® Postepy Hig Med Dosw (online), 2018; 72: 471-480
e-ISSN 1732-2693

www.phmd.pl
Review

Received: 24.07.2017
Accepted: 30.01.2018
Published: 29.05.2018

Antigens of *Actinobacillus pleuropneumoniae* and their use in the design of vaccines, especially glycoconjugates*

Antygeny *Actinobacillus pleuropneumoniae* i ich wykorzystanie w projektowaniu szczepionek, ze szczególnym uwzględnieniem preparatów glikokoniugatowych

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Summary

Actinobacillus pleuropneumoniae (further: *A. pleuropneumoniae*) is microaerophilic, Gram-negative bacteria belonging to the *Pasteurellaceae* family. This pathogenic microorganism is a major cause of porcine pleuropneumonia and fibrinous pleurisy, highly contagious diseases of the respiratory tract, affecting predominantly young pigs. Pleuropneumonia and fibrinous pleurisy can be diagnosed due to cough combined with a high mortality and the common infection route is direct transfer of bacteria by aerosol. *A. pleuropneumoniae* is a significant factor for economic losses in the swine industry all over the world. Progress made in research concerning a new potential vaccine enables the development of technology for designing safe, new candidates which could provide full protection against most of *A. pleuropneumoniae* serotypes. Several immunogenic factors of *A. pleuropneumoniae* have been found, including carbohydrate antigens, protein molecules and lipostructures. Carbohydrate antigens, as capsular polysaccharides (CPS) and lipopolysaccharides (LPS), have a special place due to their properties and wide potential. Some of these microbial structures were used to create subunit vaccines containing polysaccharide-protein conjugates, which seem to be a promising solution. The prevention methods, such as vaccines, should minimize medical expenses and financial losses. This article shows a wide repertoire of *A. pleuropneumoniae* antigens and focuses on one of the most promising strategies in vaccine design – glycoconjugate vaccines.

Keywords: ***Actinobacillus pleuropneumoniae*** **• carbohydrate antigens • conjugate • glycoconjugate • pleuropneumonia • vaccines**

*This work was supported by The National Centre for Research and Development project PBS3/A8/33/2015 "The elaboration of subunit vaccines for cattle and pigs based on recombinant Hsp60, OMP40 and LPS core antigens from Gram-negative bacteria".

GICID 01.3001.0012.0683
DOI: 10.5604/01.3001.0012.0683
Word count: 6529
Tables: –
Figures: 2
References: 112

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ETIOLOGICAL FACTOR

A. pleuropneumoniae (earlier: *Haemophilus pleuropneumoniae* [94]) is a microaerophilic, encapsulated, Gram-negative bacterium. This coccobacillus is the most important etiological agent of contagious porcine pleuropneumonia and fibrinous pleurisy [36]. It was classified to the *Actinobacillus* genus in the *Pasteurellaceae* family in 1983 [66,77]. That is the reason there are still differences in the classification and nomenclature of *A. pleuropneumoniae.* Moreover, 15 serotypes have been identified that differ in the external membrane components, environmental requirements, metabolism and pathogenicity [70,89]. Two biovars of *A. pleuropneumoniae* have been described: 13 serovars from biovar 1, which need the nicotinamide adenine dinucleotide (NAD) to grow, and 2 serovars from biovar 2, which are NADindependent. The important attribute between serotypes is a different level of *A. pleuropneumoniae* RTX-toxins expression: *A. pleuropneumoniae* RTX- toxin I (ApxI), *A. pleuropneumoniae* RTX- toxin II (ApxII), *A. pleuropneumoniae* RTX- toxin III (ApxIII) and *A. pleuropneumoniae* RTX- toxin IV (ApxIV). ApxI, ApxII and ApxIII have a lethal effect on neutrophils and macrophages [18,26]. The major feature for *A. pleuropneumoniae* is strong resistance to iron-poor environment. Many iron uptake systems have been reported in *A. pleuropneumoniae,* such as transferrin- and haemoglobin-binding proteins and ferric hydroxamate receptors [24,45,97]. It has been proven that *A. pleuropneumoniae* virulence is correlated with the ability of the bacteria to bind to the mucus and proteins secreted by the cells of the lower respiratory tract [81]. Strains unable to form biofilm are considered as less virulent and have different susceptibility to antimicrobials [5,47]. One of the most important surface molecules is a PGA (poly-N-acetylglucosamine) polysaccharide, consisting of linear chains of N-acetyl-D-glucosamine (GlcNAc), which may have relevance to the colonization of *A. pleuropneumoniae* in pig respiratory system [43]. Specific outer membrane proteins (Omps) [21,41], heat shock proteins (Hsps) [28] and superoxide dismutase (SOD) [55,92] presence contribute to intracellular resistance for *A. pleuropneumoniae* [93]. These two biovars differ in their functions and pathogenic symptoms but all 15 serotypes can cause disease [50,112]. Knowledge on immunogens and metabolic pathways is crucial for developing effective and safe vaccines.

PLEUROPNEUMONIA AND FIBRINOUS PLEURISY

Although the *A. pleuropneumoniae* have been extensively characterized, there are still significant problems with the identification of infection, treatment and finding the most effective strategies for vaccination. *A. pleuropneumoniae* contagions can be difficult to diagnose, due to the occurrence of frequent asymptomatic infections [68]. Pleuropneumonia and fibrinous pleurisy can be diagnosed due to cough combined with high mortality, especially in growing pigs, but can occur in all ages of swine. 10-16 weeks old pigs are the most susceptible with highest mortality rates. The common infection route is direct transfer of bacteria by aerosol. Animals suffer from exercise intolerance, develop a fever, loss of appetite, respiratory ailments, swelling lung and tonsils, lung with hemorrhagic, ulcerative and necrotic areas, subchronic and chronic lung lesions, presence of blood-stained froth around the nose and mouth. In the peracute type of pleuropneumonia, pigs die 24-36 hours from first clinical symptoms. In the acute form, animals may take a few days to die, go to a chronic type with lung damage or recover. It has been observed that a large percentage of miscarriages are due to *A. pleuropneumoniae* infections [20,33,44,56].

ECONOMIC AND INDUSTRIAL IMPLICATIONS

A. pleuropneumoniae infection is primary a bacterial pneumonia disease. This microorganism is the cause of 20% of disease cases. The most widespread coinfection in respiratory tract diseases is couple of *A. pleuropneumoniae* with *Pasteurella multocida* (further: *P. multocida*), which causes the atrophic rhinitis in pigs [66]. One of the major health problems in pork production is a porcine respiratory disease complex (PRDC) caused by multiple infectious agents, i.e. *A. pleuropneumoniae* and *P. multocida.* An examination performed on 212 nasal swabs obtained from swine farmed in Aguascalientes (Mexico) showed that nearly 20% of the samples were positive for *A. pleuropneumoniae* and 23% of the samples were positive for *P. multocida* [60]. In the another case, high levels of antibodies reacting with both microorganisms could suggest a synergistic effect of both *A. pleuropneumoniae* and *P. multocida* and PRDC [75,107]. It has been demonstrated that *A. pleuropneumoniae* had been diagnosed in United States in approximately 9.6% of grower-finisher operations and resulted in a total loss of $30-32 million

to the United States economy in 1995 [63]. The diagnosis of *A. pleuropneumoniae* was correlated with a 4.6% reduction in pork production [63]. *A. pleuropneumoniae* serotypes 3, 6, 8 and 15 are most frequently isolated in North America [34]. Serotypes 1, 5, 7 and 15 are the most prevalent types in Australia [56]. Serotype 2 dominates in Switzerland, Norway, Sweden [96] and Denmark [49]. In the Czech Republic, the most prevalent is serotype 9 [53]. Outbreaks of pleuropneumonia cause huge economic losses in the swine and bovine industry. Prophylaxis is the best way to avoid the expensive implications of *A. pleuropneumoniae* infections. The prevention methods, such as vaccines, should be established before pleuropneumonia occurs in the herd, to minimize medical expenses and financial losses. For instance, vaccines against *A. pleuropneumoniae* are the third most frequently used kind of vaccines in the Danish swine production (26% of dosages in 2013) [103].

VACCINATION STRATEGIES

As with other members of the *Pasteurellaceae* family, the big challenge in designing vaccines against these microorganisms is their effective transformation system [83]. Advances made in the identification of new potential vaccine candidates show interesting perspectives. Intensive research in the *A. pleuropneumoniae* vaccinationarea resulted in the development of several immunogenic preparations from bacterins based on whole bacterial cells to more sophisticated ones such as subunit vaccines, but unfortunately none of the developed vaccines provide complete protection [37,40]. One of the vaccinal candidates are live attenuated bacteria cells. This type of vaccine could undergo spontaneous reversion to a virulent form and thus endanger the vaccinated animals (especially with immunocompromised hosts). The earliest commercialized vaccines against *A. pleuropneumoniae* contained inactivated whole-cells (killed by heat or formalin). They presented a full spectrum of antigens derived from bacteria; however, antigenic determinants depend strongly on the growing conditions. The composition of such a vaccine is highly variable [29]. Moreover, the whole-cell vaccines do not offer sufficient protection against other serotypes. The quality control and comparison between different production lots is limited. The simplest strategy was using the live attenuated vaccines, such as temperature-sensitive mutants of serotype 1 strain 4074 [15], attenuated serotype 1 strain CM5 with a thinner capsule [11] or attenuated double-deletion mutant S-*8ΔclpP/apxIIC* [111]. The results show that the whole-cell vaccines reduced mortality in young herds, but did not provide protection against pneumonia [90]. The use of bacterial ghosts technology gives promising perspectives due to their functional and antigenic potential [102]. An interesting solution is oral immunization using formalin-inactivated *A. pleuropneumoniae* serotype 1 entrapped in microspheres with polymers obtained in co-spray drying process. In an immune response test, all of the pigs (9 per group) in the control group and injection group died, whereas only one pig died in oral-vaccination class. Also, microscopic examinations of lungs show that the oral-vaccine group was better protected against lung lesions, especially hemorrhagic changes [57]. Ghost vaccine is an empty whole-cell envelope obtained by the expression of PhiX174 bacteriophage lysis gene *E* [110]. Another strategy was based on using highly conserved molecules for all serotypes, such as outer membrane proteins (Omps) and outer membrane lipoproteins (Omls). Omps play key roles in infections and may be targets for vaccine studies [20]. Several Omps and Omls were characterized [21] and used in the development of subunit vaccines [72], i.e. purified Omp with hybrid liposome ISCOM adjuvant [88], acellular pentavalent vaccine Pleurostar™ (Novartis) containing ApxII, OmplA1, OmlA5, CysL1 and TfbA7 subunits [106], OmlA lipoprotein with cholera toxin and VSA adjuvant [3], purified outer membrane lipoprotein PalA and/or ApxI, ApxII toxins with Diluvac Forte adjuvant [105] and Coglapix® (Ceva Animal Health Ltd, Amersham, United Kingdom) made of ApxI, ApxII and ApxIII toxins from serotypes 1 and 2 in addition to somatic antigens of *Actinobacillus pleuropneumoniae* [52]. In another approach, conserved surface proteins of *A. pleuropneumoniae* were identified to find new vaccine candidates with bioinformatics methods. The results of the *in silico* examinations of proteins showed 39 highly conserved Omps and Omls. Three of them (APJL_0126, HbpA and OmpW) were evaluated for their usability as a vaccine. The results showed high titers of antibodies, but none of them could induce protective immunity individually. They may be used in further investigations as subunit vaccines [17]. Trimeric autotransporter adhesins (TAAs) are virulence agents to many Gram-negative bacteria that are involved in some pathogenic processes [58,76]. The functional head domain (Apa2H1) of *A. pleuropneumoniae* trimeric autotransporter adhesin was used as a component of a subunit vaccine. Results showed that immunization with Apa2H1 effected in strong production of antigen-specific antibodies, induction of Th1 and Th2 immunity and improved survival rates in tests in mouse model [79]. Molecules involved in iron uptake system are, therefore, interesting candidates for subunit vaccines, i.e. recombinant cytolysin CytA with 60 kDa transferrin-binding protein TfbA [87] and recombinant transferrin binding protein B (TbpB) [48]. Strong immunogenic properties of Apx toxins [104] have been demonstrated in many studies [46] and they are named as "second generation" vaccines. In this group vaccines containing toxins ortoxin alone can be found as well as subunit vaccines based on Apx toxin fragment, i.e. commercially available Porcilis® (Intervet, Boxmeer, The Netherlands) vaccine contains ApxI, ApxII and ApxIII toxoids with 42 kDa Omp [19,35,95] and ApxI Nterminal domain with Montanide ISA 70 adjuvant [91]. ApxII toxin is an especially promising candidate for a vaccine, due to the fact that all serotypes of *A. pleuropneumoniae* (exceptions are serotypes 10 and 14) express this protein antigen [27]. Therefore, a vaccine containing Apx toxins is serotype-independent. It has been proven that nasal immunization with M cell-targeting ligand-conjugated

ApxIIA toxin fragment induces efficient mucosal and systemic immune responses against *A. pleuropneumoniae* infection in a murine model [73]. Another approach was oral immunization using the cubic phase of monoolein and purified toxins of *A. pleuropneumoniae*. The monoolein in the cubic phase proved to be a good carrier and protective medium for toxins, and the vaccine provides good protection into weaned piglets as growing pig stage [61]. Based on a detergent wash extraction of Omps and secreted proteins, a DIVA (differentiating infected from vaccinated animals) vaccine was invented, which uses deletions in immunogenic *apxIIA* gene [65]. Analyzes indicated 75 different protein components and Apx toxins in this vaccine [13]. The internal proteins are also involved in inducing an immunological response. The NADPH sulfite reductase hemoprotein CysI was tested, which resulted in weaker symptoms of pleuropneumonia and lower mortality [109]. Many vaccines function as a mixture of different antigens, like the mixed cell-free culture supernatant of *A. pleuropneumoniae* [32], *A. pleuropneumoniae ΔapxIIA* mutant [65] or sodium deoxycholate extraction of *A. pleuropneumoniae* serotype 2 and 9 cultures induced by iron restriction [32]. The most sophisticated methods of vaccine design use genetics, i.e. genomic expression library immunization (ELI). This method has been used in experiments against *A. pleuropneumoniae* with mice [9,59]. Another approach showed that DNA vaccine encoding type IV pilin of *A. pleuropneumoniae* induces strong immune response but gives limited protection (only 30%) against *A. pleuropneumoniae* serotype 2 [64].

GLYCOCONJUGATE VACCINES

Carbohydrate structures present on *A. pleuropneumoniae* cell surface such as CPS and O-antigen and core region from LPS were also identified as potential vaccine candidates, due to their immunogenic potential [8]. LPS is thermostable glycolipid molecule composed of lipid A anchored in the outer membrane, the core oligosaccharide built of 2-keto-3-deoxyoctulosonic acid (further: Kdo) and heptose residues, and the O-antigen, a polysaccharide consisting of repeating units [69]. Strain differences are defined by monosaccharide units and sequence, linkages and non-carbohydrate substitutions, presenting the diversity associated with this part of LPS [67]. Carbohydrate antigens expressed by *A. pleuropneumoniae* [98] are similar to antigens expressed by other mucosal pathogens, such as *P. multocida* [38,99,100]. The glycoform A of *P. multocida* core is conserved in all *P. multocida* strains and presented by other pathogens in the *Pasteurellaceae* family (*A. pleuropneumoniae* and

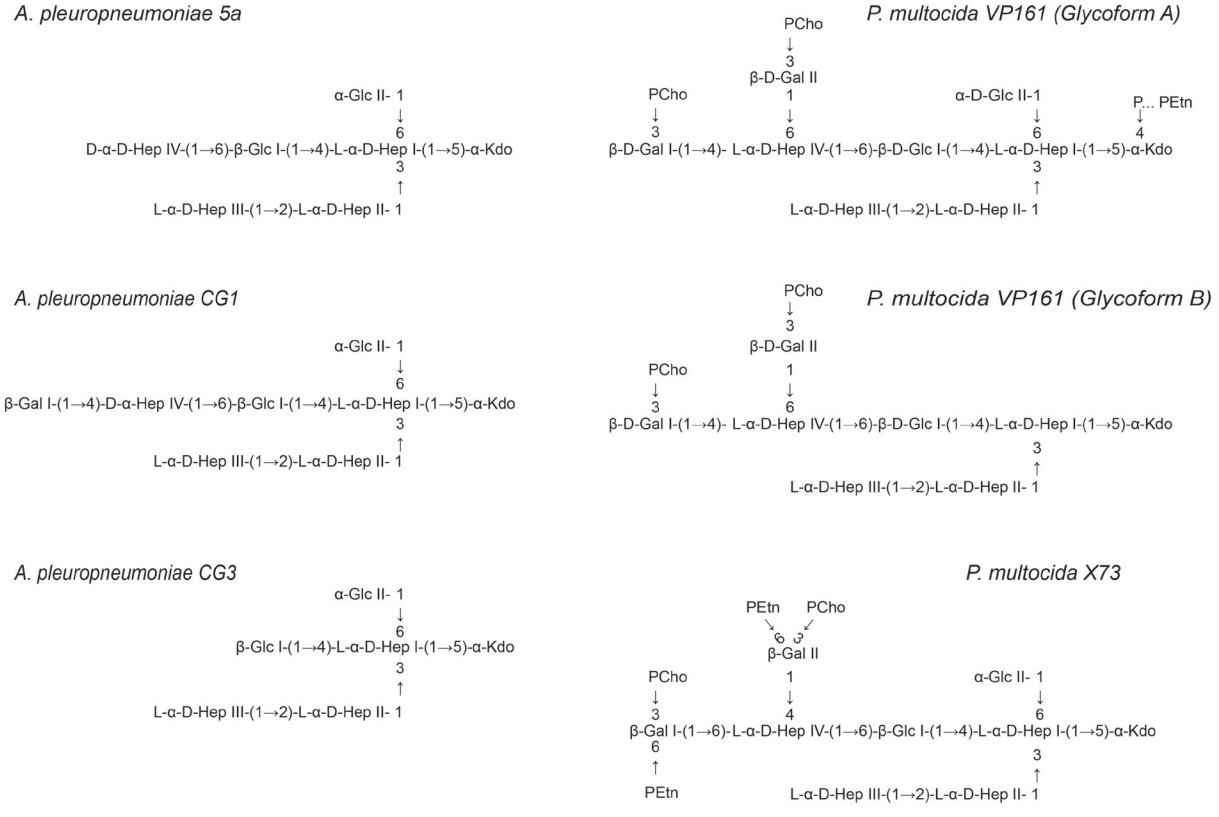

Fig. 1. Schematic presentation of the six *A. pleuropneumoniae* and *P. multocida* LPS core structures. Residues are Kdo, 2-keto-3-deoxyoctulosonic acid; P, phosphate; PCho, phosphocholine; PEtn, phosphoethanolamine; Gal, galactose; Glc, glucose; Hep, heptose [38,80,81,82,99,100]

Mannheimia haemolytica) [101] (Fig. 1). The similarity of structures may result in high cross-reactivity of sera obtained by immunization with carbohydrate-based vaccines.

Endotoxins (lipopolysaccharides, LPS) are responsible for several clinical manifestations of Gram-negative bacterial infections. They induce the production of many types of septic shock mediators [1]. CPS (K-antigen), much like to LPS O-antigen, has high antigenic properties. The variability of CPS is also used for serotyping of encapsulated bacteria. The structures of CPSs can be classified into three diverse groups: the first group, defined by sequences of glycosidically linked oligosaccharide units (5a, 5b and 10 serotypes). The second group is comprised of the polymers of oligosaccharide units joined through phosphate linkages (1, 4, 12 serotypes) and the third group – teichoic acid-like chains with repeating glycosylglycitol units joined through phosphate diester linkages (2, 3, 6, 7, 8, 9 and 11 serotypes) [74]. It has been reported that bacterial CPSs are involved in biofilm formation [47]. LPS takes part in adherence process of bacteria to respiratory tract cells, thus being a good target for the potential vaccine. The use of carbohydrate surface antigens is attractive due to the good accessibility for immune system cells. The high heterogeneity of CPS and LPS in different serotypes of *A. pleuropneumoniae* and other bacteria makes it difficult to design universal vaccine against pleuropneumonia [74,82]. Many methods of producing glycoconjugate vaccines have been developed (Fig. 2).

In vaccinology, pigs were found to have greatly increased IgG antibodies against CPS and LPS, and sera were opsonic in phagocytic tests. The pigs exhibited significantly greater weight increase and less mortality [15,16]. It was shown that LPS can interact with Omps and exotoxins, i.e. *Bordetella pertussis* adenylate cyclase toxin (CyaA) [12,86], leukotoxin (Lkt) from *Mannheimia haemolytica* [54], meaning that these antigens are exposed in the context of these proteins on the surface of bacterial cells. Proteins can act as carrier molecules for polysaccharides as well as the antigens (eliciting strong immune response); thus, covalently linking these molecules could provide a positive immunogenic effect of the new vaccine. The possibility of multiple of approaches would merit discussing. Two of the glycoconjugate vaccines were prepared from de-O-acylated LPS and CPS from serotype 1 by coupling to *A. pleuropneumoniae* haemolysin protein by using adipic acid dihydrazide as the spacer. Both sera induced by aforementioned vaccines were opsonic in a phagocytosis assay [15]. In another

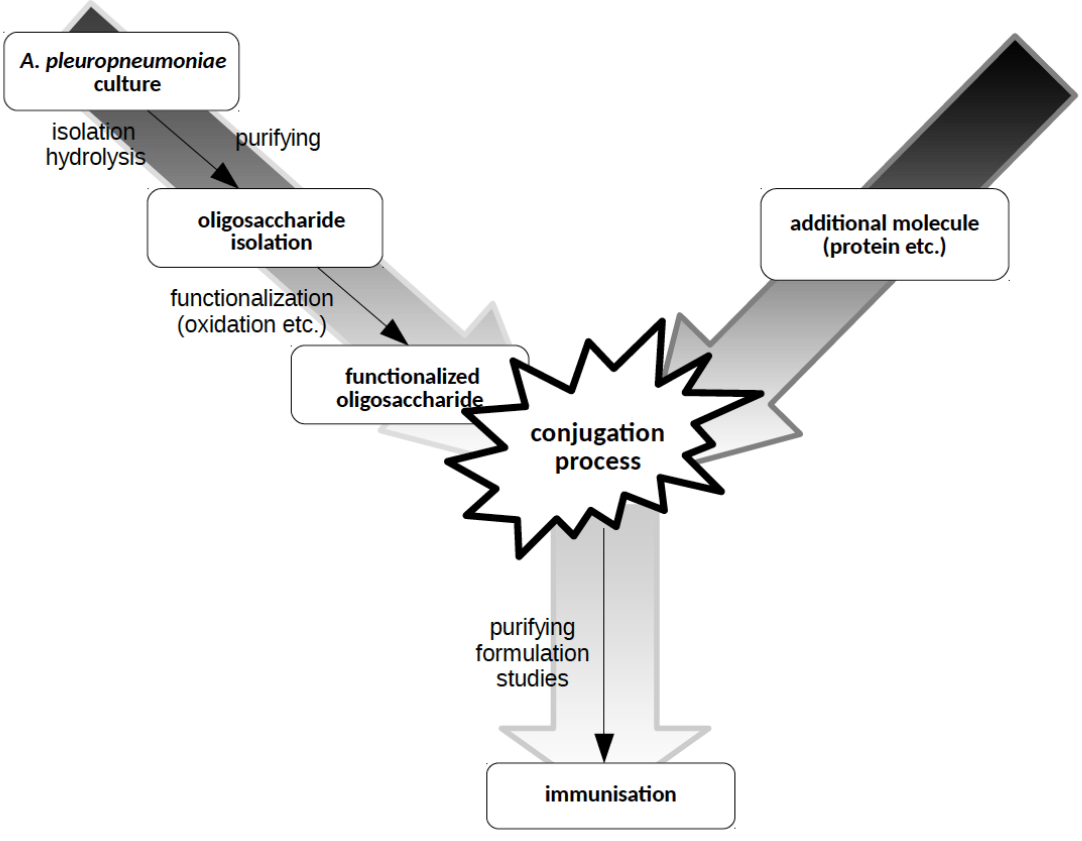

Fig. 2. Schematic presentation of *A. pleuropneumoniae* glycoconjugate vaccine development

variant of the vaccine, the purified capsule from capsulated *A. pleuropneumoniae* serotype 5 was conjugated to bovine serum albumin (BSA) through an adipic acid dihydrazide spacer. Protection provided by antiserum to capsule was similar to the level of protection provided by vaccination with killed bacteria [42]. The other approach was to use the anionic fraction of *A. pleuropneumoniae* serotype 1 saline extract (ANEX) that presents protective properties in combination with appropriate adjuvant and contains carbohydrate antigens and Omps [108]. The protective efficacy of cell-free antigen carbohydrate vaccine was studied. The mice given monoclonal antibodies to capsular antigen of serotype 5 had a 70% survival rate against a challenge with a *A. pleuropneumoniae* homologous serotype. Furthermore, mice given antibodies against ApxI toxin and capsular antigen of serotype 5 were fully protected against this serotype [71]. Pigs vaccinated with *A. pleuropneumoniae* 5b capsular polysaccharide conjugated with tetanus toxoid (TT) had reduced the mass ratio of the affected to unaffected lung tissue compared to animals in the control groups. The results showed that CPS-TT conjugate vaccination had protective efficacy against pulmonary lesions and lethality caused by *A. pleuropneumoniae* serotype 5b infection [4]. Conjugate vaccines were also prepared with bovine serum albumin (BSA) as a protein carrier. Induced immunological response was serotypespecific and there was no crossserotype protection against all serotypes of *A. pleuropneumoniae* in vaccinated mice. The highest protection (80%; $P<0.05$) was observed in immunization with adjuvant [84]. Analogous studies have been made on pigs. Results showed that protection was obtained when pigs were immunized with a detoxified LPS as well as with commercial whole-cell bacterins [85]. One of the currently available vaccines is SUIVAC APP (Dyntec Ltd, Terezin, Czech Republic), which occurs in two variants: SUIVAC APP ID (intradermal version) and SUIVAC APP IM (intramuscular version). Except lipopolysaccharides, mentioned preparations contain inactivated bacterium *A. pleuropneumoniae* (serotypes 2 and 9), ApxI, ApxII, ApxIII toxoids and Omps [25]. The immune responses induced by commercial vaccine SUIVAC APP administered intramuscularly and experimental vaccine containing alternative doses of antigen given intradermally were compared. The response reached the highest level in piglets immunized intramuscularly and intradermally by three-time diluted doses. The secondary response in these animals was significantly higher than in group immunized intradermally with two- and four-time concentrated doses. It is worth pointing out, that the highest concentration of antibodies induced by intramuscular route elicited a significantly lower level of protection [10]. The breakthrough could be a technology based on bacterial protein glycosylation system, especially with NGT protein (cytoplasmic *N*-linked glycosylating enzyme). Potential application may have *N*-linked glucose-based conjugate against *A. pleuropneumoniae. Ngt* operon is highly conservative among *A. pleuropneumoniae* strains and it could favor the engineering of a glycoconjugate against multiple serovars [22].

WHY GLYCOCONJUGATE VACCINES?

Carbohydrates and glycolipids present on the surface of bacterial cells can be used as antigens for vaccine development. LPS nonspecifically activates B cells and macrophages, and less so T cells. LPS is bound in blood by LPS binding protein (LBP). This protein mediates the transfer of LPS on the cluster of differentiation 14 (CD14) cell surface receptor presented on macrophages and neutrophils. CD14 molecule mediates the interaction of LPS with a target receptor, which is the Toll-like receptor 4 (TLR4). LPS *in vivo* often causes a septic shock. Lipid A is responsible for the toxic properties of the molecule, while the O-specific chain is responsible for the immunomodulatory and antigenic features [30,67]. The immunogenicity potential of weak T-independent antigens can be increased by a combination with protein carriers, which provide T-cell help [23]. Bacterial polysaccharides induce a protective immune response in healthy adult organisms, but they are weakly immunogenic in infants and the elderly. Covalent linking a carbohydrate antigen to a protein results in long lasting memory, even among infant groups. In general, standalone polysaccharides used as a vaccine elicit low affinity in immunoglobulin class M (IgM) independent of T-cell response, with no Major Histocompatibility Complex Class II (MHCII) CD4 cell interaction. This route does not lead to the formation of B-cell response and does not generate an immune memory. This disadvantage is eliminated in protein-polysaccharide glycoconjugate vaccines. Carbohydrate-protein molecules bind to the B-cell receptor of polysaccharide-specific preB cells. After it is absorbed by the endosome, the protein is digested to release peptide epitopes, which are presented in the context of MHCII to the CD4 T-cell receptors. Activated T-cell release cytokines such as IL4 and IL2 to stimulate B-cell maturation to memory B-cells and induce immunoglobulin class switching from IgM to polysaccharide-specific IgG [2,31,78]. Carbohydrate antigens conjugated to proteins were noted as "the most abundant structurally diverse class of molecules in nature" [39]. The effectiveness of glycoconjugate vaccine prophylaxis is limited by the optimal presentation of carbohydrate epitopes, conjugation chemistry, molecule construction and age of the vaccinated organism [6,7,51]. An understanding of the mechanisms involved in glycoconjugate immunization is crucial in the design of new vaccines against contemporary infections.

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The authors have no potential conflicts of interest to declare.