) assay following the instructions of the manufacturer (Thermo Fisher). Immuno-reactivity in each serum was determined after reading the plate at OD₄₀₅ in a plate reader.

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**Author Contributions**

N.L.P., G.G.-D. and H.N. performed experiments. N.L.P., C.M.S., M.S. and M.F.F. wrote the paper. E.V. performed statistical analysis on the results. All the authors interpreted data and reviewed the manuscript.

**Additional Information**

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