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Characterization of the key antigenic components of pertussis vaccine based on outer membrane vesicles

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ABSTRACT

Pertussis has resurged during the last two decades in different countries. In particular in the 2010–2013 period large outbreaks were detected in US, Australia, UK and The Netherlands with significant mortality in infants. The epidemiological situation of pertussis points out the need to develop new vaccines and in this regard we previously developed a new vaccine based on outer membrane vesicles (OMVs) which have been shown to be safe and to induce protection in mice. Here we have further investigated the properties of OMVs vaccines; in particular we studied the contribution of pertussis toxin (PTx) and pertactin (Prn) in OMVs-mediated protection against pertussis. PTx-deficient OMVs and Prn-deficient OMVs were obtained from defective *Bordetella pertussis* mutants. The absence of PTx or Prn did compromise the protective capacity of the OMVs formulated as Tdap vaccine. Whereas the protective efficacy of the PTx-deficient OMVs in mice was comparable to Prn-deficient OMVs, the protective capacity of both of them was significantly impaired when it was compared with the wild type OMVs. Interestingly, using OMVs obtained from a *B. pertussis* strain which does not express any of the virulence factors but expresses the avirulent phenotype; we observed that the protective ability of such OMVs was lower than that of OMVs obtained from virulent *B. pertussis* phase. However, it was surprising that although the protective capacity of avirulent OMVs was lower, they were still protective in the used mice model. These results allow us to hypothesize that OMVs from avirulent phase shares protective components with all OMVs assayed. Using an immune proteomic strategy we identified some common components that could play an important role in protection against pertussis.

1. Introduction

Despite relatively high vaccination rates, in recent years the respiratory disease named pertussis or whooping cough has came roaring back to infect people in high numbers. Indeed, pertussis is now recognized as a frequent infection not only in newborn and infants, but also in adults [1–3]. The increase in pertussis cases has been mostly attributed to waning vaccine-induced immunity, the switch from whole cell vaccines to acellular vaccines (aP) and pathogen adaptation [4]. Despite the causes, the epidemiological situation has led to intensified research on the design of new vaccines capable of conferring both long-lasting immunity and protection against different strain genotypes. Recently, we have demonstrated that outer membrane vesicles (OMVs) derived from *Bordetella pertussis* can protect against intranasal pertussis challenge when administered in a mouse infection model [5]. OMVs seem to have important advantages in vaccine development over the currently used aP since they are capable of conferring both long-lasting immunity and protection against different strain genotypes, eliciting IFNγ response, that have been associated with a better protective profile [6,7]. To further study the mechanisms involved in OMVs-mediated protection and in particular the contribution of some virulence factors in the protective efficacy, we investigated the role of pertussis toxin (PTx) and pertactin (Prn), which are present in the OMVs, derived from wild type *B. pertussis* [22]. PTx and Prn have shown to be protective antigens

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of *B. pertussis* and are used as essential components of acellular pertussis vaccines [8–10]. The PTx is a multimeric AB subunit protein in which A monomeric subunit expresses toxic activity through ADP ribosylation of the membrane-bound G_i-likeproteins, leading to the blockage of certain transmembrane signaling processes and eventually to cell intoxication. The B pentameric subunit binds receptors on the surface of eukaryotic cells and allows the toxic A subunit to reach its intracellular targets [11]. Several studies indicate that pertussis toxin possesses adjuvant properties able to potentiate local and systemic Ab responses, enhancing IgE, IgA, and IgG production [12,13] and promoting Th1- and Th2-type responses to co-administered Ags [14,15]. More recently, Nasso et al. using a genetically detoxified variant of pertussis toxin have shown that it promotes a Th1/Th17 response by an IL10 dependent mechanism [16].

PRN is a surface protein, which contains a RGD motif (Arg–Gly–Asp) involved in the attachment of *B. pertussis* to mammalian cells [11]. Anti-pertactin antibodies were found to be crucial for *B. pertussis* phagocytosis [17]. These data are consistent with field studies showing that the levels of pertactin antibodies correlate with protection [18].

In this study, we used PTx or Prn-deficient OMVs to analyze their immunogenicity, and protective efficacy in the well-accepted murine model of *B. pertussis* infection. Moreover, we analyzed whether the expression of all virulence factors is necessary to induce protection against pertussis. To this aim, we obtained OMVs from a *B. pertussis* strain blocked in the avirulent phase, a phase well characterized by the absence of all the virulence factors dependent on the BvgAS two component system but still able to express a whole set of new proteins known as avirulence factors.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. pertussis Tohama phase I strain, the Argentinean clinical isolate *Bp*106 [7,19] and the mutant strains defective in PTx [20], Prn [21] or BvgS protein [22] expression were used throughout this study. The *B. pertussis* strains were grown on Bordet Gengou (BG) agar medium (Difco, Houston, US) supplemented with 10% (v/v) defibrinated sheep's blood (BGA plates) [19].

2.2. Isolation of outer membrane vesicles (OMVs)

OMVs from bacterial cells we obtained according to the previously described method [23,24]. OMVs were negatively stained and examined by transmission electron microscopy as explained elsewhere [23,24]. Protein content was estimated by the Lowry method [25] using bovine serum albumin as standard.

2.3. Cloning and expression of the recombinant subunit A of pertussis toxin (PTxA), Fimbriae 2 (Fim2) and GroEL like protein

As previously stated for the PTxA subunit [7], the PCR product of each coding sequence obtained using Pfx Platinum polymerase (0.5 units, Invitrogen) was cloned into pET-TOPO 200 vector according to the manufacturer's instructions (Invitrogen). The recombinant clones were confirmed by PCR and sequenced. The recombinant pET-TOPO 200 vectors containing each coding sequence were first transformed into TOP10 *Escherichia coli* competent cells and then into expression host cells, BL21Star. For protein expression, selected clones were grown in LB medium containing km (50 μg/ml) and IPTG 1 mM.

Each recombinant protein was purified using Ni-NTA columns (Invitrogen) equilibrated in denaturing lysis buffer (Invitrogen) according with the manufacturer's protocols. Recombinant protein purity was judged by SDS-PAGE. The identity of the expressed protein was confirmed by MALDI-TOF mass spectrometry [7]. Search and identification of peptides were performed with a licensed Version of MASCOT software (Matrix Science at http://matrixscience.com), in a database containing the 3436 accession number entries derived from the complete *B. pertussis* genome sequence (downloaded from http://www.ncbi.nlm.nih.gov/). The MASCOT search parameters: (i) species, Bacteria (Eubacteria); (ii) allowed number of missed cleavages (only for trypsin digestion) 1; (iii) variable post-translational modifications, methionine oxidation; (iv) fixed modifications, carbamidomethylation; (v) peptide tolerance, ±50 ppm; (vi) peptide charge, +; and (vii) monoisotopic peptide masses, were used to search the database, allowing a molecular mass range for 1-DE analyses of ±15%. Only significant hits as defined by MASCOT probability analysis were considered. The sequence coverage % for the identified proteins was higher than 37%.

2.4. Production of a polyclonal serum against His6-PTx S1, His6-Fim2, His6- GroEl and Prn

To obtain specific sera against each recombinant protein and Prn (NIBSC), groups of 3 mice obtained from the Instituto Biológico Argentino (Biol. SAIC, Argentina) were immunized intraperitoneally twice, at intervals of 14 days, with 5 μg of each immunogen each time. $Al(OH)_3$ was used as an adjuvant. Fourteen days after the last immunization, mice were bled, and the serum was separated in order to assess by immunoblotting the presence of specific antibodies against the recombinant proteins.

2.5. Gel electrophoresis and immunoblots

OMV proteins were separated by SDS-PAGE and then transferred onto PVDF (Immobilon P, Millipore) at constant voltage (100 V). After the transfer, PVDF membranes were probed with either a polyclonal anti-PTx S1 antibody (diluted 1:300), an anti-Prn antibody (diluted 1:500), an anti-Fim antibody (diluted 1:500) or an anti GroEl (diluted 1:500), followed by incubation with anti-mouse IgG conjugated with alkaline phosphatase (AP) at a 1:1000 dilution. Nitroblue tetrazolium (NBT)—5-bromo-4-chloro-3-indolylphosphate (BCIP) was used as the AP substrate according to the manufacturers protocol (Promega).

2.6. Formulation of acellular vaccines containing OMVs, $Tdap_{OMVs}$

The vesicle preparations were detoxified with formalin (0.37% at 37 °C overnight). To use the OMVs from wild type bacteria or their deficient mutants as a combined acellular vaccine, vesicles (1.75 μg of total OMVs protein), were formulated with diphtheria (1 to 3 Lf/dose with an output of 0.1 UIA/ml serum) and tetanus toxoids (5 to 7 Lf/dose with a power greater than or equal to 2 UIA/ml serum) in the amounts described below, besides aluminum hydroxide did not exceed 1.25 mg/dose.

2.7. Active immunization and intranasal challenge

Animal protocol using female BALB/c mice (4 weeks of age) was performed as previously described [19]. Groups of 5 mice were immunized systemically (i.p) with Tdap vaccines containing specific OMVs (1.75 μg of total OMV protein that contains approximately 47.1 ng ± 11.2 ng of the complete PTx protein) or 1/10 human dose (HD) of commercial TdaP (Boostrix™ that contains as pertussis components PTx 8 μg, FHA 8 μg and Prn 2.5 μg per human dose) using a two-dose schedule over a period of 2 weeks. Mice were challenged 2 weeks after the second immunization by nasal inoculation with a sublethal dose (10^6–10^8 CFU 40 μl^{-1}) of Tohama phase I.

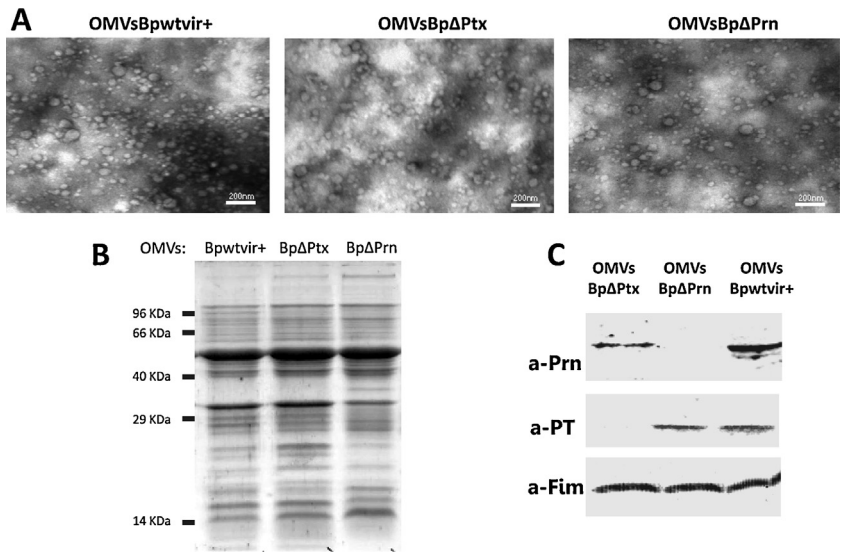

Fig. 1. (A) Transmission electron microscopy image of negatively stained outer membrane vesicles (OMVs) obtained from: *Bordetella pertussis* Tohama I (OMVsBpwtvir+), mutant *B. pertussis* Tohama I not expressing pertussis toxin (OMVsBpΔPtx) and mutant *B. pertussis* Tohama I not expressing pertactin (OMVsBpΔPrn) (scale bar: 200 nm). (B) SDS-PAGE 12.5% w/v of OMVsBpwtvir+, OMVsBpΔPtx and OMVsBpΔPrn. Molecular weights are indicated at the left. (C) Immunoblots of OMVsBpwtvir+, OMVsBpΔPtx and OMVsBpΔPrn using anti-pertactin, anti-pertussis toxin subunit 1, and anti-fimbrial protein polyclonal mouse antibodies.

In all cases, bacterial counts were performed 7 days after the challenge. Lungs were aseptically removed, homogenized in the sterile PBS, serially diluted and plated on BGA plates to determine bacterial recoveries. At least three independent experiments were performed in each case.

2.8. Immuno-proteomics assays

OMV samples were subjected to 1D gel electrophoresis in triplicate as described previously [24]. For immunoblotting serum sample from mice immunized with the different OMVs studied here were collected both before vaccination and the day before challenge. The unstained 1-D gels were electroblotted onto polyvinylidene difluoride membranes which were then incubated with sera from each treatment (1/300 dilution) and the immunoreactivity was finally detected as above described. Membranes were then scanned, and the profile of immuno-reactive proteins was matched to 1D gel images of the same sample and selected protein bands were excised from the stained 1D gels, digested in-situ with trypsin and subjected to mass spectrometry fingerprinting. Immunoreactive proteins present in the vesicles were identified as previously reported by us [19,26].

2.9. Expression of inflammatory markers upon systemic delivery of OMVs

In order to assess the pro-inflammatory capacity of the different formulations, blood samples were collected 4 h after each immunization by submandibular bleeding and the sera were then separated. Serum IL-6 was measured by ELISA using BD OptiEIA (BD Biosciences) following manufacturers instructions.

2.10. Statistical analysis

Before applying the statistical methods described bellow we evaluated the normality of the data by using Shapiro–Wilk test (http://scistatcalc.blogspot.com.ar/2013/10/shapiro-wilk-test-calculator.html). After verifying that our data follows a normal distribution, we statistically analyzed them by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPadPrims®). Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Isolation and characterization of the OMVs obtained from parental and PTx or Prn defective B. pertussis mutants

The OMVs were obtained from parental and both PTx or Prn *B. pertussis* defective mutant cells following the previously described procedure. The samples obtained were negatively stained and examined with an electron microscope (Fig. 1A). In all cases the size range was consistent from batch to batch and similar to previously described OMV preparations [24,27]. To further characterize the OMVs 1D electrophoresis and immunoblottings were performed using specific antibodies against PTx and Prn. As positive control OMVs obtained from wild type bacteria were used. No major changes in the electrophoretic profile of the OMVs obtained from defective strains were detected (Fig. 1B). While all OMVs studied expressed fimbriae, Ptx or Prn expression was not observed in PTx or Prn defective *B. pertussis* mutants, respectively (Fig. 1C).

3.2. Protection against intranasal B. pertussis challenge after vaccination with B. pertussis OMVs

To evaluate the protection capacity of OMVs from PTx defective *B. pertussis* mutant (OMVsBpΔPtx) and Prn defective *B. pertussis* mutant (OMVsBpΔPrn), against *B. pertussis* challenge, the murine model of intranasal *B. pertussis* challenge was used. In particular, the effect of two administrations of each type of OMVs on subsequent *B. pertussis* (10^7 CFU per 40 μl) colonization by after was quantified. These results were compared with those obtained in mice immunized with OMVs from wild type bacteria in the virulent phase (OMVsBpwtvir+) (Fig. 2). All OMVs used as vaccines were detoxified by formalin treatment and formulated with tetanus and diphtheria toxoids as it was described in the materials and methods section. These vaccines were here named $Tdap_{OMVsBpwtvir+}$, $Tdap_{OMVsBp\Delta Ptx}$ or $Tdap_{OMVsBp\Delta Prn}$. Animals vaccinated twice with PBS were used as negative control. Significant differences in lung bacterial counts between $Tdap_{OMVsBpwtvir+}$ immunized animals and control group were observed ($p < 0.001$)

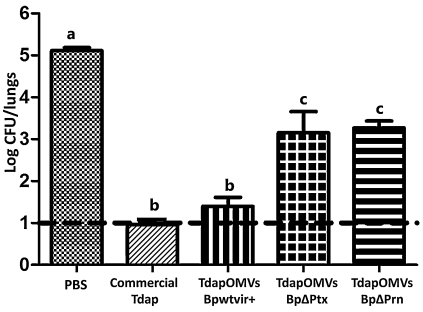

Fig. 2. Effect of systemic (i.p.) immunization with TdapOMVsBpwtvir+, TdapOMVBpΔPtx or TdapOMVBpΔPrn in the mouse intranasal challenge model. Commercial Tdap vaccine (1/10 of the human dose) was used as a positive control. *B. pertussis* Tohama was used as challenge bacteria (5×10^6 CFU 40 μl^{-1}). Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of four mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. The number of bacteria recovered from mouse lungs are expressed as the log 10 means ± standard errors (error bars) of colony forming units per ml. Statistical analysis was performed by Bonferroni test. Different letters indicate significant differences with $p < 0.001$.

(Fig. 2). We here confirmed our previous results showing that the number of lung colonies recovered from mice immunized with $Tdap_{OMVsBp\Delta Ptx}$ vaccine at day 7-post challenge were lower than those from PBS treated animals but higher than those from $Tdap_{OMVsBpwtvir+}$ immunized mice [7] (Fig. 2). Similar results were obtained with the $Tdap_{OMVsBp\Delta Prn}$. The OMVs derived from Prn defective mutant decreased their protective capacity in approximately two logs, being an important determinant about OMVs protective capacity (Fig. 2).

The protection level of the OMVsBpwtvir+ vaccine against *B. pertussis* infection was not achieved by immunization with either the $Tdap_{OMVsBp\Delta Ptx}$ or the $Tdap_{OMVsBp\Delta Prn}$ vaccines. The data confirm the importance of Ptx and Prn in eliciting a protective anti-pertussis response.

3.3. Protection against intranasal B. pertussis challenge after vaccination with OMVs from B. pertussis blocked in the avirulent phase

Since virulence factors have been considered as main protective antigens to the formulation of pertussis vaccines, we aimed to evaluate the protective capacity of vaccines containing the OMVs derived from *B. pertussis* blocked in the avirulent phase (OMVsBpvir−). To this goal, OMVs were obtained from a BvgS defective *B. pertussis* mutant. The characterization of OMVsBpvir− evidenced no differences in their size distribution evaluated by electron microscopy (Fig. 3A) but some changes in the electrophoretic profile were detected even though major components are shared with OMVsBpvir+ (Fig. 3B). The lack of expression of the main virulence factors was evidenced by immunoblotting (Fig. 3C). Upon characterization, assays using the intranasal *B. pertussis* challenge were performed. In particular, the effect of two administrations of OMVsBpvir−, on subsequent colonization by *B. pertussis* Tohama strain (2×10^7 CFU per 40 μl) was analyzed (Fig. 4). The results were once again compared with those obtained in mice immunized with the OMVsBpwtvir+ from *B. pertussis* Tohama phase I strain (Fig. 4). All OMVs used as vaccines were detoxified by formalin treatment and formulated with tetanus and diphtheria toxoids as it was described in the materials and methods section. These vaccines were here named $Tdap_{OMVsBpwtvir+}$ or $Tdap_{OMVsBpvir-}$. While $Tdap_{OMVsBpwtvir+}$ was used as a positive control, treatment with PBS served as a negative control. Significant differences in lung bacterial counts between $Tdap_{OMVsBpwtvir+}$-immunized animals

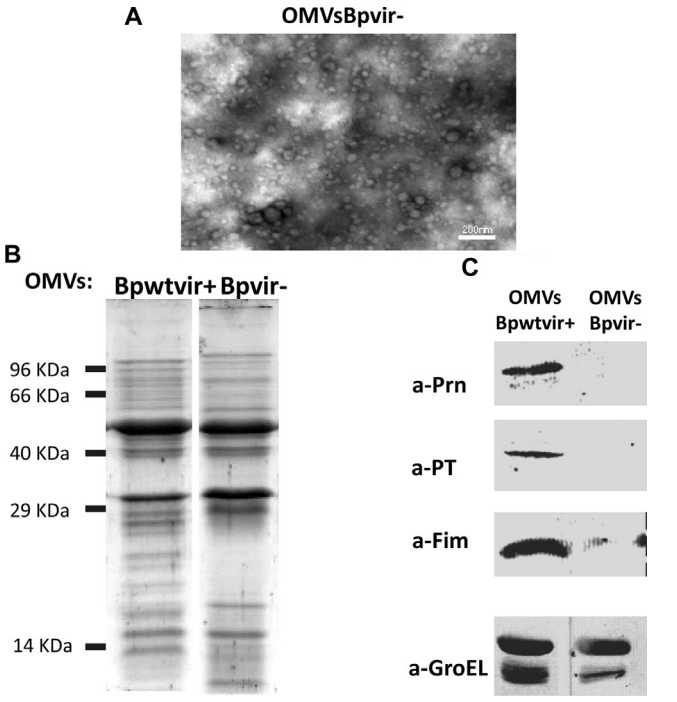

Fig. 3. (A) TEM image of negatively stained (OMVs) obtained from mutant *Bordetella pertussis* Tohama I blocked in a-virulence phase (OMVsBpvir−) (scale bar: 200 nm). (B) SDS-PAGE 12.5% w/v of OMVsBpwtvir+ and OMVsBpvir−. Molecular weights are indicated at the left. (C) Immunoblots of OMVsBpwtvir+ and OMVsBpvir− with anti-pertactin, anti-pertussis toxin subunit 1, anti-fimbrial protein and anti-GroEL specif polyclonal mouse antibodies.

and the negative control group were observed ($p < 0.001$) (Fig. 4) in concordance with our previous studies [7]. As expected, in mice immunized with $Tdap_{OMVsBpwtvir+}$ vaccine the number of lung-recovered colonies at day 7-post challenge dropped at least 4 orders of magnitude in relation to the non-immunized mice (Fig. 4). Interestingly, protection against *B. pertussis* challenge was also achieved with $Tdap_{OMVsBpvir-}$ immunization. Though the protective capacity of $Tdap_{OMVsBpvir-}$ is lower than that of the $Tdap_{OMVsBpwtvir+}$, the number of lung-recovered colonies at day 7-post challenge still dropped at least 1.5 orders of magnitude compared to the non-immunized mice (Fig. 4).

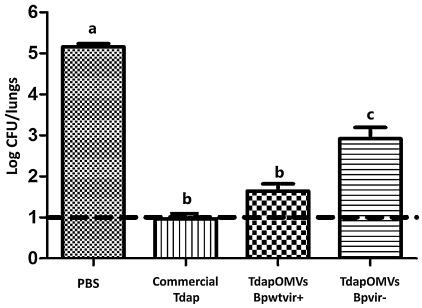

Fig. 4. Effect of systemic (i.p) immunization with TdapOMVsBpwtvir+ and TdapOMVsBpvir− in the mouse intranasal challenge model. Commercial Tdap vaccine (1/10 of the human dose) was used as a positive control. *B. pertussis* Tohama was used as challenge bacteria (5×10^6 CFU per 40 μl). Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of four mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log 10 means ± standard errors (error bars) of colony forming units per ml. Statistical analysis was performed by Bonferroni test. Different letters indicate significant differences with $p < 0.001$.

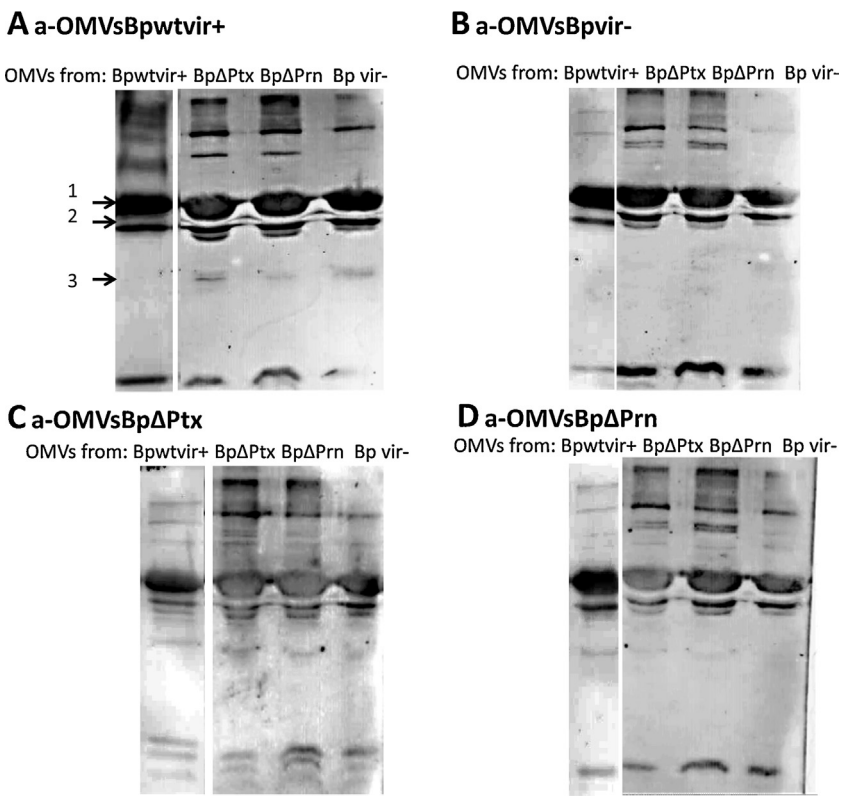

Fig. 5. Immunnobots of OMVsBpwtvir+, OMVs BpΔPtx, OMVs BpΔPrn and OMVsBpvir− using induced polyclonal mouse antibodies against OMVsBpwtvir+ (A), OMVsBpvir− (B), OMVBpΔPtx (C) or OMV BpΔPrn (D).

In order to evaluate whether the observed protective capacity of all the tested *B. pertussis* OMVs against *B. pertussis* Tohama strain could be extended to other strain/isolate of *B. pertussis*, we repeated the protection experiments with the different OMVs vaccine but using for challenge the previously characterized Argentinean clinical isolates *Bp*106 [7,19,26]. The results (Supplementary Table S1) show that under the experimental conditions employed, the protective capacity detected for the different OMVs is independent of the strain/isolate used in the challenge.

3.4. IL-6 levels after immunization

Usually after systemic immunization, a rise in pro-inflammatory cytokines can be detected. The levels are related with the pro-inflammatory capacity of the formulation employed. IL-6 is among the pro-inflammatory cytokines usually employed as indicators of this activity [28]. In our case, formulations containing any of the OMVs used in this study induced levels of IL-6 comparable to the PBS or the commercial Tdap treatment (Supplementary material Fig. S1).

3.5. Immuno-proteomics assays

We used immuno-proteomics as a method to identify potentially cross-reactive antigens among the different OMVs used here. This was done by examining the reactivity of sera induced by each type of OMV vaccines against the 4 different OMVs used here: OMVsBpΔPtx, OMVsBpΔPrn, OMVsBpwtvir+ and OMVsBpvir−. Naïve sera were used as negative controls (not shown). The blots for each sample sera were analyzed for bands showing common immuno-reactivity among the 4 types of OMVs.

Fig. 5 shows the blots with the serum-reactive protein bands detected in the different OMVs used. We confirmed the presence of several antigens in the OMVsBpvir− that are recognized by the sera from mice immunized with the other OMV variants, clearly pointing out the existence of several common antigens among all OMVs irrespectively of the virulence status of the strain from which they derive. The identity of these antigens was determined after selected immuno-reactive bands were excised from the 1D electrophoretic gels and then analyzed by mass spectrometry. Bands 1, 2 and 3 in Panel of Fig. 5 were identified as GroEL-like protein (band 1, panel A) and OMPc proteins (bands 2 and 3, panel A), both of them very abundant in the outer membrane. Regarding GroEL protein, MS identification was confirmed by immunodetection using both a GroEL-specific antibody, which evidenced that this protein is present in the different OMVs preparations (Fig. 6 panel A), and also using as antigen the purified recombinant GroEL protein which was recognized by the specific antibodies induced by the different OMVs (Fig. 6 panel B).

4. Discussion

The resurgence of the important respiratory disease named pertussis or whooping cough has been mainly attributed to the waning of vaccine-induced protective immunity over the time as well as to pathogen adaptation to the vaccine induced immunity [4]. Therefore, development of a new generation of pertussis formulations capable to overcome the weaknesses associated to the current vaccines is required. Previously we have demonstrated that the outer membrane vesicles obtained from *B. pertussis* are good vaccine candidates that could successfully avoid the drawbacks of available formulations [5,22].

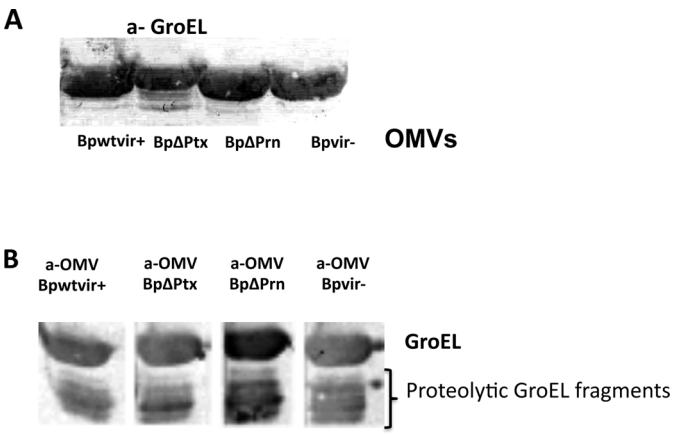

Fig. 6. (A) Immunnobots of OMVsBpwtvir+, OMVs BpΔPtx, OMVs BpΔPrn and OMVsBpvir− using polyclonal mouse sera against recombinant GroEL protein. (B) Immunnobots of purified recombinant GroEL protein using polyclonal mouse sera against OMVsBpwtvir+, OMVBpΔPtx, OMV BpΔPrn or OMVsBpvir−.

While the OMVs-induced protective immune responses have been previously reported by us [5,24], the contributions of bacterial factors in the OMVs-mediated response remained to be investigated in more detail. In our previous work, we assessed the contribution of the pertussis toxin (PTx) in the protection afforded by OMV vaccination [7]. Here we confirmed our previous results regarding the importance of PTx in OMV protective capacity. To extend the analysis to pertactin (Prn), protection levels of OMVs derived from a Prn defective *B. pertussis* mutant were compared to the parental OMVs strain and also to those OMVs in which PTx is not present.

Using the murine model of intranasal pertussis challenge, we showed that the absence of Prn in the OMVs is responsible for their decreased protective capacity (Fig. 2), pointing out once again the major contribution of Prn to the anti-pertussis response. The current observation about Prn protective effects is consistent with its early inclusion in the 3 component acellular vaccine. Several studies in both animals and humans have indicated that Prn can elicit protective antibodies [18,29]. It was observed that anti-pertactin antibodies are crucial for *B. pertussis* phagocytosis, which was also correlated with protection against the disease [17]. Furthermore it was reported that Prn contains an Arg–Gly–Asp (RGD) motif, which is implicated in ligand-receptor interactions in eukaryotes. It has been demonstrated that this motif is involved in the Prn-mediated attachment of *B. pertussis* to mammalian cells [30,31].

Recently it was reported an increase in the prevalence of *B. pertussis* isolates not producing pertactin in populations with high aP vaccination coverage. Although *B. pertussis* isolates lacking the production of aP vaccine antigens had already been isolated, they had never reached such a high prevalence [32–34]. The appearance and spread of PRN-deficient isolates in populations vaccinated with the current commercial aP have elicited a deep concern in the health system since it was hypothesized that PRN-deficient clinical isolates present an advantage in an aP-vaccine primed immunity. Our results seem to be in line with this idea since they reinforce the known function of Prn in protection. However, this important role of Prn could have a negative outcome such as the selection of Prn deficient isolates, especially in the context of its inclusion in vaccines with a few components in high dose. Although it is essential to address whether the acellular vaccines containing components in high dose are responsible for the emergence and spread of aP antigen-deficient isolates, it is urgent as well to assess the impact of Prn deficient isolates on aP vaccine effectiveness.

Based on our results showing the role of some particular virulence factors in OMVs-induced protective responses we decided to evaluate if the lack of all virulence factors may abrogate OMVs protective capacity. To this end, OMVs derived from *B. pertussis* blocked in the avirulent phase were obtained. This approach seemed appropriate to our goal, since on one hand *B. pertussis* blocked in the avirulent phase does not expresses any of the virulence factors and, on the other hand, the avirulent factors characteristic of this phase seem to behave as poor immunogens [35]. Surprisingly, OMVs from *B. pertussis* blocked in the avirulent phase were still protective against the infection, as judged by the significant 2 logs decrease ($p < 0.001$) (Fig. 4) in lung bacterial counts between avirulent OMVs-immunized mice and the negative control group. Even though this protection level is lower than the 4 logs decrease observed with OMVs from *B. pertussis* Tohama phase I strain, the above described effect is elicited by avirulent components that have not been so far reported as capable of conferring protection against *B. pertussis*. In addition, the toxicity of these OMVs seems to be as low as the other OMVs, according to the measured production levels of the pro-inflammatory IL-6 just after OMVs vaccination (Fig. S1). In addition, the OMVs used in these studies induced a moderate rise in the levels of the pro-inflammatory IL-6 in serum just after OMVs vaccination (Fig. S1), without differences among the different OMVs. Although this is a partial indicator of its pro-inflammatory capacity, in principle the presence of different virulence factors in each type of OMV were not reflected in the capacity to induce a systemic proinflammatory response.

Altogether the results presented here evidence that OMVs derived from *B. pertussis* exhibit a basal level of protection against *B. pertussis* irrespectively of the expression of one or more virulence factors. Therefore, it is tempting to speculate that protective efficacy observed with all OMVs may be attributed to common factors that are expressed in all bacterial strains used.

In order to identify such common components that could have a role in the protection we performed an immune-proteomics assays using as antigen the four different OMVs here studied and the different mouse sera induced by those OMVs. Several components were detected to be present in all the different OMV preparations analyzed and the identities of three immuno-reactive bands were determined. These included well-studied antigens such as OMPc and GroEL like protein. OMPc previously named OMP P contains conserved regions common to the neisseria [36]. Recently, it was demonstrated for other pathogen that OmpC is a promising vaccine target [37]. Regarding GroEL like protein, it was reported that in neonatal mice this protein provided little protection against an aerosol challenge with *B. pertussis* [38]. Furthermore, antibodies to this protein were elicited in mice by a standard diphtheria–tetanus–pertussis (DTP) vaccine but not by an experimental acellular pertussis vaccine.

According to the results shown here, protective capacity of OMVs is contributed by several components, including PRN and PT. Not only OMV composition is very complex, but also includes several factors common to both the virulent and the avirulent bacterial phases. It is of major importance to fully identify the high number of components present in OMVs and also to determine their individual roles in building up the remarkable OMV protective capacity. We can anticipate that this OMV feature may constitute a very positive vaccine quality, inasmuch as the selective pressure on bacterial circulating populations will not be centered in a single or a few components, making more difficult the emergence of escape variants, as was observed for acellular vaccines made with purified protein antigens.

The detailed investigation of the biological properties of such antigens in the OMV context may provide the required candidates for the development of second generation acellular pertussis vaccines.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.08.084.

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