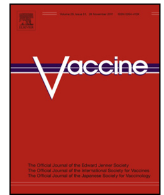
Vaccine 31 (2013) 5262–5268

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Outer membrane vesicles derived from *Bordetella parapertussis* as an acellular vaccine against *Bordetella parapertussis* and *Bordetella pertussis* infection

D. Bottero [a,1], M.E. Gaillard [a,1], A. Errea [b], G. Moreno [b], E. Zurita [a], L. Pianciola [c], M. Rumbo [b], D. Hozbor [a,*]

[a] Laborartorio VacSal, Instituto de Biotecnología y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CCT La Plata CONICET, Argentina
[b] Laboratorio de Investigaciones del Sistema Inmune, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina
[c] Laboratorio Central Subsecretaría de Salud de Neuquén, Neuquén, Argentina

A R T I C L E I N F O

Article history:
Received 28 May 2013
Received in revised form 12 August 2013
Accepted 21 August 2013
Available online 5 September 2013

Keywords:
Bordetella parapertussis
Bordetella pertussis
Acellular vaccine
OMVs
Protection
Whooping cough

A B S T R A C T

Bordetella parapertussis, a close related species of *B. pertussis*, can also cause the disease named pertussis or whooping cough. The number of cases caused by this related pathogen has risen sustained in the last years. The widely used cellular (wP) or acellular (aP) pertussis vaccines have little or no efficacy against *B. parapertussis.* In an effort to devise an effective acellular vaccine against *B. parapertussis* infection, outer membrane vesicles (OMVs) were obtained from *B. parapertussis*. Proteomic analysis of the resulting OMVs, designated OMVsBpp, evidenced the presence of several surface immunogens including pertactin. The characterized OMVsBpp were used in murine *B. parapertussis* intranasal challenge model to examine their protective capacity when administered by systemic route. Immunized BALB/c mice were challenged with sublethal doses of *B. parapertussis.* Significant differences between immunized animals and the negative control group were observed ($p < 0.001$). OMVsBpp protected against *B. parapertussis* infection, whereas current commercial aP vaccine showed little protection against such pathogen. More interestingly, protection induced by OMVsBpp against *B. pertussis* was comparable to our previously designed vaccine consisting in OMVs derived from *B. pertussis* (OMVsBp). For these experiments we used as a positive control the current commercial aP vaccine in high dose. As expected aP offered protection against *B. pertussis* in mice. Altogether the results presented here showed that the OMVs from *B. parapertussis* are an attractive vaccine candidate to protect against whooping cough induced by *B. parapertussis* but also by *B. pertussis*.

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1. Introduction

Whooping cough (or pertussis) is a current public health problem and a major cause of death in children, even in countries with high vaccination coverage [1–3]. The estimations of the World Health Organization (WHO) indicate that in 2008, there were about 16 million cases of pertussis worldwide, 95% of them in developing countries, and that about 195,000 children died because of the disease [4]. This disease is mainly caused by the bacteria *Bordetella pertussis* but also by *B. parapertussis*. Though *B. parapertussis* is identified as other causal agent of the disease, its impact in whooping cough is underestimated [5]. The reasons for this may be the fact that *B. parapertussis* infection is not easily distinguished from *B. pertussis* infection by symptoms and sometimes it is not laboratory confirmed [6]. Furthermore, the lack of obligatory surveillance for *B. parapertussis* also contributes to the underestimation of *B. parapertussis* infection. Despite this general situation, in some countries, a rise in infections caused by this pathogen has been documented. Recently, Cherry and Seaton reported that *B. parapertussis* infections are more common than previously realized [7]. Pertussis cases in which *B. parapertussis* was the etiologic agent were notified in Italy, Germany, and Sweden [6,8–10]. Moreover, in different efficacy trials, *B. parapertussis* accounted for between 2.1% and 25% of the culture-positive cases [8–12]. Data from some provinces of Argentina indicate that *B. parapertussis* accounted 27% of the culture-positive cases. In western Finland enhanced surveillance in the period of 1994–1997 with use of culture and PCR showed *B. parapertussis* infections as more prevalent than previously

* Corresponding author. Tel.: +54 221 422 9777; fax: +54 221 422 6947.
E-mail addresses: hozbor@biol.unlp.edu.ar, hozbor.daniela@gmail.com (D. Hozbor).

[1] These authors have equally contributed to the work.

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http://dx.doi.org/10.1016/j.vaccine.2013.08.059

documented [12]. Similar frequency of *B. parapertussis* has been found in St. Petersburg [13]. In a study conducted in Pakistan between 2005 and 2009, among 802 suspected cases of pertussis, the etiologic agent could be retrieved in 7.4% of the cases and identified by culture, being in 92% of the cases *B. parapertussis* [14]. The majority of these cases are thought to be pertussis vaccine failures. In fact there are some reports that suggest that both pertussis cellular or acellular vaccine have no or limited ability to protect against *B. parapertussis* [15]. Khelef et al. showed that immunization with different antigens derived from *B. pertussis* induce no protection against *B. parapertussis* in mice [16]. Stehr et al. reported that the cross-protective efficacy in children of acellular vaccine and the whole-cell pertussis vaccine was 31% and 6%, respectively [11].

The novel live attenuated whole cell pertussis vaccine BPZE1 recently developed by Locht s group seems to protects in mouse model against both *B. pertussis* and *B. parapertussis* infections [17]. However, in this case protection of this whole cell vaccine is induced by nasal route that though is an important route for *B. pertussis* since it mimics natural infection, its use is not currently widespread.

In an effort to develop acellular vaccines that are also effective in protecting against *B. parapertussis* infection by systemic route, we examined the protective effects of vaccines prepared with outer membrane vesicles (OMVs) derived from *B. parapertussis*. The acellular vaccine containing the OMVs from *B. parapertussis* induced protective immunity against *B. parapertussis.* Furthermore, this formulation protected also against *B. pertussis*, suggesting that the here developed acellular vaccine might prevent whooping cough caused by both species of *Bordetella*. We also confirmed here that the current acellular pertussis vaccines offer little protective immunity against *B. parapertussis* in the mouse model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. pertussis Tohama phase I strain (CIP 8132), the WHO reference strain *B. pertussis* 18323 [18] and the Argentinean clinical isolate *B. parapertussis* AR729, were used throughout this study. *B. pertussis* and *B. parapertussis* strains were grown in Bordet-Gengou agar (BGA, Difco) supplemented with 1% glycerol, Bacto peptone (Difco) 10 g/L and 10% (v/v) defibrinated sheep blood and incubated at 36 °C or Stainer-Scholte liquid medium (SS) as indicated previously [19].

2.2. Isolation of outer membrane vesicles (OMVs)

To obtain OMVs from bacterial cells we used the method previously described by us [20–22]. The obtained OMVs were stored with glycerol 1% and sodium azide 0.001% at 4 °C. OMVs were negatively stained and then examined with an electron microscope [21].

2.3. Protein assay

Protein content was estimated by the Bradford method using bovine serum albumin as standard [23].

2.4. 2D electrophoresis associated to mass spectrometry

Proteins that constitute the membrane vesicles have been separated and detected in electrophoresis runs in two dimensions (2D electrophoresis). Proteins present in the vesicles were identified by mass spectrometry associated with 2D electrophoresis as previously reported by us [24,25].

2.5. Lipopolysaccharide (LPS) extraction and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

LPS from OMVs of *B. parapertussis* were solubilized in Laemmli sample buffer [26] and heated at 100 °C for 10 min. Twenty-five micrograms of proteinase K in 10 μl of Laemmli sample buffer were added per 50 μl of OMVs suspension. The mixtures were incubated in a water bath at 60 °C for 1 h with occasional vortexing. Proteinase K-treated samples were applied to gels. Electrophoresis was performed at room temperature and constant voltage. The LPS was visualized by the BioRad silver-staining technique.

Outer membrane vesicles from *B. pertussis* and *B. parapertussis* were obtained with the procedure described above at least 10 times and in all cases the size, morphology and surface immunogens were similar.

3. Formulation of acellular vaccines

To use the OMVs as acellular vaccines (hereafter referred as OMVs vaccine), the vesicles preparations were mixed with aluminum hydroxide as adjuvant (0.2 mg/ml) and detoxified with formalin (0.37% at 37 °C overnight).

To use the OMVs as combined acellular vaccines (hereafter referred as combined vaccine or TdapOMVs), vesicles (3 μg of total OMV protein), were formulated with diphtheria (1–3 Lf/dose with an output of 0.1 UIA/ml serum) and tetanus toxoids (5–7 Lf/dose with a power greater than or equal to 2 UIA/ml serum) in the amounts described below, besides aluminum hydroxide that not exceed 1.25 mg/dose.

3.1. Active immunization and intranasal challenge

Four week-old female BALB/c mice were used for all assays. Mice were obtained from Biol SAIC, Argentina. Animal immunization protocol was performed as we previously described [25].

Groups of BALB/c mice were immunized with the formulations described above. Immunization protocols comprised a two-dose schedule over a period of 2 weeks. Mice were challenged 2 weeks after the second immunization by nasal challenge with sub-lethal dose (10^6–10^8 CFU 40 μl^{-1}) of *B. pertussis* 18323 strain or *B. parapertussis* AR729 as indicated in the figures. Lungs of challenged mice were collected for bacterial counts at 7days after the challenge. The lungs were aseptically removed, homogenized in sterile PBS, were serially diluted, and then plated on Bordet–Gengou plates supplemented with defibrinated sheep blood to determine bacterial recoveries. At least three independent experiments were performed in each case.

3.2. Mouse weight gain test (MWG)

The MWG-test was carried out using groups of 8 Balb/c mice (15–20 g) which were vaccinated with detoxified with 37% formalin 20 h at 37 °C OMVs obtained from *B. pertussis* and *B. parapertussis* strains. In all cases control group received an equal volume of sterile PBS. Animals were observed for 7 days and body weight was recorded after 16 h, 3 and 7 days. Vaccines were considered non-toxic when passing the WHO and EP requirements [21].

3.3. Expression of inflammatory markers upon systemic delivery of OMVs

In order to have an additional indication of pro-inflammatory capacity of the different formulations, serum was collected 4 h after each immunization by submandibular bleeding and serum

separation. Serum IL-6 was measured by ELISA using BD OptiEIA (BD Biosciences) following manufacturers instructions.

3.4. Statistical analysis

Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPadPrims®). Differences were considered to be significant when $p < 0.05$.

4. Results

4.1. OMVs isolation

The OMVs were obtained from *B. parapertussis* AR729 cells following the procedure described above. The samples obtained were negatively stained and examined with an electron microscope (Fig. 1A). Size range was consistent from batch to batch and similar to previously described OMV preparations [21,22]. To go further in the OMVs characterization we performed 2D electrophoresis associated to matrix-assisted laser desorption ionization–time-of-flight mass spectrometry analysis in order to identify some of the proteins present in the OMVs obtained from *B. parapertussis*. The identified proteins (Fig. 1B) were correlated on the basis of their predicted locations within the bacterial cell using the PSORTb algorithm, which predicts the subcellular locations of proteins in gram-negative bacteria according to the presence or absence of leader peptides, homologies to known proteins, transmembrane domains, and outer membrane anchoring motifs. Some of the identified proteins that had cytoplasmic location such as EF-Tu, 60-kDa chaperonin and some dehydrogenases had been associated to membranes in related microorganisms such as *Neisseria meningitides* [27]. As we described previously for *B. pertussis* OMVs samples [21] some of the proteins separated by 2D electrophoresis were present as multiple spots exhibiting variability in pI values (horizontal spot patterns, Fig. 1B). Charge variants included EF-Tu (Fig. 1B, spot 7), 60-kDa chaperonin (Fig. 1B, spot 5), outer membrane porin protein precursor (Fig. 1B, spot 15), and serine protease (Fig. 1B, spot 6). These may represent natural isoforms or an artifact caused by sample preparation for two-dimensional electrophoresis. Serum resistance protein was resolved also in multiple spots of differing masses and pIs, suggesting possible protein processing, degradation, and/or modifications (Fig. 1B). The predominant outer membrane proteins detected were those that have been well established as the major components of the outer membrane, namely, pertactin, outer membrane porin precursor, outer membrane protein OmpQ. Others proteins identified in the OMVs would be involved in carbohydrate metabolism and some of them

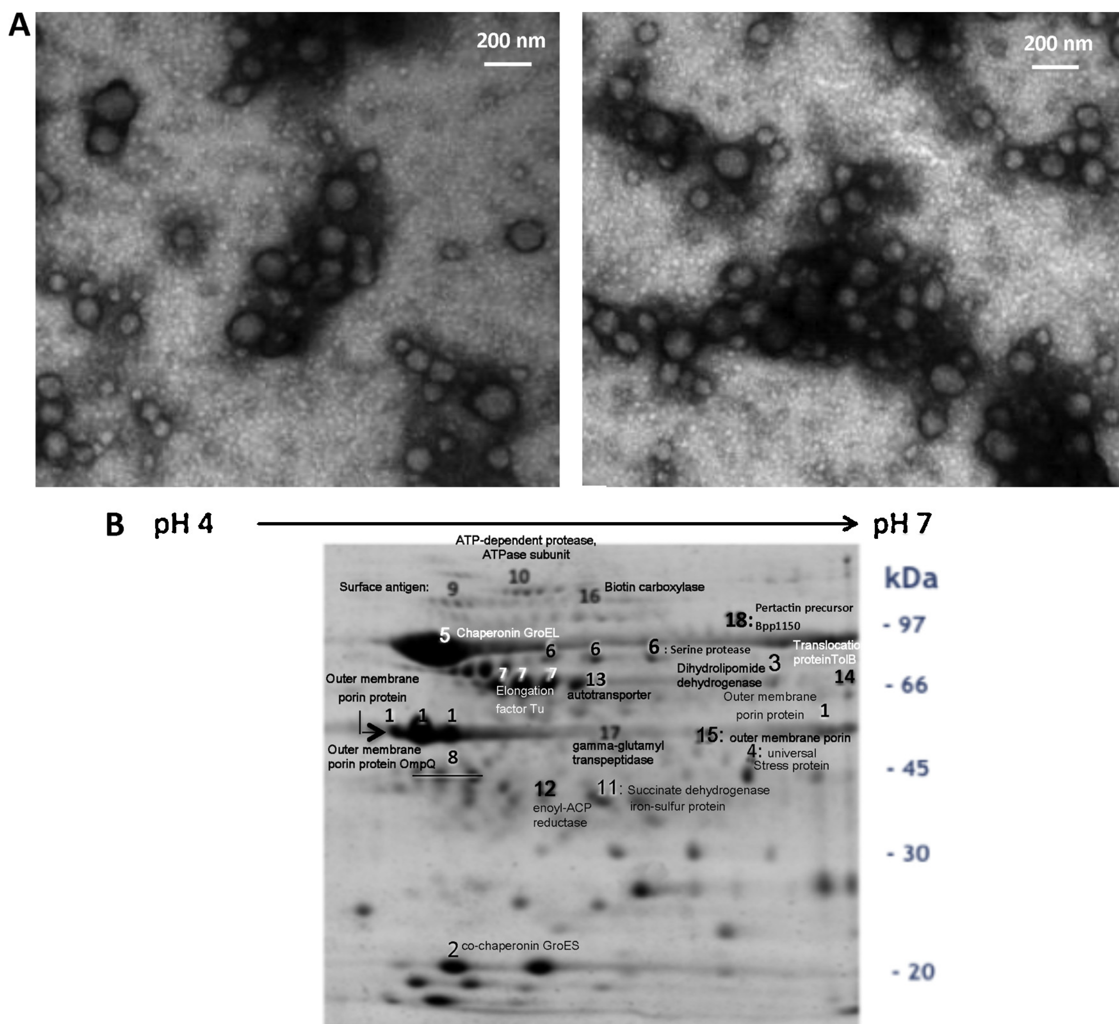

Fig. 1. Panel A Negatively stained *B. parapertussis* OMVs examined with an electron microscope. Panel B Proteome reference map of OMVs derived from *B. parapertussis*. Proteins samples were separated by IEF at pH 4–7 in the first dimension and then by 12.5% SDS-PAGE in the second dimension. Protein spots were visualized by colloidal Coomassie staining. The spot numbers refer to the identified peptide subunits by matrix-assisted laser desorption ionization–time of flight mass spectrometry, which are described in the figure.

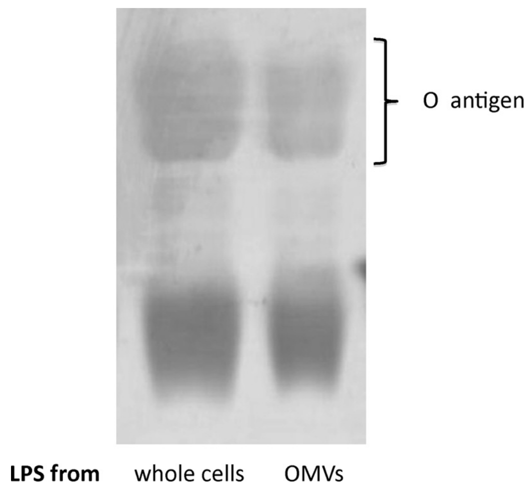

Fig. 2. Lipopolysaccharide extraction from OMV and its SDS-PAGE profiles. LPS was visualized by the BioRad silver-staining technique.

were recently described in other Gram-negative bacteria as having roles in pathogenicity [28] and immunogenicity [29].

As expected, the profiles of *B. parapertussis* LPS present in the OMVs preparation has an O-Ag structure consisting of a homopolymer of 2,3-dideoxy-2,3-di-N-acetylgalactosaminuronic acid (Fig. 2). This molecule was described as having a penta-acylated lipid A [30–32].

Protection against intranasal *B. parapertussis* challenge after vaccination with OMVs obtained from *B. parapertussis*.

To evaluate the protection capacity against *B. parapertussis* infection induced by the OMVs prepared from *B. parapertussis* (OMVsBpp), animal assays using intranasal *B. parapertussis* challenge were performed. In particular, the effect on subsequent colonization by *B. parapertussis* (10^7 CFU 40 μl^{-1}) after two administrations of OMVsBpp was analyzed. These results were compared with those obtained in mice immunized with commercial aP vaccine formulated using antigens from *B. pertussis* (Fig. 3). Mice immunized with OMVs obtained from *B. pe*rtussis Tohama phase I strain were also included in the experiment (OMVsBp). All OMVs used as vaccines were detoxified by formalin treatment. Animals vaccinated twice with PBS were used as negative control. Significant differences in lung bacterial counts between OMVsBpp immunized animals and control group were observed ($p < 0.001$, Fig. 3). In mice immunized with OMVsBpp vaccine the number of lung recovered colonies at day 7-post challenge dropped below to the detection limit of the assay, which means a difference of at least 3 orders of magnitude in relation to the counting in non-immunized mice (Fig. 3) where more than 10^4 CFU per lung were recovered. The significant differences in lung bacterial counts between OMVsBpp immunized animals and control group were also observed at day 5-post challenge (more than 2 logs difference $p < 0.001$, not shown).

The magnitude of protection against *B. parapertussis* induced by the vaccine containing the OMVsBpp was not achieved by immunization with either the commercial aP or the OMVsBp vaccines.

Protection against intranasal *B. pertussis* challenge after vaccination with OMVs obtained from *B. parapertussis*.

To evaluate the protection capacity against *B. pertussis* infection induced by the vaccines containing the OMVsBpp, animal assays using intranasal *B. pertussis* challenge were performed. In particular, the effect on subsequent colonization by *B. pertussis* 18323 strain (2×10^7 CFU per 40 μl) after two administrations of OMVsBpp was analyzed. The results were compared with those obtained in mice immunized with the OMVs obtained from *B. pertussis* Tohama phase I strain. All OMVs used as vaccines were detoxified by formalin treatment. While commercial *B. pertussis* acellular vaccine (Tdap) in high dose was used as a positive control, treatment with PBS was used as negative control. Significant differences in lung bacterial counts between immunized animals and negative control group were observed ($p < 0.001$, Fig. 4).

In concordance with our previous studies, adequate elimination rates were observed in mice immunized with OMVsBp vaccine [21,22]. Interestingly, similar levels of protection against *B. pertussis* challenge were also achieved with OMVsBpp immunization. As expected in mice immunized with commercial Tdap vaccine the number of lung-recovered colonies at day 7-post challenge

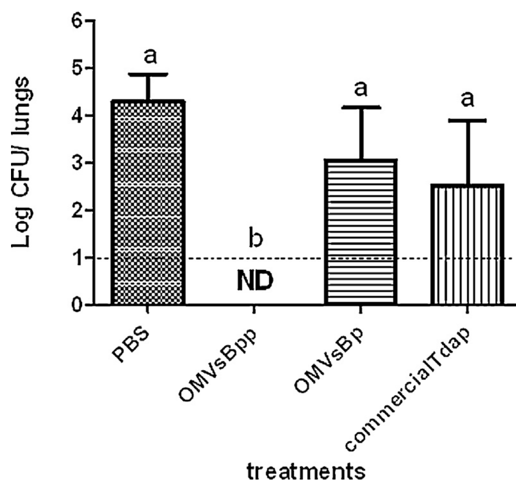

Fig. 3. Lung clearance efficiency of *B. parapertussis* OMVs (3 μg per dose) in intransal challenge mice model. *B. parapertussis* AR729 was used as challenge bacteria (1×107 CFU 40 μl^{-1}). Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of five mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log ± SD. ND not detected. Different letters were used to indicate statistical differences with $p < 0.001$. No differences were observed between conditions marked with the same letter. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

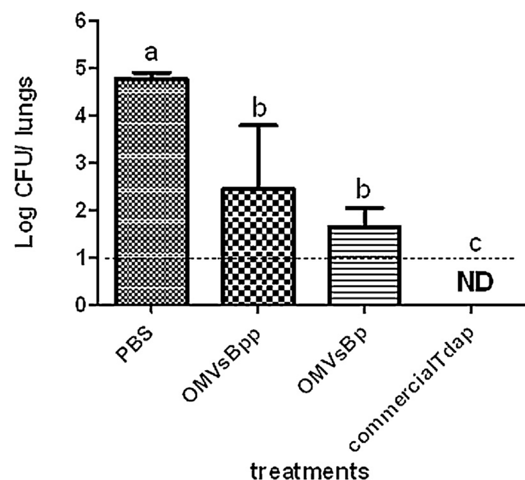

Fig. 4. Lung clearance efficiency of *B. parapertussis* OMVs (3 μg per dose) in intransal challenge mice model. *B. pertussis* 18323 was used as challenge bacteria (2×107 CFU 40 μl^{-1}). Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of five mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log ± SD. ND not detected. Different letters were used to indicate statistical differences with $p < 0.001$. No differences were observed between conditions marked with the same letter. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

dropped at least 4 orders of magnitude in relation to the counting in non-immunized mice (Fig. 4). In mice immunized with PBS the number of recovered colonies from lungs was much higher (10^5 CFU per lung). Similar results were obtained when mice were challenged with *B. pertussis* Tohama phase I strain instead of *B. pertussis* 18323 (Supplementary Fig. 1).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.08.059.

4.2. Protective capacity of combined acellular vaccines containing OMVs from Bordetella

The protection capacity against *B. parapertussis* or *B. pertussis* infection induced by the combined vaccine was evaluated in animal assays using intranasal bacterial challenge. In particular, colonization by *B. parapertussis* (1×10^7 CFU per 40 μl^{-1}) or *B. pertussis* (2×10^7 CFU 40 μl^{-1}) after two administrations of combined OMVs vaccines. All the immunogens 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 named here TdapOMVsBpp, TdapOMVsBp or Tdap(OMVsBp + OMVsBpp). Animals vaccinated twice with PBS were used as negative control.

Significant differences in lung *B. parapertussis* bacterial counts between immunized animals with TdapOMVsBpp or Tdap(OMVsBp + OMVsBpp) and control group were observed ($p < 0.001$, Fig. 5). TdapOMVsBp offered little protection against *B. parapertussis*.

Adequate elimination rates ($p < 0.001$) were also observed when mice were challenged with *B. pertussis* 18323 and immunized with TdapOMVsBpp, TdapOMVsBp or Tdap (OMVsBp + OMVsBpp) vaccines. In mice immunized with TdapOMVsBpp treatment the number of lung recovered colonies at day 7-post challenge dropped at least 3 orders of magnitude in relation to the counting in non-immunized mice (Fig. 6). In mice immunized with PBS the number of recovered colonies from lungs was high (10^5 CFU per lung).

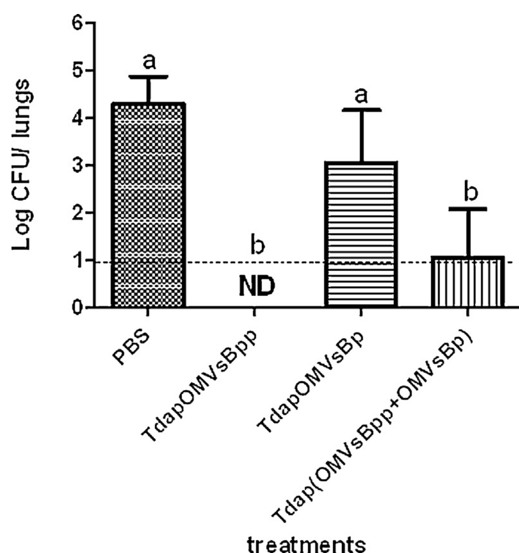

Fig. 5. Lung clearance efficiency of Tdap vaccines containing OMVs from Bordetella in intransal challenge mice model. *B. parapertussis* AR729 was used as challenge bacteria (1×107 CFU 40 μl^{-1}). Results from one representative experiment are shown. Results depicted are means of five mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log ± SD. ND not detected. Different letters were used to indicate statistical differences with $p < 0.001$. No differences were observed between conditions marked with the same letter. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

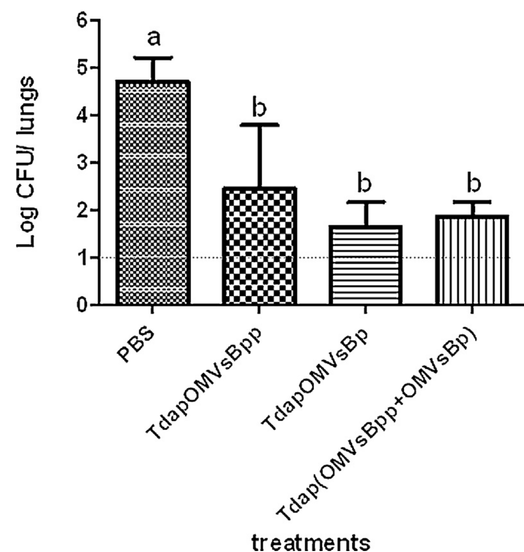

Fig. 6. Lung clearance efficiency of Tdap vaccines containing OMVs from Bordetella in intransal challenge mice model. *B. pertussis* was used as challenge bacteria (2×107 CFU 40 μl^{-1}). Results from one representative experiment are shown. Results depicted are means of five mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log ± SD. Different letters were used to indicate statistical differences with $p < 0.001$. No differences were observed between conditions marked with the same letter. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

5. Safety of the acellular vaccines containing the OMVs

5.1. Mouse weight gain test

The mouse weight gain test is usually employed to measure the toxicity of pertussis formulations. Mice were weighed 16 h, 3 and 7 days after immunization. All the formulations here used for vaccination resulted in no alteration in the weight gain curve, meaning that the formulations were non toxic.

5.2. 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 [33]. In our case, formulations containing OMVsBpp induced levels of IL-6 comparable to the PBS or the commercial Tdap treatment (Fig. 7). Moreover Tdap OMVsBpp vaccine induced lower levels of IL-6 (approx. 2 fold less) than formulations containing OMVsBp (Fig. 7). This may be related to the lower pro-inflammatory capacity of LPS from *B. parapertussis* compared with LOS from *B. pertussis* [34].

6. Discussion

The *B. pertussis* and *B. parapertussis* epidemiological data encourages the design of new vaccines that achieve better protection levels against both *Bordetella* species. In line with this idea different formulations that induce protection against both species were already proposed: the live attenuated *B. pertussis* nasal vaccines [35], whole cell vaccines containing both *B. pertussis* and *B. parapertussis* [36], or supplementation of aP vaccines with *B. parapertussis* protective antigens [37]. Here we present a novel acellular vaccine candidate, the outer membrane vesicles derived from *B. parapertussis* that not only exhibits protective capacity against both species of *Bordetella* but also has an important biotechnological advantage

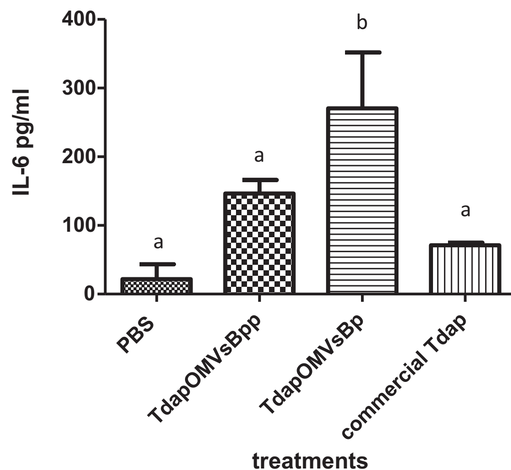

Fig. 7. Levels of serum IL-6 after immunization. Four hours after immunization serum was collected and IL6 was measured by ELISA. Results show mean and standard deviations of individual measurements. Control group was treated with PBS. Different letters were used to indicate statistical differences with $p < 0.001$. No differences were observed between conditions marked with the same letter. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

over purified immunoprotective proteins. The OMVs isolation procedure consists in a protocol of simple steps that avoid the need for costly infrastructure to support the purification of each of the antigens included in the current acellular pertussis vaccines.

As described in our previous work, vesicle formation from cell pellets could be induced by sonication [20–22]. Following this methodology, OMVs from *B. parapertussis* AR729 strain were obtained. In electron microscopic observations (Fig. 1), OMVs from *B. parapertussis* strain were visible as nanosphere vesicles with variable sizes ranging from 50–150 nm in diameter, which are similar to OMVs obtained from other *Bordetella* strains [20–22]. The protein profiles of these OMVs analyzed by 2D gel electrophoresis showed the presence of well-known surface immunogens such as PRN, among several protein components. The wide spectrum of antigens that constitute the OMVs is an additional advantage of this kind of formulation over the current acellular vaccines, which contain only a few bacterial proteins because of recently observed vaccine-induced antigenic shifts and adaptations. Such concerns are heightened by the recent discovery of circulating strains of *B. pertussis* and also *B. parapertussis* that are either deficient in antigens included in the acellular vaccines, [38,39] or present alterations in the ptx promoter that result in increased production of pertussis toxin [40]. In addition, the OMVs contain several innate immune response activating components such as outer membrane proteins (OMPs) and lipopolysaccharide (LPS) that exert adjuvant capacity [41]. Moreover, the presence of the *B. parapertussis* LPS in the OMVs seems to be a critical feature for conferring protection against *B. parapertussis* [37].

We carried out at least 10 independent replicates of the OMVsBpp isolation and characterization procedure. In all cases similar morphology, size distribution and presence of surface immunogens were observed, indicating the relative robustness of the OMVs isolation procedure.

The protective capacity induced by the OMVs prepared from *B. parapertussis*, was here demonstrated using the intranasal bacterial challenge mouse model. The magnitude of protection against *B. parapertussis* achieved with the OMVsBpp systemic immunization was neither observed with commercial acellular pertussis vaccine nor with OMVs obtained from *B. pertussis* Tohama phase I strain. While in mice immunized with those pertussis vaccines the number of recovered colonies from lungs was high (approx. 10^3 CFU per lung), in mice immunized with OMVsBpp (3 μg), the number of lung recovered colonies of *B. pertussis* at day 7-post challenge dropped at least 4 orders of magnitude in relation to the counting in non immunized mice (Fig. 3). The data presented here support the use of OMVs derived from *B. parapertussis* strain to clear efficiently *B. parapertussis* from the lungs of mice.

Another attractive feature is the protective capacity exhibited by the OMVsBpp against *B. pertussis* infection. Protection against *B. pertussis* induced by OMVsBpp was comparable to that induced by two injections of our previous designed vaccine consisting in OMVs derived from *B. pertussis*. As expected the commercial pertussis acellular vaccine used in high dose as positive control offered good protection level against *B. pertussis*. Significant differences in lung bacterial counts between all immunized animals and control group were observed ($p < 0.001$, Fig. 4). The results obtained with OMVsBpp was not anticipated, since so far there were no reports of *B. parapertussis* derived vaccines that confer efficient protection against *B. pertussis*. Komatsu et al. have tested a whole cell *B. parapertussis* vaccine that confers good protection against *B. parapertussis* challenge, but is not protective against *B. pertussis* challenge [42].

Regarding cross protection, only recently a novel live attenuated whole cell pertussis vaccine BPZE1 developed by Locht s group seems to protects in mouse model against both *B. pertussis* and *B. parapertussis* infections [17]. This interesting result is in agreement with the observation that in the mouse model reciprocal protection was observed after infection with *B. pertussis* or *B. parapertussis* [43].

We also showed here that the protective capacity of OMVsBpp against both *B. parapertussis* and *B. pertussis* was maintained when the vesicles were formulated as combined vaccine with tetanus and diphtheria toxoids (Figs. 5 and 6), making them suitable for application in the usual combined formulations.

The mouse weight gain test widely used to measure the toxicity of pertussis formulations showed that the vaccines containing OMVsBpp resulted in no alteration in the weight gain curve, meaning that all formulations were non toxic. In the same line, early production of the pro-inflammatory IL-6 after OMVBpp vaccination was low. Although there is LPS present in the OMV formulation, in the case of *B. parapertussis*, this molecule has been shown to be a very weak agonist of TLR4-dependent response [34,44].

Altogether, data presented here supports the use of OMVs obtained from *B. parapertussis* strain as a good and safe formulation to improve the control of whooping cough caused by both *B. pertussis* and *B. parapertussis*.

Acknowledgements

This work was supported by ANCPyT and CICBA (Argentina) grants to DFH and MR. DFH is a member of the Scientific Career of CICBA. MR and GM are members of the Scientific Career of CONICET, DB and MEG are supported by CICBA. AE and EZ have fellowships from CONICET.

References

[1] Guiso N. Whooping cough from infants to adults. Bull Acad Natl Med 2008;192:1437–49 (discussion 49–52).

[2] von Konig CH, Halperin S, Riffelmann M, Guiso N. Pertussis of adults and infants. Lancet Infect Dis 2002;2:744–50.

[3] Galanis E, King AS, Varughese P, Halperin SA. Changing epidemiology and emerging risk groups for pertussis. CMAJ 2006;174:451–2.

[4] WHO. Immunization surveillance, assessment and monitoring; 2008 http://www.who.int/immunization_monitoring/diseases/pertussis/en/

[5] Watanabe M, Nagai M. Whooping cough due to *Bordetella parapertussis*: an unresolved problem. Expert Rev Anti Infect Ther 2004;2:447–54.

[6] Heininger U, Stehr K, Schmitt-Grohe S, Lorenz C, Rost R, Christenson PD, et al. Clinical characteristics of illness caused by *Bordetella parapertussis* compared with illness caused by *Bordetella pertussis*. Pediatr Infect Dis J 1994;13:306–9.

[7] Cherry JD, Seaton BL. Patterns of *Bordetella parapertussis* respiratory illnesses: 2008–2010. Clin Infect Dis 2012;54:534–7.

[8] Liese JG, Renner C, Stojanov S, Belohradsky BH. Clinical and epidemiological picture of *B. pertussis* and *B. parapertussis* infections after introduction of acellular pertussis vaccines. Arch Dis Child 2003;88:684–7.
[9] Mastrantonio P, Stefanelli P, Giuliano M. *Bordetella parapertussis* infection in children: epidemiology, clinical symptoms, and molecular characteristics of isolates. J Clin Microbiol 1998;36:999–1002.
[10] Bergfors E, Trollfors B, Taranger J, Lagergard T, Sundh V, Zackrisson G. Parapertussis and pertussis: differences and similarities in incidence, clinical course, and antibody responses. Int J Infect Dis 1999;3:140–6.
[11] Stehr K, Cherry JD, Heininger U, Schmitt-Grohe S, Uberall M, Laussucq S, et al. A comparative efficacy trial in Germany in infants who received either the Lederle/Takeda acellular pertussis component DTP (DTaP) vaccine, the Lederle whole-cell component DTP vaccine, or DT vaccine. Pediatrics 1998;101:1–11.
[12] He Q, Viljanen MK, Arvilommi H, Aittanen B, Mertsola J. Whooping cough caused by *Bordetella pertussis* and *Bordetella parapertussis* in an immunized population. JAMA 1998;280:635–7.
[13] Kurova N, Njamkepo E, Brun D, Tseneva G, Guiso N. Monitoring of *Bordetella* isolates circulating in Saint Petersburg, Russia between 2001 and 2009. Res Microbiol 2010;161:810–5.
[14] Bokhari H, Said F, Syed MA, Mughal A, Kazi YF, Heuvelman K, et al. Whooping cough in Pakistan: *Bordetella pertussis* vs *Bordetella parapertussis* in 2005–2009. Scand J Infect Dis 2011;43:818–20.
[15] Long GH, Karanikas AT, Harvill ET, Read AF, Hudson PJ. Acellular pertussis vaccination facilitates *Bordetella parapertussis* infection in a rodent model of bordetellosis. Proc Biol Sci 2010;277:2017–25.
[16] Khelef N, Danve B, Quentin-Millet MJ, Guiso N. *Bordetella pertussis* and *Bordetella parapertussis*: two immunologically distinct species. Infect Immun 1993;61:486–90.
[17] Mielcarek N, Debrie AS, Raze D, Bertout J, Rouanet C, Younes AB, et al. Live attenuated *B.* pertussis as a single-dose nasal vaccine against whooping cough. PLoS Pathog 2006;2:e65.
[18] WHO-World Health Organization. Expert committee on biological standardization WHO/BS/2011.2158 english only; 2011. p. 1–64.
[19] Stainer DW, Scholte MJ. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J Gen Microbiol 1970;63:211–20.
[20] Hozbor D, Rodriguez ME, Fernandez J, Lagares A, Guiso N, Yantorno O. Release of outer membrane vesicles from *Bordetella pertussis*. Curr Microbiol 1999;38:273–8.
[21] Roberts R, Moreno G, Bottero D, Gaillard ME, Fingermann M, Graieb A, et al. Outer membrane vesicles as acellular vaccine against pertussis. Vaccine 2008;26:4639–46.
[22] Asensio CJ, Gaillard ME, Moreno G, Bottero D, Zurita E, Rumbo M, et al. Outer membrane vesicles obtained from *Bordetella pertussis* Tohama expressing the lipid A deacylase PagL as a novel acellular vaccine candidate. Vaccine 2010;29:1649–56.
[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[24] Bottero D, Gaillard ME, Basile LA, Fritz M, Hozbor DF. Genotypic and phenotypic characterization of *Bordetella pertussis* strains used in different vaccine formulations in Latin America. J Appl Microbiol 2012;112:1266–76.
[25] Bottero D, Gaillard ME, Fingermann M, Weltman G, Fernandez J, Sisti F, et al. Pulsed-field gel electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical *Bordetella pertussis* strains. Clin Vaccine Immunol 2007;14:1490–8.
[26] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[27] Ferrari G, Garaguso I, Adu-Bobie J, Doro F, Taddei AR, Biolchi A, et al. Outer membrane vesicles from group B *Neisseria meningitidis* delta *gna33* mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. Proteomics 2006;6:1856–66.
[28] Delvecchio VG, Connolly JP, Alefantis TG, Walz A, Quan MA, Patra G, et al. Proteomic profiling and identification of immunodominant spore antigens of *Bacillus anthracis Bacillus cereus*, and *Bacillus thuringiensis*. Appl Environ Microbiol 2006;72:6355–63.
[29] Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca Jr S, Geary SJ. Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (lpd), in Mycoplasma gallisepticum through in vivo screening of transposon mutants. Infect Immun 2006;74:931–9.
[30] Caroff M, Aussel L, Zarrouk H, Martin A, Richards JC, Therisod H, et al. Structural variability and originality of the *Bordetella* endotoxins. J Endotoxin Res 2001;7:63–8.
[31] Di Fabio JL, Caroff M, Karibian D, Richards JC, Perry MB. Characterization of the common antigenic lipopolysaccharide O-chains produced by *Bordetella bronchiseptica* and *Bordetella parapertussis*. FEMS Microbiol Lett 1992;76:275–81.
[32] Wolfe DN, Goebel EM, Bjornstad ON, Restif O, Harvill ET. The O antigen enables *Bordetella parapertussis* to avoid *Bordetella pertussis*-induced immunity. Infect Immun 2007;75:4972–9.
[33] Geurtsen J, Banus HA, Gremmer ER, Ferguson H, de la Fonteyne-Blankestijn LJ, Vermeulen JP, et al. Lipopolysaccharide analogs improve efficacy of acellular pertussis vaccine and reduce type I hypersensitivity in mice. Clin Vaccine Immunol 2007;14:821–9.
[34] Wolfe DN, Buboltz AM, Harvill ET. Inefficient Toll-like receptor-4 stimulation enables *Bordetella parapertussis* to avoid host F immunity. PLoS ONE 2009;4:e4280.
[35] Mielcarek N, Debrie AS, Raze D, Quatannens J, Engle J, Goldman WE, et al. Attenuated *Bordetella pertussis*: new live vaccines for intranasal immunisation. Vaccine 2006;24(Suppl. 2):S2–54.
[36] Burian V, Kubatova E, Kratochvilova M, Burianova-Vysoka B. New combined alum hydroxide adsorbed diphtheria-tetanus-pertussis-vaccine with parapertussis component I. Reactogenicity and serological efficacy. J Hyg Epidemiol Microbiol Immunol 1970;14:143–7.
[37] Zhang X, Goebel EM, Rodriguez ME, Preston A, Harvill ET. The O antigen is a critical antigen for the development of a protective immune response to *Bordetella parapertussis*. Infect Immun 2009;77:5050–8.
[38] Bouchez V, Brun D, Cantinelli T, Dore G, Njamkepo E, Guiso N. First report and detailed characterization of *B. pertussis* isolates not expressing pertussis toxin or pertactin. Vaccine 2009;27:6034–41.
[39] Hegerle N, Paris AS, Brun D, Dore G, Njamkepo E, Guillot S, et al. Evolution of French *Bordetella pertussis* and *Bordetella parapertussis* isolates: increase of Bordetellae not expressing pertactin. Clin Microbiol Infect 2012;18:E340–6.
[40] Mooi FR, van Loo IH, van Gent M, He Q, Bart MJ, Heuvelman KJ, et al. *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 2009;15:1206–13.
[41] Moshiri A, Dashtbani-Roozbehani A, Najar Peerayeh S, Siadat SD. Outer membrane vesicle: a macromolecule with multifunctional activity. Hum Vaccin Immunother 2012;8:953–5.
[42] Komatsu E, Yamaguchi F, Eguchi M, Watanabe M. Protective effects of vaccines against *Bordetella parapertussis* in a mouse intranasal challenge model. Vaccine 2010;28:4362–8.
[43] Watanabe M, Nagai M. Reciprocal protective immunity against *Bordetella pertussis* and *Bordetella parapertussis* in a murine model of respiratory infection. Infect Immun 2001;69:6981–6.
[44] Mann PB, Wolfe D, Latz E, Golenbock D, Preston A, Harvill ET. Comparative toll-like receptor 4-mediated innate host defense to Bordetella infection. Infect Immun 2005;73:8144–52.