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Bordetella pertussis and outer membrane vesicles

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ABSTRACT

Bordetella pertussis is the causative agent of a respiratory infection called pertussis (whooping cough) that can be fatal in newborns and infants. The pathogen produces a variety of antigenic compounds which alone or simultaneously can damage various host cells. Despite the availability of pertussis vaccines and high vaccination coverage around the world, a resurgence of the disease has been observed in many countries. Reasons for the increase in pertussis cases may include increased awareness, improved diagnostic techniques, low vaccine efficacy, especially acellular vaccines, and waning immunity. Many efforts have been made to develop more effective strategies to fight against *B. pertussis* and one of the strategies is the use of outer membrane vesicles (OMVs) in vaccine formulations. OMVs are attracting great interest as vaccine platforms since they can carry immunogenic structures such as toxins and LPS. Many studies have been carried out with OMVs from different *B. pertussis* strains and they revealed promising results in the animal challenge and human preclinical model. However, the composition of OMVs differs in terms of isolation and purification methods, strains, culture, and stress conditions. Although the vesicles from *B. pertussis* represent an attractive pertussis vaccine candidate, further studies are needed to advance clinical research for next-generation pertussis vaccines. This review summarizes general information about pertussis, the history of vaccines against the disease, and the immune response to these vaccines, with a focus on OMVs. We discuss progress in developing an OMV-based pertussis vaccine platform and highlight successful applications as well as potential challenges and gaps.

KEYWORDS

Bordetella pertussis; outer membrane vesicle; acellular pertussis vaccine; whole-cell pertussis vaccine

1. Introduction

Bordetella pertussis is a non-sporulating, aerobic, Gram-negative coccobacillus that can grow rapidly by attaching to the epithelial surfaces of the trachea and bronchi in the human respiratory system [1]. This pathogen is the causative agent of whooping cough (pertussis), which is an acute respiratory disease with a severe course, mostly seen in newborns and young children, more recently, in adolescents and adults. The disease is characterized by choking-like breaths after severe coughing attacks followed by vomiting [2,3]. This disease, which can cause brain damage due to oxygen deprivation and is fatal, especially in infants younger than one year of age, was known to be one of the main causes of infant morbidity and mortality before the development of the pertussis vaccine [4]. The incidence of the disease was significantly reduced with the Expanded Programme on Immunization (EPI) initiated in 1974 and the National Vaccination Campaigns started in the 1980s [5,6], but widespread studies in recent years have revealed that pertussis cases started to increase again [7–9]. According to World Health Organization (WHO), more than 151,000 cases of pertussis were recorded worldwide in 2018 [10]. About 42,000 cases were reported in Europe in 2017, with five countries (Germany, the Netherlands, Poland, Spain, and the United Kingdom) accounting for 76% of all case reports. Patients aged 14 years and older in particular were found to account for 67% of the total incidence of the disease [11]. Since pertussis is an endemic disease with an epidemic peak every 3–5 years and frequent outbreaks, adolescents and adults are now included in immunization practices [12]. As a matter of fact, in recent years there has been a shift in the profile of age groups where the disease is common, from infants and young children to adolescents and adults [2,9]. The most important reasons for the reemergence of pertussis are a decrease in the effectiveness of the vaccines resulting in waning vaccine-induced immunity over time, genetic changes in circulating strains, improved diagnosis of the disease due to the development of molecular techniques, and a corresponding increase in awareness of the infection in society [13–15]. It is very important to highlight two important issues here: First, the immune response elicited by the acellular pertussis vaccine (aP) used in most countries is less robust than that elicited by the whole-cell pertussis vaccine (wP) because these two vaccines elicit a different Th response [16]. In addition, although aP can provide protection against the disease, this protection quickly wears off and does not prevent colonization or transmission of the pathogen [17–19]. Second, changes in the genotypic characteristics and antigenic structure of circulating *B. pertussis*

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strains have also been described, leading researchers to question the protection provided by commercial vaccines. Vaccine antigen alleles expressed by circulating bacteria differ significantly from those expressed by strains used in vaccine production [20]. There have been reports of the emergence of strains that do not express one or more components of pertussis vaccine, particularly pertactin, a membrane-bound transporter, and an increase in pertactin-deficient strains causing pertussis has been observed in several countries [21–26]. As suggested by Rumbo and Hozbor (2014) [16], the observation that pertactin-deficient isolates have been detected only in regions where aP vaccines have been used may suggest that aP vaccination has led to the spread of strains that have a selection advantage in vaccinated human populations.

2. Whooping cough (pertussis)

After the nasopharyngeal colonization by *B. pertussis*, incubation lasts 9–10 days, and after this period, the clinical findings specific to pertussis appear [27]. There are three clinically distinct stages of the disease: the catarrhal stage (flu-like period), the paroxysmal stage (spasmodic cough period), and the convalescent stage (recovery period). The catarrhal stage is the stage when the bacteria colonize, and it is characterized by cough, runny nose, low fever, and chills. In this stage, which lasts about 1–2 weeks, the risk of transmission of the disease through particles produced by saliva and sneezing is highest [28]. The paroxysmal stage is characterized by a disease-specific deep breathing sound followed by spasmodic coughing attacks. During these attacks, facial bruising may occur due to insufficient breathing, and nausea and vomiting may also develop at the end of the attack [29,30]. It lasts approximately 2–4 weeks and during this period, *B. pertussis* cells are rarely found in cultures, and antimicrobial agents cannot affect the course of the disease [31,32]. It is known that the symptoms and some complications during this period are associated with the effect of well-known toxins belonging to the bacterium. While lymphocytosis begins at the end of the catarrhal phase, leukocytosis reaches its peak during the paroxysmal phase [30]. The convalescent phase lasts 1–3 weeks, and the symptoms specific to the disease gradually decrease during this period. Serious complications such as bronchopneumonia and acute encephalopathy, which can sometimes be fatal, may occur. Hemorrhages and hernias may appear in the intraocular, conjunctiva, skin, and mucous membranes with the force of coughing [27,30].

Nasopharyngeal culture and polymerase chain reaction (PCR) can provide laboratory confirmation of pertussis diagnosis. However, this difficult and slow-growing microorganism requires special culture media to grow, and cultures are usually positive only after 3 to 7 days [30]. In adults, cultures are often negative (96%) until the diagnosis is suspected, and the overall culture sensitivity is only between 20% and 40%. PCR is more sensitive and specific than culture but is not commonly performed [33,34]. Pertussis treatment is largely supportive, including oxygen, aspiration, hydration, and avoidance of respiratory irritants. Parenteral nutrition may be necessary as the disease tends to have a prolonged course. Hospitalization is indicated in patients with overlapping pneumonia, hypoxia, central nervous system complications, or who cannot tolerate oral nutrition and hydration [35]. Patients younger than one year of age are at the greatest risk for morbidity and mortality as they are not yet fully vaccinated and should be hospitalized regardless of symptoms. Newborns should be admitted to the intensive care unit because life-threatening cardiopulmonary complications and unexpected cardiac arrest may occur [35]. Although treatment with antibiotics does not significantly shorten the duration of the disease, antibiotics can limit the severity of the infection when treatment begins in the catarrhal phase. Antibiotics have not been proven to be more effective when used in the paroxysmal phase, but they may reduce the transmission and spread of the disease [32]. Erythromycin is the first-line treatment for pertussis and other antibiotics such as azithromycin and clarithromycin are alternative treatment options. Trimethoprim-sulfamethoxazole has been used as an alternative in patients with macrolide allergy, but its efficacy has not been proven yet [30]. In patients receiving antibiotic treatment, isolation should be continued for at least 5 days after initiation of treatment. Post-exposure prophylaxis with erythromycin is recommended for all household contacts [35].

3. Virulence factors of *Bordetella pertussis*

Pertussis is caused by the coordinated interaction of the virulence factors of *B. pertussis*, whose expression is mainly controlled by the regulatory signal transduction system called BvgAS [36]. BvgA is a DNA-binding response regulator while BvgS is a transmembrane sensor kinase that responds to the environment. For a long time, the system was considered as a molecular on-off switch between two distinct phases, called Bvg+ and Bvg-. However, it was revealed that BvgAS acts a rheostat and directly or indirectly regulates hundreds of genes in different ways, resulting in at least three distinct phenotypic phases (Bvg+, Bvg-, and Bvg^{i}) (Figure 1) [38–40]. Research shows that conformational changes of BvgA within its periplasmic Venus flytrap domains are responsible for the transition between these modes (VTF1 and VTF2) [41,42]. Studies have

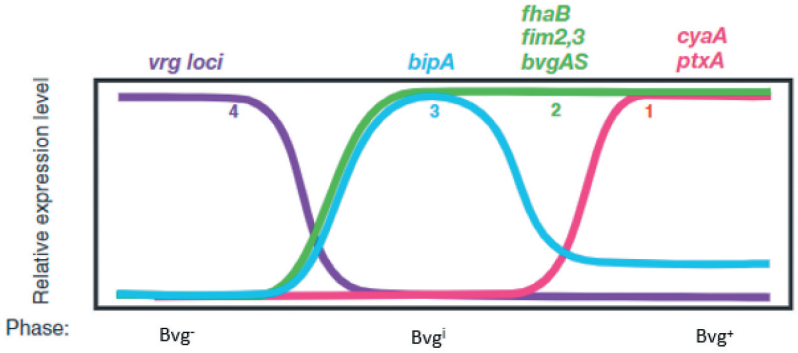

Figure 1. The BvgAS signal transduction system and temporal gene regulation (Adapted from [37]).

revealed that expression of *bvgAS* can be activated (Bvg+) when *B. pertussis* is grown at 37°C in the absence of $MgSO_4$ or nicotinic acid while exposure to certain modulators such as $MgSO_4$, low temperature, and nicotinic acid induces the transition to Bvg- phase [36]. When the pathogen is grown in the presence of low concentration of modulators, an intermediate phase (Bvg^i) is observed. Moreover, Bvg^i phase can also occur within the first few hours of switching from Bvg- to Bvg+ [43]. In Bvg+ phase, signaling inputs are detected through BvgS, resulting in the phosphorylation of BvgA that activates maximal expression of virulence-activated genes (*vags*) which are required for pathogenesis and colonization [44]. Bvg+ phase is also characterized by the lack of expression of BvgAS-repressed genes (*vrgs*). During this process, not all promoters are activated simultaneously, so some genes such as *fha* are expressed early while others such as *ptx* and *cya* are activated later [40,45]. Under Bvg- conditions, the activity of BvgS is reduced and BvgA remains unphosphorylated, which in turn causes maximal expression of *vrgs*. A recent study revealed new genes that play roles in metabolic pathways and are significantly upregulated in the Bvg- phase, suggesting a possible role of this phase in bacterial survival and transmission [44]. In Bvg^i phase, the expression of adhesins and the absence of toxins have been observed, as well as the expression of some highly immunogenic and surface-exposed factors like an outer membrane protein named BipA [46]. Although the Bvg^i phase is commonly characterized by the presence of some *vags* and the absence of Bvg-repressed genes, there is still a void of information about the potential of this phase that requires further investigation.

During disease, filamentous hemagglutinin (FHA) and fimbriae (FIM) allow bacteria to attach to ciliated epithelial cells in the trachea, while pertactin (PRN), serum resistance protein (BrkA), and tracheal colonization factor (TCF) act as autotransporters [47]. The toxins that cause damage to ciliated epithelial cells and alveolar macrophages, followed by lymphocytosis, are pertussis toxin (PT), adenylate cyclase-hemolysin (AC-Hly), tracheal cytotoxin (TCT), and lipooligosaccharide (LOS) layer.

FHA, located on the surface of the cell, is a filamentous and highly immunogenic protein with a molecular weight of 220 kDa and a β-helix structure. FHA, which is involved in the attachment of the microorganism to the upper respiratory tract and the initiation of colonization, can self-aggregate and this protein has no known toxic effect. PRN, a 69 kDa membrane protein, also allows the bacterium to attach to the host cell [48]. FIM is a 5–6 nm long, helical protein containing two complete turns and five repeat units and plays a role in colonization. *B. pertussis* produces two types of fimbriae, serotype 2 and serotype 3. These serotypes are mainly composed of Fim2 (22.5 kDa) and Fim3 (22 kDa) units. In addition to these, there is another 40 kDa unit called FimD in the fimbriae that acts as an adhesin [48,49]. PT consists of five different subunits called S1-S5 with a total molecular weight of 117 kDa and is an important virulence factor in the colonization step. PT is found in the extracellular fluid as well as on the bacterial cell during *in vivo* and *in vitro* growth of *B. pertussis*. PT consists of an A component with biological activity (ADP-ribosyl transferase activity) and a B component that provides binding to the host cell membrane. The B component is responsible for the interaction of the toxin with the target cell receptors while the S1 unit, called the A component, has an enzymatic effect and is responsible for the biological activity of the toxin. Therefore, the toxin is classified in the A-B type bacterial toxin group [48,50]. AC-Hly plays a role in bacterial invasion of mammalian cells, and it exhibits hemolytic and cytotoxic activities [51]. Dermonecrotic toxin (DNT) which is the first *B. pertussis* toxin discovered causes tissue damage in the respiratory tract by interacting with TCT and LOS. It causes polynucleation by stimulating DNA and protein synthesis without cell division. It also inhibits Na±K+ ATPase activity, resulting in the depolarization of muscle fibers [48]. TCT damages ciliated cells in the respiratory tract and *in vitro* studies reveal that it inhibits DNA synthesis in tracheal epithelial cells and causes the production of interleukin-1 and

nitric oxide. *B. pertussis* LOS lacks O-antigen and consists of two types of lipid structures called A and X. Lipid A of *B. pertussis* LOS is nontoxic but has many biological activities, acting in synergy with other toxins [48]. BrkA enables *Bordetella* to acquire resistance to host serum by reducing C3 and C4 deposition on the bacterial surface, although the exact mechanism is not yet known [52]. TCF is responsible for the colonization of the pathogen in tracheal cells through an Arg-Gly-Asp motif allowing it to attach to host integrins, and it is both cell-associated and secreted [28,48]. In summary, *B. pertussis* is a microorganism that has the necessary equipment to attach to the cell, escape the host's protection mechanism, and damage the host's respiratory tract [53].

Despite the rapid developments in *B. pertussis* research, especially in the last 20 years, not much is known about its pathogenesis. For instance, while some researchers have suggested that TCT may be responsible for the paroxysmal cough, the underlying mechanism has not been clearly defined. In addition, the interactions of virulence factors such as PT, LOS, and TCT, that determine the clinical progression and spectrum of the disease in different age groups, and the synergistic effect resulting from these interactions need to be studied more [43,54]. Further studies on the pathogenesis of *B. pertussis* may provide the basis for the design of novel antimicrobial agents that interfere with the newly identified virulence mechanisms.

4. Pertussis vaccines

Especially with the worldwide vaccination process against pertussis in the 1940s, various whole-cell and acellular vaccine compositions against the disease have been developed to date. The effort to create an effective vaccination against pertussis started in the 1910s after Bordet-Gengou was successful in growing *B. pertussis* as a single colony on the medium [55]. The first pertussis vaccine was licensed in the United States in 1914 and contained a suspension of heat- or formalin-killed *B. pertussis* cells (wP) combined with diphtheria and tetanus toxoids (DTwP) [56]. To enhance its effect, the vaccine was enriched with aluminum hydroxide as an adjuvant. As a result of these vaccination processes, a significant decrease in the incidence of the disease was observed. Side effects of the wP vaccine include fever, headache, high-pitched crying, hyperirritation, pain at the injection site, nausea, erythema, and swelling. However, a small percentage of vaccinated children may also experience more serious adverse effects such as convulsions and brain damage [13]. This has led to interruptions in the pertussis vaccination program in some countries and difficulties in immunization programs. In fact, pertussis outbreaks have been observed in countries such as Japan and England, with the rejection of vaccination in the 1970s due to the aforementioned side effects [57,58]. At this point, it is worth recalling that the WHO position paper on pertussis vaccines provides excellent safety data for wP vaccines, and the studies have shown no association between wP vaccines and infant deaths [6,58]. Today, these wP vaccines are still used in some low- or low-middle-income countries [59] (Figure 2). Although the wP vaccine is usually combined with diphtheria toxoid and tetanus toxoid in these countries, some vaccines are also combined with other vaccines routinely administered in infancy, such as *Haemophilus influenzae* type b (Hib), hepatitis B (HBV), and inactivated poliovirus (IPV) [5].

Due to the adverse effects caused by wP vaccines, the first cell-free pertussis vaccine (aP) prepared using formalin-treated FHA and formalin-inactivated PT of *B. pertussis* bacteria was developed and put into practice in Japan in 1981 [60]. After further research, PT-9K/129 G, a non-harmful form of PT, was obtained through genetic modification and used in aP vaccines. In the newly developed vaccines, other virulence factors,

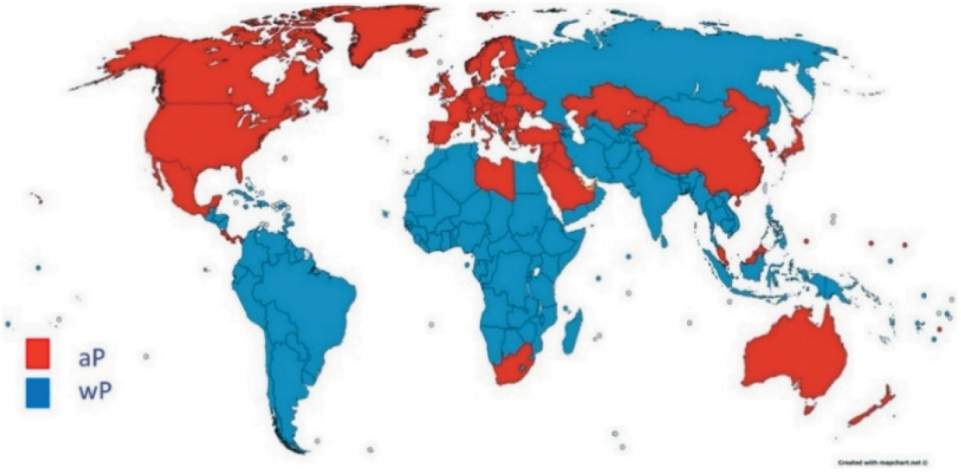

Figure 2. Countries using combination vaccines containing acellular pertussis (aP) and whole-cell pertussis (wP) in 2016 (Red: aP vaccines; Blue: wP vaccines) (Adopted from [59]).

including PRN and FIM, were used in addition to PT and FHA. In subsequent studies, different aP vaccines were developed containing different amounts and varieties of virulence factors and adjuvants [58]. Since these vaccines do not contain LOS, the severe side effects usually associated with wP vaccines are not observed with their use. It is usually administered together with diphtheria and tetanus toxoids (DtaP), as in the case of DTwP [58,61]. aP vaccines consist of few numbers of pertussis antigens and are as immunogenic or even more immunogenic than wP vaccines, possibly due to the higher amounts of bacterial-specific antigens. However, the inclusion of a limited number of antigens in aP vaccines resulted in a shorter duration of protection [62,63].

In *B. pertussis* infection, the first immediate response is by airway mucosal dendritic cells (AMDCs) and alveolar macrophages, which recognize *B. pertussis* cells and secrete cytokines and chemokines, leading to the recruitment of cells of the innate immune system [64]. While natural killer cells enhance the antimicrobial activity of macrophages through interferon-gamma (IFN-γ) secretion, dendritic cells present antigens to naïve T cells, which in turn differentiate into T helper (Th) 1, and Th17 cells. The secretion of IFN-γ and interleukin 17 (IL-17) is provided by Th1 and Th17 cells, respectively, which in turn activate macrophages and neutrophils, resulting in bacterial clearance from the host airways [3,64,65]. Meanwhile, differentiation of activated B cells into plasma cells takes place and pathogen-specific immunoglobulin (Ig) 2a/c and/or IgA antibodies are produced [64,66]. Natural infection also triggers a strong Th17 response with IL17 production, contributing to the immunity conferred by *B. pertussis* infection [67,68]. Moreover, tissue-resident memory T cells producing IFN-γ and/or IL17 were observed in animal models following the infection, possibly contributing to long-term immunity to *B. pertussis* [69].

When vaccine-induced immunity is considered, the immune responses to the administration of wP and aP vaccines are quite different. Although both vaccines induce strong *B. pertussis* antigen-specific IgG responses, the patterns of class switching differ, leading to IgG1 subclass production in aP vaccination and predominantly IgG2a/b/c production in wP vaccine immunization [70,71]. Moreover, high levels of the IgG4 subclass are observed after booster immunizations with aP vaccines, which is considered a possible reason for a suboptimal inflammatory response and impaired phagocytosis [63]. Besides humoral response, another major difference between aP and wP vaccines is in the polarization of CD4+ *T*-cell responses, which predominantly elicit a strong Th2 response in aP vaccination, whereas wP vaccines stimulate a significant level of Th1 response along with IFN-γ and IL17 production like natural infection [3]. Furthermore, a recent study compared the immune responses of the individuals who were initially primed with aP or wP vaccines and subsequently received a booster dose of aP [72]. The study revealed that individuals originally primed with aP vaccines demonstrated a strong Th2 response with increased IL4 production, decreased levels of IFN-γ and IL17, and a deficient ex vivo capacity for memory cell expansion, whereas wP-primed individuals exhibited a strong Th1 response with high levels of IFN-γ and IL17. *T*-cell-specific differences in polarization and proliferation of *T*-cell responses due to initial doses are thought to be present, despite repeated aP boosters.

Considering the immune responses stimulated by aP and wP vaccines, immunization with aP vaccines appears to be sufficient for protection against the disease, but Th1 and Th17 responses are likely required to provide better protection in terms of the clearance of *B. pertussis* cells from the respiratory tract and prevention of pathogen transmission. Based on this, extensive studies are being conducted to develop next-generation pertussis vaccines designed to elicit a broad and sustained immune response involving Th1, Th2, and Th17 responses [73]. Four main strategies are being pursued: (1) formulation with different adjuvants, (2) addition of new vaccine antigens to aP vaccines, (3) development of attenuated vaccines, and (4) vaccine preparation with pathogen-derived structures like outer membrane vesicles (OMVs) [73]. As known, alum is the most commonly used adjuvant in many commercial vaccines, including pertussis vaccines, and it mainly promotes a Th2-type immune response, which is not sufficient to prevent *B. pertussis* colonization [64]. Thus, new adjuvants, including CpG oligodeoxynucleotides, toll-like receptor (TLR) agonists, and curdlan that boost humoral immunity or counterbalance Th1/Th2 responses have been tested [74–76]. In addition to adjuvants, studies have been conducted to discover new vaccine candidates or to evaluate the potential of known virulence factors of *B. pertussis* that are not included in commercial pertussis vaccines [77]. Proteomic studies have revealed novel antigenic proteins, including Vag8, SphB1, iron superoxide dismutase, AfuA, and IRP1–3 that their protective capacities have been demonstrated in mice, suggesting that they should be included in aP vaccines to enhance protection [78–82]. In addition, known virulence factors such as AC-Hly and BrkA have also been evaluated and suggested to be combined with aP vaccines to enhance protection against the disease [83,84]. Although the addition of new antigens can enhance the effectiveness of the current aP vaccines, the development of vaccines with attenuated pathogens that mimic the infection without causing disease may be a better solution in terms of a broad and balanced immune response. Therefore, researchers have constructed a live attenuated *B. pertussis* strain called

BPZE1 through some modifications. They first replaced the *ampG* gene, required for the transportation of TCT to the cytosol, by *Escherichia coli ampG* gene by allelic exchange to reduce the TCT activity. Secondly, the *ptx* operon was deleted and the mutated version of the operon was inserted to provide an enzymatically inactive toxin. Lastly, the *dnt* gene coding DNT was deleted by allelic exchange [85]. After the construction of the strain, they formulated the strain for intranasal administration and obtained promising results in preclinical studies. Then, Phase 1 clinical trials (ClinicalTrials.gov, NCT01188512) were conducted with healthy adult volunteers, the results of which successfully demonstrated the safety profile and immune response against pertussis antigens [86]. Furthermore, a Phase 2 trial (ClinicalTrials.gov, NCT03541499) was conducted with healthy adults, which was completed in February 2022, but has not yet been published. Another live vaccine called GamLPV has completed Phase 1 trials (ClinicalTrials.gov, NCT03137927) with a successful safety profile, and now the Phase 1/2 trial is ongoing (ClinicalTrials.gov, NCT04036526) [87]. These clinical trials for both vaccines also highlight the route of vaccine delivery that intranasal immunization can also be an effective approach to enhance immunity, particularly the mucosal immune response. The last strategy is the use OMVs, which are non-replicative vesicles released by microorganisms and consist mainly of bacterial outer membrane components along with other antigenic factors such as toxins and enzymes [88]. They are highly immunogenic and may generate long-lasting immunity against diseases. Studies of OMVs as vaccine products specifically for administration against meningitis B disease were quickly initiated, and following the success of vaccination with the OMV-based MeNZB vaccine in controlling an outbreak of *Neisseria meningitidis* B (MenB) in New Zealand in 2013, the vaccine containing OMVs, named as Bexsero, has been licensed for protection against many strains of MenB, paving the way for the development of OMV-based vaccines against other infectious diseases [89].

5. Outer membrane vesicles of *Bordetella pertussis*

It is known that many cells, from prokaryotes to eukaryotes, release various types of membrane vesicles to the external environment [90]. The naturally released OMV of Gram-negative bacteria has been known for a long time, but studies have shown that these vesicles are also produced by Gram-positive bacteria, mycobacteria, and archaea [91–94].

OMVs are naturally released during the growth of Gram-negative bacteria, and this release has been associated with various biological functions [95,96]. In 1965, the secretion of cell-free LPS from *Escherichia coli* was observed and the spherical membrane structures in these cell-free LPS samples were imaged by electron microscopy in 1966, suggesting the formation of vesicles from the outer membrane [97,98]. Then, Chatterjee and Das discovered OMVs in 1967 by studying the cell wall structure of *Vibrio cholera in vitro*, and to date, OMVs have been observed to be formed by a wide variety of Gram-negative bacteria[99–101]. The presence of OMVs in patients with meningococcal infections led researchers to believe that these structures are important in the pathogenesis of bacteria [102]. Many subsequent studies have shown that OMVs play a role in increasing the survival of bacteria in harsh environments and delivering virulence factors and DNA to host cells [100,101]. For several bacteria, OMV participates in biofilm formation, thereby enabling bacterial survival in hosts or soil [103,104]. Kadurugamuwa and Beveridge (1995) demonstrated that OMV production also helps bacteria in the defense against antibiotics [105]. The possibility of gene transfer between bacteria via OMVs has been reported for a variety of bacteria, including the transfer of antibiotic resistance [106,107]. Occasionally, OMVs may also occur as a byproduct of an imbalance between cell growth and outer membrane synthesis, resulting in the release of excess membrane material as OMVs [40]. Although OMVs are spontaneously released into the environment, some factors such as heat, limited amino acid supply, or antibiotics can enhance their production [105,108,109]. Moreover, various methods including detergents, sonication, and genetic manipulation are used to increase the release OMVs for industrial use along with the various extraction methods [110,111].

The first pieces of evidence OMVs in *B. pertussis* culture were provided in the studies of Lane (1968) and Morse and Morse (1970) [112,113]. In the study by Lane (1968), electron microscopy was used to visualize small vesicles on the cell wall of *B. pertussis* and it was suggested that toxin release might be associated with these vesicles (Figure 3). In the study by Morse and Morse (1970), two distinct extracellular elements were recognized in the culture of *B. pertussis* grown in the Hedley-Wright medium, and they suggested that one of these elements may be 'vesicles' as proposed in the study by Lane (1968). In 1990, *B. pertussis* vesicles were artificially obtained by sonication and used as vaccine antigens against the intracerebral mouse challenge model [114]. Then, Hozbor et al. (1999) published a study comparing the protein content in OMVs obtained naturally from culture supernatants and artificially by sonication of *B. pertussis* cells [115]. The results revealed a similar protein composition between the naturally and artificially derived vesicles. They also confirmed the presence of AC-Hly toxin in the vesicles in addition to LOS. Another study also

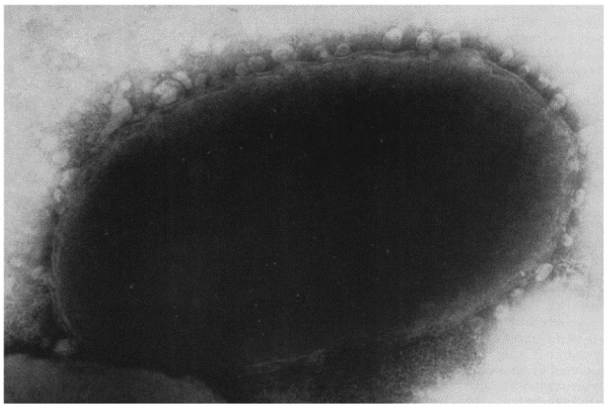

Figure 3. Examination of *B. pertussis* vesicles using electron microscopy (Adopted from [112]).

proved its presence in the OMVs, possibly on the external surface, and the study also provided the first evidence of *in vivo* production of OMVs recorded in an autopsy airway sample from a fatal case, suggesting a possible role in pathogenesis [116].s

Gasperini et al. (2017) investigated the physiopathological role of OMVs naturally released by *B. pertussis* in a human ciliated airway cell model and isolated vesicles from both Bvg- and Bvg+ phases [117]. The study revealed that OMVs derived from Bvg+ phase interacted with A549 and Calu-3 cells followed by internalization, suggesting potential roles of virulence factors for the first attachment of vesicles, which has also been observed in other pathogens [118–120]. Moreover, a tenfold increase in protein content was observed between Bvg+ and Bvg- phases and the OMVs contained PT as an active and functional ingredient. However, unlike the previously mentioned studies, they failed to detect AC-Hly among the OMV components, which was attributed to the variations in the methods to obtain vesicles. In the study, proteomic analysis of OMVs also demonstrated the presence of proteins responsible for iron storage and uptake, and further analysis revealed the intrinsic ability of OMVs of *B. pertussis* to load iron and restore bacterial growth in the absence of iron in growth media. Therefore, a role for OMVs as iron reservoirs is proposed.

To characterize OMVs of *B. pertussis* as vaccine candidates, several proteomic studies were performed with different strains grown mainly in the Stainer-Scholte medium (SS) used for commercial vaccine production. In a study, OMVs were recovered from the *B. pertussis* Tohama (CIP8132) strain by sonication of the bacterial suspension and characterized by 2-dimensional electrophoresis (2-DE) followed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis, resulting in 43 proteins along with PT and AC-Hly [121]. In another study, OMVs from the *B. pertussis* B1917 strain (omvP), the heat-inactivated B1917 strain (wP), and the acellular vaccine (aP) were compared with respect to their protein composition using 2-DE followed by Liquid Chromatography (LC)-MS/MS [71]. The results revealed the identification of a total of 332 and 268 proteins in wP and omvP, respectively. While the major abundant proteins were Vag8 and BrkA autotransporters in omvP, FHA and GroEL were found abundant in wP. In a recent study, *B. pertussis* Tohama I-derivatives BP536 and BP537 were used as representatives of Bvg+ and Bvg- phases, respectively, to reveal any variations in protein contents and adhesion ability of OMVs in virulent and avirulent states [122]. The SDS PAGE analysis revealed differences in the number and abundance of high molecular weight proteins in the Bvg+ phase, while an intense protein band was more apparent in the Bvg- phase that has slight similarity with an outer membrane porin protein according to the peptide mass fingerprinting identification. Comparative proteomic analysis of BP536 and BP537 revealed variations in the biological replicates, possibly due to sensitive regulation of the Bvg system in response to slight environmental changes. The analysis revealed that about 64% of the total protein were specific for Bvg+ OMVs. Moreover, the adherence ability of OMVs from the Bvg+ phase was more evident than that of in Bvg- OMVs, because only Bvg+ OMVs were able to interact with A549 cells and adhere to human respiratory cells. In this study, proteomic analysis was also performed to reveal possible vaccine candidates including BrkA, Vag8, TcfA, SphB1, BipA, and BfrD.

In summary, proteomic studies revealed the presence of various virulence factors and proteins involved in the attachment of OMVs to host cells. However, they also showed that the protein composition of *B. pertussis* OMVs may vary due to distinct phases and differences in OMV production or isolation methods as well as applied proteomic strategies including sample preparation and analysis. Moreover, the influence of growth medium was also confirmed, and the availability of some nutritional elements such as zinc and iron could affect the protein pattern of OMVs, although the influence of the medium on the efficiency of OMV production was not established [123].

6. OMV-based pertussis vaccines

The increase in pertussis cases in countries with a high rate of vaccination in recent years has raised questions about the protection of currently used vaccines while creating the need to develop more effective vaccines against the disease. One of the new vaccine strategies is the use of OMVs from *B. pertussis* since most of the known virulence factors are components of OMVs or at least associated with vesicles.

In 2008, the first study using OMVs from *B. pertussis* was conducted to investigate the vaccine potential of the vesicles in the murine intranasal challenge model

[121]. After the isolation of OMVs from *B. pertussis* Tohama CIP 8132 cells, mice were immunized by two different routes: intraperitoneal and intranasal. In intraperitoneal immunization, the killed whole-cell bacteria and OMVs were first detoxified with formalin and then emulsified with aluminum hydroxide (Alum) as an adjuvant. On the other hand, no adjuvant was used in intranasal immunization for both detoxified killed whole-cell bacteria and detoxified OMVs. After the challenge assay, bacterial counting at the lungs collected at specific time intervals revealed that intraperitoneal immunization with OMVs provided similar protection against *B. pertussis* as immunization with the wP vaccine compared with the group immunized with PBS. Moreover, intranasal immunization triggered the upregulation of innate immune response markers including IL-6, CCL20, and TNF-α in the lungs and also conferred protection against challenge compared with the control group. In a recent study, intranasal immunization of mice with OMV vaccines was performed to evaluate the mucosal immune response in detail by comparing it with subcutaneous OMV immunization [124]. Both intranasal and subcutaneous immunization elicited a broad immune response, including a Th1/Th17 response and multiple antibody subclasses. Moreover, intranasal immunization with OMVs resulted in increased titers of nasal and pulmonary IgA antibodies along with a high amount of IgG- and IgA-producing plasma cells. Also, only intranasal immunization prevented bacterial colonization of the nasal cavity, trachea, and lungs. The results suggest that intranasal OMV immunization can result in both a strong systemic and mucosal immunity profile along with the bacterial clearance from the lungs and the respiratory tract, suggesting a potential for prevention of transmission. Moreover, intranasal OMV vaccines can be used in a spray-dried form, which was recently studied by Kanojia et al. (2018) [125]. They developed a spray-dried OMV vaccine with improved thermostability, a comparable immune response to liquid OMV vaccine, and protection against *B. pertussis*. Thus, this study reveals the advantages of thermostable spray-dried nasal OMV vaccines in terms of worldwide distribution and application without any need for a cold chain.

OMVs naturally contain endotoxic LOS derived from the bacterial outer membrane that could stimulate excessive production of pro-inflammatory cytokines in the host, which is a possible explanation for the reactogenic nature of wP vaccines [126]. To reduce the toxicity encountered with LOS, a modifying enzyme called PagL has been used through genetic manipulations. This enzyme is responsible for the hydrolysis of the ester bond at the third position of Lipid A, resulting in the modulation of Lipid A recognition by the TLR4/MD-2 receptor [127]. In a study, a recombinant *B. pertussis* strain carrying a *pagL*-expressing vector was used since the *pagL* open reading frame in *B. pertussis* was not functional due to a frameshift unlike in *B. parapertussis* and *B. bronchiseptica* [128]. OMVs from the recombinant strain (OMV_{pagL}) and OMVs from the wild-type strain showed similar size distribution, morphology, and protein composition, proposing that PagL expression did not affect the tested parameters on *B. pertussis* OMVs. Moreover, they were both shown to confer protection in the mouse intranasal challenge model. However, it has been emphasized that OMV_{pagL} showed less reactogenicity and less weight loss in animals, and a diminished pro-inflammatory response was also induced by these OMVs. A further study by the same group was conducted by formulating OMV_{pagL} with diphtheria and tetanus toxoids ($Tdap_{OMVPagL}$) to resemble the commercial pertussis vaccines [129]. A good safety profile of $Tdap_{OMVPagL}$ was presented in the study and it conferred protection against bacterial challenge performed with various strains including vaccine strain and circulating isolate. In addition, the analysis revealed that both Th1 and Th2 responses were induced by $Tdap_{OMVPagL}$, indicating balanced immunity, while the commercial pertussis vaccine mostly activated Th2 and weak Th1 response.

OMVs of *B. pertussis* generally contain well-known virulence factors of the pathogen, whose individual contribution to vaccine efficacy has been questioned. In 2014, Ormazábal and colleagues investigated the role of PT and PRN in OMVs in terms of protection against intranasal *B. pertussis* challenge [130]. They isolated vesicles from the PT- or PRN-deficient mutants, followed by formulation as the Tdap vaccine. Compared with the group immunized with OMVs from the isolated wild-type strain, the vaccine formulations containing OMVs from PT or PRN deficient strains did not elicit a protective response as much as the wild-type OMVs, indicating the critical role of these virulent factors in protection to be conferred by *B. pertussis* OMVs. Furthermore, a recent study investigated the role of virulence-associated outer membrane proteins (virOMPs), especially BrkA and Vag8, in OMV vaccination by comparing OMVs from Bvg- and Bvg+ phases of *B. pertussis* [131]. The results revealed that Bvg+ OMVs provided stronger protection against bacterial challenge than Bvg- OMVs and that virOMP levels were positively related to the efficacy of the OMV vaccine. However, the protective capacity of OMVs derived from BrkA- or Vag8- deficient constructs was equal to the capacity of wild-type OMVs with respect to bacterial colonization and mixed Th1/Th2/Th17 responses, indicating that OMV-induced immunity is not solely dependent on these individual antigens, the absence of which might be compensated for by other antigenic proteins.

Although PRN seems to be an important virulence factor, an increase in circulating *B. pertussis* strains that

do not express PRN has been reported, proposing a selective advantage for these strains in an aP-vaccine primed immunity [130]. Therefore, Zurita et al. (2019) investigated whether OMVs could be effective in controlling infections caused by PRN(-) isolates and they revealed that the OMV formulation provided higher protective efficacy against the PRN(-) isolates than a commercial aP vaccine [132]. Also, the induction of CD4 T cells in the lungs, indicating a long-term protective immunity has been demonstrated through the immunization with OMVs. Another recent study used *B. pertussis* OMV obtained from biofilm (OMV_{biof}) or planktonic (OMV_{plank}) lifestyle to compare their protective efficacy against clinical isolates of PRN(+) and PRN(-) *B. pertussis*. SDS-PAGE analysis displayed similar protein profiles between OMV_{biof} and OMV_{plank}, and OMV_{biof} was found to be more efficient in eliminating PRN(-) isolates and inducing high antibody titers with high avidity, suggesting that OMVs from biofilm structures can also elicit immune responses, as they can reflect the composition and physiology of *B. pertussis* during *in vivo* infection [133].

Many studies have displayed the great potential of *B. pertussis* OMVs as vaccine candidates, and further studies have been conducted to characterize the immune response against OMVs in detail. Raeven et al. (2015) determined the systemic humoral response by characterizing pertussis-specific antibody levels and antibody subclasses after subcutaneous immunization of mice with heat-inactivated *B. pertussis* (wPV), OMVs (omvPV), and a commercial acellular pertussis vaccine (aPV) containing PT, PRN, and FHA [71]. While IgG1, IgG2a, IgG2b, and IgG3 subclasses were generated in both wPV and omvPV vaccinations, the level of responses was higher in omvPV vaccination. IgG1 was predominant in aPV vaccination, whereas natural infection resulted mainly in the formation of IgG2a and IgG2b. Overall, the study demonstrated distinct humoral responses with quantitative and qualitative differences between these vaccine formulations, suggesting a more balanced response only in omvPV-induced immunity. In their study, Bottero et al. (2016) identified an OMVs-based vaccine candidate (TdapOMVsBp) that is protective and safe in mice. They demonstrated production of IFN-γ and IL −17 in spleen cells isolated from OMV-immunized mice, and high IgG antibody levels were detected after OMV vaccination. The safety of the vaccine candidate was evaluated using a mouse weight gain assay, and after 16 hours, little weight loss was observed in mice immunized with TdapOMVsBp, compared to mice immunized with wP. To evaluate the safety of their new vaccine candidate in humans, the researchers also performed in vitro human whole blood (WB) IL −6 release assay and showed that TdapOMVsBp, was protective not only in mice but also in humans. The cytokine release levels of their vaccine candidate were significantly lower than those of the commercial wP vaccine. In this study, no cytokine-stimulating activity of the aP vaccine was detected, which was expected since aP vaccines contain trace amounts of LOS [134]. While the OMV-based vaccine produced a higher IgG2a/IgG1 ratio than the commercial aP vaccine, the titers of PT-specific IgG after aP immunization were higher than the OMV-based vaccine. In addition, OMV- and wP-induced serum antibodies demonstrated opsonization of *B. pertussis*, which was not the case with the aP-induced serum. Based on all these features, the researchers suggested that this vaccine candidate is one of the good candidates for third generation vaccines. In a recent study, Raeven and colleagues (2016) demonstrated that increased production of IL-5, IL-13, IL-17A, IL-10, and IFN-γ was observed in the splenocytes of OMV- and wP- immunized mice compared with naïve mice, indicating a mixed Th1/Th17/Th2 response in contrast to the Th2-biased immune response elicited by aP vaccines [135]. Besides higher IgG titers that was a possible explanation for faster *B. pertussis* clearance from the lungs, the induction of pro-inflammatory cytokines (IL-6, IL-1α, and IL-1β) and chemokines (CXCL1 and CXCL10) was lower in OMV immunized mice than in wP immunized mice. Moreover, the transcriptome analysis revealed the induction of many genes related to the type I and type II IFN signaling pathway was more dominant in OMV vaccination, whereas wP vaccination exclusively induced only 13 genes. They concluded that the OMV vaccine provided equivalent protection in terms of bacterial colonization together with enhanced induction of protective antibodies and milder inflammatory responses [135]. Recently, another study investigated whether OMVs could activate the inflammasome pathway, a critical component of the innate immune system, in macrophages, and the mechanisms underlying this activation were analyzed [136]. In general, inflammasomes are first primed followed by the upregulation of caspase-1, NLRP3, and pro-IL -1β and then activated. In this study, the results showed that OMVs induced canonical inflammasome priming and activation in both murine and human macrophages along with enhanced IL-1β secretion. Moreover, GFP-ASC speck oligomerization was observed in macrophages, a hallmark of the canonical inflammasome. Non-canonical inflammasome activation was also triggered through OMVs after sensing intracellular LOS by caspase-11 [136].

7. Concluding remarks

Despite high vaccination coverage worldwide, pertussis is still an important public health problem with frequent outbreaks. Therefore, in addition to recommended strategies like frequent booster vaccinations, studies have been conducted to develop more effective solutions in terms of next-generation pertussis vaccines. Although new adjuvants and antigenic factors have been characterized, more complex strategies may be required to provide broad and efficient immune response with a balanced humoral and cellular response. At this point, OMV-based vaccines draw attention as they serve as a valuable carrier containing the main immunogenic components of pathogens such as LPS, toxins, and adhesins in their native form. Because of their pathogen-associated molecular patterns recognized by Toll like receptors and other pattern recognition receptors, they also elicit an innate immune response. In addition, their small size allows them to be easily processed by antigen-presenting cells. Although they are considered attractive vaccine candidates, some challenges including endotoxin level and different antigen patterns resulting from OMV isolation techniques may limit the wide application of OMVs.

Up to date, OMVs from *B. pertussis* seem to be a promising platform for pertussis vaccines, but there are still variations in vaccine composition in terms of the amount or presence of virulence factors. Therefore, culture conditions, strains, extraction methods, and purification steps should be standardized for proper vaccine manufacturing. Nevertheless, the potential of these vesicles as vaccine candidates is undisputed.

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