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EXPERT REVIEWS

Resurgence of pertussis calls for re-evaluation of pertussis animal models

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Arno AJ van der Ark*[1], Daniela F Hozbor[2], Claire JP Boog[1], Bernard Metz[1], Germie PJM van den Dobbelsteen[1,3] and Cécile ACM van Els[1]

[1]*Unit for Vaccinology, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, PO Box 1, 3720 AL Bilthoven, The Netherlands*
[2]*Facultad de Ciencias Exactas, Instituto de Biotechnologia y Biologia Molecular, Universidad Nacional de La Plata, CCT La Plata CONICET, Calle 115 entre 49 y 50, Edificio Ex Liceo Interno 39, 1900, La Plata, Argentina*
[3]*Innovation & Discovery Laboratory, Crucell Holland, Archimedesweg 4-6, 2333 CN Leiden, The Netherlands*
**Author for correspondence*
Tel.: +31 30 274 3218
arno.van.der.ark@rivm.nl

Pertussis has recently re-emerged in well-vaccinated populations most likely due to a combination of pathogen adaptation and waning of vaccine-induced pertussis immunity. Changes in genomic content of the etiologic agent, *Bordetella pertussis,* observed in the postvaccination era can have a bearing on the efficacy of vaccines currently in use. Moreover, protective immune responses in vaccinees wane gradually depending on their originally induced size and breadth, and memory responses may not be as regularly boosted by circulating strains as was the case in the prevaccination era. This pertussis scenario asks for new, improved vaccines with at least a longer duration of protection. Pertussis vaccine research, development and postmarketing surveillance require re-evaluation and innovation of the currently available pertussis animal models, with emphasis on the use of circulating *B. pertussis* strains.

Keywords: animal models • disease and pathogen • immune modulation • pathogen adaptation • re-emerging pertussis • vaccine effectiveness • virulence

Whooping cough, or pertussis, has attracted renewed interest because of its recent resurgence in highly vaccinated populations. This respiratory disease is still one of the leading causes of vaccine preventable deaths in infants under 1 year of age. The WHO estimated that in 2008 approximately 16 million cases of pertussis occurred worldwide, 95% of which were in developing countries, and that approximately 195,000 children died from the disease [1]. Pertussis is a highly contagious disease caused by the Gram-negative rod *Bordetella pertussis*, which binds to the ciliated epithelial cells in the human nasopharynx of the upper respiratory tract. The disease is characterized by three stages, a catarrhal, paroxysmal and convalescence stage, and derives its name from the 'whoop' sound made from the inspiration of air after a cough. Disease presentation varies with age and history of previous infection or vaccination. Young infants can present apnea and cyanosis, with or without other disease symptoms. Adults and adolescents can exhibit only mild symptoms or have the typical prolonged paroxysmal cough. In all persons, the cough can continue for months [2]. A small proportion of pertussis is caused by the closely related *Bordetella parapertussis* and mild respiratory disease sporadically by *Bordetella bronchiseptica*. Recently, it has been reported that infection with *B. holmesii* may induce symptoms similar to pertussis [3].

B. pertussis and *B. parapertussis* are independent derivatives of *B. bronchiseptica*-like ancestors, and evolved as human-specific pathogens. These three species, known as classical *Bordetellae,* express a wide array of virulence antigens to colonize the respiratory tract of their natural hosts, to impair the function of immune mechanisms and/or to invade host tissues [4]. The expression levels of the majority of these virulence antigens are controlled by one master two-component regulatory system, BvgS/BvgA. BvgS is a transmembrane sensor protein that can phosphorylate itself and BvgA. The system controls the adherence of Bordetella (*bvg*$^+$), the forming of biofilms (*bvg*^{i}) and survival outside the host (*bvg*$^-$) in response to environmental stimuli [5]. The repression of the regulated genes is dependent upon the third gene, *bvgR* [6]. Progressive loss and inactivation of *B. pertussis* genes has been seen over the past 60 years and may have altered pathogen virulence due to loss of regulatory or control functions [7]. Furthermore, although the human *Bordetellae* are considered as genetically monomorphic, there is increasing evidence for accumulation of

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genetic variation in *B. pertussis*, such as through expansion of insertion sequence elements and SNPs [4,8,9]. This process may underlie selection of *B. pertussis* strains that are more adapted to survive in dense and immune human populations.

Pertussis vaccines & quality control

To date, there are two different types of licensed pertussis vaccines, the first generation being whole cell vaccines (WCVs), which are gradually being replaced by the second generation of acellular vaccines (ACVs). Both types of vaccines differ considerably in composition, side effects and steering of immune responses, but also greatly with respect to their release requirements after production. WCVs contain killed bacteria and cause more adverse reactions than ACVs, which contain one to five purified virulence factors, being: pertactin (Prn), filamentous hemagglutinin (FHA), two fimbriae (Fim) serotypes (Fim 2 and 3) and chemically detoxified pertussis toxin (Ptx). These ACV products, whichare available from diverse manufacturers, differ not only in the number of components but also in their concentrations, detoxification methods, their degree of adsorption to different adjuvants and consequently in their immunogenicity.

WCVs and ACVs induce different types of humoral, as well as cellular, immune responses in human and animals [10,11]. WCV induce a T-helper cell response of a predominant type 1 (Th1) cytokine profile, and a broad antibody response against a whole range of surface antigens yet moderate or low levels against the major protective antigens, such as Ptx and Prn [12]. ACV induces a more Th2-cytokine dominated cellular immune response and high antibody responses against the vaccine antigens involved [13–15]. The short-term efficacy of both vaccines is regarded similarly high, but neither type of vaccine has a satisfactory long-term duration of protection. Protection after vaccination with WCV is estimated at 4–12 years while protection by pediatric ACV may last 5–7 years, depending on the quality of the vaccine and immunization schedule in use [16,17]. For release of vaccines, national and international authorities require the performance of extensive quality control testing, including a potency test before each vaccine batch is released for human use.

Currently, the potency of WCV is assessed by an intracerebral mouse protection test (ic-MPT) based on the Kendrick test [18] but this test is not applicable for ACV batch release. In Japan, China and Korea, a modified ic-MPT (suckling mice test) is used to assess the potency of ACV with varying pertussis antigen compositions [19]. In Europe and North America, consistency in production of licensed ACV is demonstrated by immunogenicity assays instead of by assessing potency [20,21]. Recently, recommendations to assure the quality, safety and efficacy of ACV were published by the WHO [201]. In this document, details are provided for the modified ic-MPT for potency testing of ACV. Simultaneously, the WHO recommends use of the intranasal mouse challenge model (in-MCM) in nonclinical testing for registration of new vaccine candidates. The in-MCM is known as a valid research model in vaccine research and development [22–24]. The WHO does not recommend this model for routine potency testing [201].

Resurgence of pertussis

Mass vaccination programs against pertussis with WCV in the 1950s led to a dramatic decrease in mortality and morbidity among children. Pertussis virtually disappeared in the industrialized world during the 1970s and 1980s, and herd immunity was initially observed [25]. Clearly, pertussis vaccination prevents severe illness and reduces infection- and transmission rates but not to the same extent as seen for other vaccine preventable childhood diseases [26,27]. In addition to reducing its incidence, 50–60 years of vaccination either with WCV and/or ACV profoundly changed the epidemiology of pertussis. Infants too young to be fully immunized have become the most vulnerable group at highest risk for severe clinical disease, whereas adolescents and adults, previously protected, have become populations susceptible to mild disease (Figure 1) [28,29]. Since the 1990s, a steady increase in the number of pertussis cases was observed in various countries with successful and long-lasting vaccination programs, such as Argentina, Finland, Norway, Spain, Switzerland, Israel, Canada, Australia, the USA and The Netherlands [30–34]. In these countries, different types of pertussis vaccines were being used with proven efficacy in clinical trials. Improved diagnostics and/or increased awareness have been implicated in the observed increased notifications and, to some extent, differences in efficacy of the various vaccines and/or national vaccination schemes in use could also play a role. However, the two key factors held responsible for the pertussis resurgence in vaccinated populations are, on the one hand, the appearance of new *B. pertussis* strain variants, and on the other hand, the gradual loss of vaccine-induced protective immunity in vaccinees, in an era of less circulation and natural boosting (Figure 1). The relative impact of these factors in the resurgence of pertussis is under investigation and may differ between subpopulations and countries [35–38].

Pathogen adaptation in immune populations

Coinciding with the observed increase of pertussis in various vaccinating countries, antigenic mutations were noted in circulating *B. pertussis* strains all over the world [32,39,40]. In The Netherlands, a country with an overall high vaccination coverage of more than 95%, the rise in pertussis notifications in the 1990s concurred highly with the switch in the Ptx promoter (*ptxP*) region from the *ptxP1* allele to the *ptxP3* allele [41]. Earlier antigenic drift from the Ptx S1 subunit PtxA2 to PtxA1, and from Prn1 to Prn2, had no significant effects on the pertussis incidence, but could have contributed to the reduced long-term effectiveness of the WCV in use [41,42]. To date, the most prominent circulating strains are typed *PtxA1/Prn2/Fim2-2* or *-/Fim3-2*, as opposed to those in the prevaccination era that were predominantly *PtxA2/Prn1/Fim2-1* or *-/Fim3-1* and are still the base for our first- and second-generation vaccines (Figure 1) [31,32,40,43,44]. In addition to the above allelic shifts, multiple other SNPs were revealed by comparative genomics of strains isolated before or after the introduction of vaccination, that might be linked to successful transmission in vaccinated populations [8]. Hence, comparative genomics data suggest that *B. pertussis*, despite its high population homogeneity, is evolving in response to

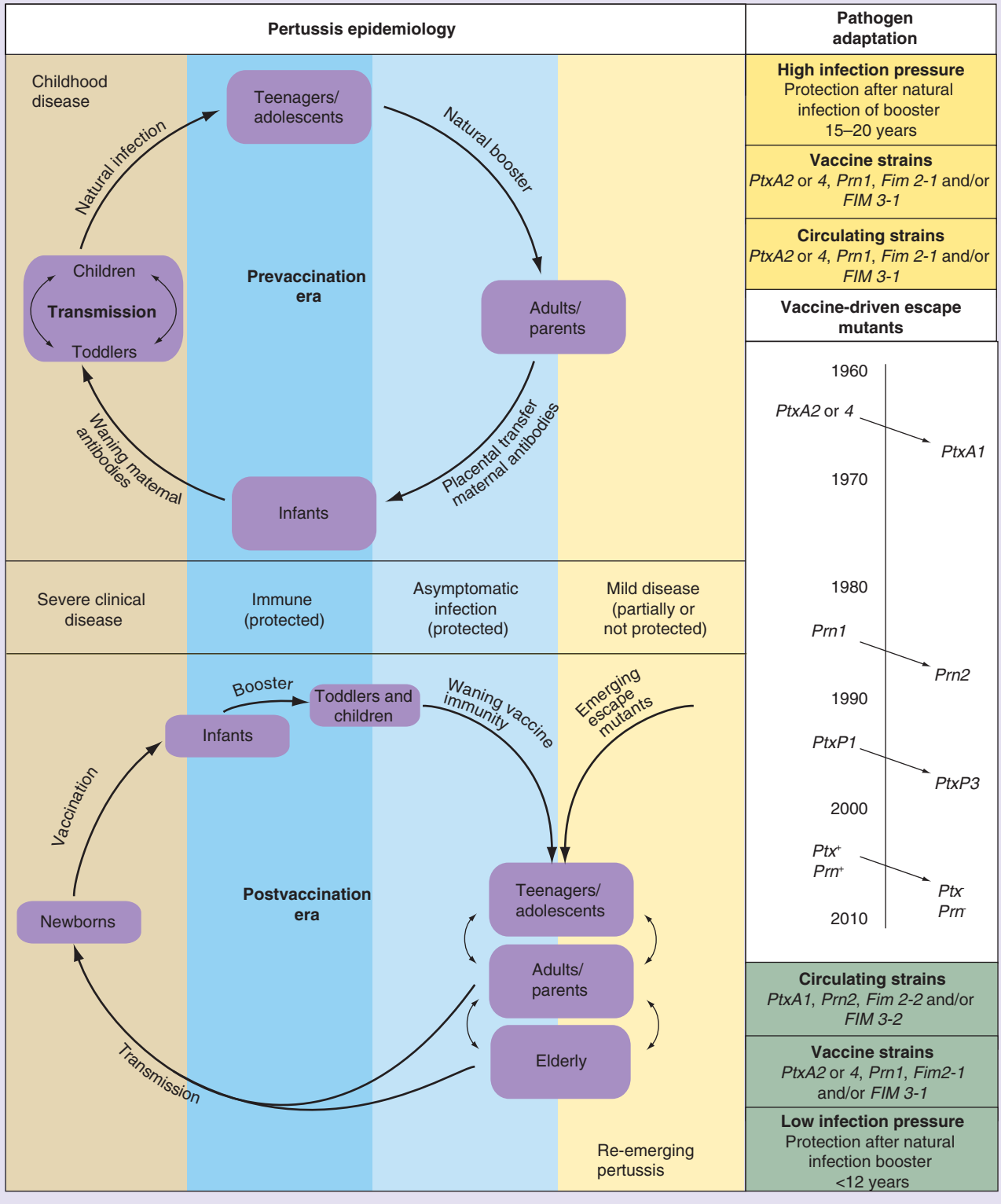

Figure 1. Changes in pertussis epidemiology and evolution of *Bordetella pertussis* since the introduction of mass vaccination programs. Pertussis changed from a classical childhood disease in the prevaccination era to a disease that affects all ages except recently vaccinated groups. *B. pertussis* resurged in well-vaccinated populations largely due to waning of immunity and pathogen adaptation (left panel). Genomic and antigenic shifts in vaccine antigens Ptx, Prn and Fim occurred in time, causing an increased antigenic mismatch between vaccines in use and circulating strains (right panel).

vaccination pressure, resulting in expansion of clones carrying new variants of genes encoding immunogenicity and pathogenicity-associated antigens. The fact that new variant *B. pertussis* strains are more often isolated from vaccinated than from nonvaccinated patients further supports this notion [45,46]. Immune pressure on *B. pertussis* antigens can eventually even result in functional inactivation or entire deletion of the genes coding for them, as has been described for *ptx* and *prn* [45]. Prn deficiency does not seem to be such a rare phenomenon. In France, Bouchez *et al.* noted that a significant percentage (5.6%) of the strains from studied hospitalized newborns below 6 months of age did not produce Prn [45]. Recently, Otsuka *et al* reported that Prn-deficient strains have significantly increased in *B. pertussis* populations since the early 2000s in Japan [47]. Both countries have molecular surveillance systems in place and report stable pertussis vaccination coverage for primary series [47,202,203].

Antigenic mismatches between pertussis vaccine strains and circulating vaccine-evasive *B. pertussis* strains may alter the effectiveness of vaccine-induced immune responses through escape from immune recognition, as will be discussed below. However, adaptation may also affect strain virulence properties or the biological impact of antigens, as is under investigation by several research groups. In preliminary studies, Prn-deficient strains were found to have competing growth advantage *in vitro* [47], but reduced multiplication in adult mice [45]. By producing 1.6-times more of the virulence factor and immune-modulating agent, Ptx, the emerging *ptxP3* strains may alter the course or severity of an infection before the immune response can become effective [41]. Allelic shifts therefore may indeed have provided a selection bias on pathogen colonization, survival and offspring. Taken together, the genetic and antigenic drift of *B. pertussis* in well-vaccinated populations is an acknowledged fact, and is one of the factors jeopardizing the efficacy of current pertussis vaccines. In this, overall gain of advantageous biological properties and loss of immunologic recognition of circulating strains occur side by side.

Induction & maintenance of pertussis immunity

The general view of human pertussis-specific immune mechanisms is that they protect against disease rather than against infection, and are not long-lived. In the search for a correlate of protection, pertussis-specific serological responses have mostly been studied. Antipertussis antibodies can minimize the infection and its symptoms by preventing bacterial adherence to the respiratory epithelium by neutralizing bacterial toxins and by removing bacteria through opsonization and complement-mediated killing [11,23,48]. Human pertussis vaccination induces initial high levels of specific IgG antibodies [49–52] and pertussis infection also induces specific IgA [53]. Although the presence of certain specificities of antipertussis antibodies has been associated with protective immunity in a household exposure setting and higher levels are generally regarded as more protective, no accepted threshold of specific antibodies to a single pertussis antigen, such as for tetanus or diphtheria toxins, is established as a correlate of protection. In a mouse model, protection against infection was shown in the absence of detectable antibodies [54,55], suggesting some kind of protection by recall of immunologic memory. In such case, pertussis specific memory B cells, which can be enumerated in immunized humans and mice [56–59], are triggered to provide a rapid rise in the number of plasma cells and in levels of antibodies with improved affinity.

Because T cells highly regulate antibody responses and are responsible for the production of antimicrobial cytokines, a wide interest also exists for human and murine T-cell responses specific for *B. pertussis* antigens after vaccination or infection. These responses have been associated with a variety of functional cytokines [11,13,60,61]. Recovery from whooping cough in older infants is associated with induction of T lymphocytes producing Th1-type cytokines IFN-γ and IL-2 [61]. Studies in very young children (2 months of age) show that natural infection with *B. pertussis* or a first administration of WCV can promote maturation of Th1-type responses [61,62]. By contrast, the widely used ACV vaccines also induce Th2-type cytokine (IL-4 and IL-5) responses to concomitant vaccine antigens in infants [60,62], becoming more pronounced when given closer to birth [63,64]. Although suggested earlier, current infant immunization with ACV does not seem to be a risk factor for the development of atopic disease in children [65,66], despite similarities in Th2 polarization of vaccine-induced and allergic T-cell responses. Recently, in a systems-level approach, it was demonstrated that the Th2 component of ACV-induced responses was counterbalanced by parallel Th2-antagonistic and antimicrobial immunity, which was either not present or actively downregulated within the Th2-polarized responses associated with allergic inflammation [67]. However, strong Th2 polarization of ACV-induced T-cell immunity might be an unfavorable effect, as it has been implied as a potential cause of local reactions to booster vaccinations [68,69]. In addition to Th1 cytokines [23], IL-17 has recently been identified as another protective cytokine in preclinical models of pertussis vaccination and challenge [70–72]. IL-17 has also been implied in *in vivo* and *in vitro* programming of human T-cell responses by *B. pertussis* [73,74]. However, IL-17 and any other proinflammatory cytokines could mediate immunopathology in the lung. Probably to avoid this, pertussis T-cell responses are strongly controlled by regulatory T cells [11].

Clearly, both humoral and cell-mediated immune responses readily react to *B. pertussis* antigens, but so far no single specificity, type or level of effector mechanism has been implied as a preclinical or clinical correlate of protection. Likely, protection involves a threshold of mucosal and systemic immunity to prevent colonization of *B. pertussis* in the nasopharynx and early production of bacterial toxins. The duration of the protected status then depends on the initially induced level of immunity and its subsequent natural contraction and maintenance in time. While for some classical examples of viruses maintenance of protective immunity may last for decades with or without boosting [75–77], this clearly seems not to be the case for pertussis.

Mechanisms underlying progressive loss of protective immunity against pertussis

Although initially induced at a sufficiently protective level, acquired pertussis-specific immunity is progressively lost with time, as

indicated by epidemiological data. Vaccinees may become at risk of natural infection with currently circulating *B. pertussis* strains as early as 2 years after vaccination, as suggested for children after preschool booster by De Greeff *et al.* [78]. Hence the duration of protection against pertussis compared with other vaccine-preventable diseases is relatively short, implying that pertussis-specific immunological memory mechanisms are not effective in the long term. Ready induction of specific serum antibody levels after vaccination or infection, as well as their rapid fall, are well documented in humans and mice [13,52,56–59,79–83]. Recently, Hendrikx *et al.* reported that despite waning of antibody titers several years after primary or preschool booster vaccination, children may have detectable circulating pertussis-specific memory B cells [57]. Moreover, levels of these Ptx, FHA and Prn specific memory B cell populations can be shown to rise shortly after a booster vaccination, yet their waning is also observed within 1 year [81,84]. This exemplifies that natural expansion and contraction of human pertussis-specific B-cell mechanisms occur after vaccination. Little knowledge is available on the long-term behavior of human pertussis-specific T-cell responses. Based on ^{3}H-thymidine incorporation, pertussis-specific cellular responses were found to wane 3–4 years after their induction by primary pertussis vaccination of infants, which in part seemed masked by ongoing natural boosting through subclinical infections [13]. With the benefits of a pathogen-specified environment and accessibility of various tissue samples, long-term persistence of immune mechanisms to pertussis can also be studied in mice. Such analysis revealed a lack of prolonged maintenance of mouse splenic memory B-cell populations specific for Ptx, FHA and Prn [58], as opposed to third-party vaccine antigen [85], suggesting a possible weakness in the self-renewal capacity of the pertussis antigen-induced memory B cells. On the other hand, follow-up of pertussis vaccine-induced murine T-cell responses suggested their prolonged persistence, but more importantly emphasized the sustained Th1 versus Th2 cytokine imprinting of cell-mediated immune responses induced by WCV and ACV, respectively [van Els C, unpublished data] [55]. More long-term clinical and preclinical studies on the characteristics of specific memory T and B-cell populations following pertussis vaccination or infection are needed to shed more light on their typical rise and contraction, but more so on the mechanisms responsible for the progressive loss of protective immunity to pertussis [86].

Basically, defects in durable immunological protection to circulating *B. pertussis* strains may arise at two different encounters between the host immune system and pertussis antigens. First, during the primary immune response to WCV or ACV, the initial clonal burst size, the differentiation of various lymphocyte specificities and their maintenance into a memory phase may be suboptimal. ACV antigens have been studied for immune-modulating properties in primary immune responses and were implied to have an effect on immunogenicity of coadministered vaccine components [87]. McGuirk *et al.* studied the *in vivo* immunomodulatory effect of detoxified Ptx when formulated with other antigens and did not find any effect [88]. On the other hand, FHA was found to suppress *in vitro* IL-12 production and to enhance IL-6 and IL-10 production by macrophages [89]. In mice, maintenance of memory B-cell populations to ACV antigens was found to be compromised compared with other vaccine antigens [58,90]. Hence, ACV antigens may have intrinsic properties affecting the primary immune response. This might not become critical in the first few years after vaccination but only after some natural waning.

Second, when encountering live *B. pertussis* the secondary immune response may lack efficiency. At this level, various mechanisms of evasion of protective immunity can be envisaged. The *B. pertussis* genus has acquired various evolutionary properties able to evade the innate phagocyte system as an important effector arm of adaptive immune responses. Induction of innate inflammatory chemokines, cytokines and of other immune mediators is needed for phagocytosis and bacterial killing. Ptx delays neutrophil recruitment and bacterial killing by inhibiting the production of early chemokines (RANTES, MCP-1, MIP-1 and IL-8) [91–93]. In addition, FHA, adenylate cyclase hemolysin (AC-Hly) and tracheal cytotoxin, as well as antibodies to lipopolysaccharide and surface-localized adherence factors interfere with recruitment, bacterial uptake, bactericidal activity and cell survival of monocytes and neutrophils [90]. Furthermore, lipopolysaccharide, CyaA, tracheal cytotoxin, type III secretion system, Ptx and (indirectly) FHA can subvert innate Toll-like receptor signaling pathways, resulting in inefficient antigen presentation and production of proinflammatory cytokines by DCs, important for the recall of memory T cells [71,72,94]. Ongoing evolutionary adaptation of *B. pertussis* in immune populations could lead to selection of variants even more efficient in escaping recall immunity. Whether allelic variants of PtxS1 or Prn have differential capacity to undermine secondary immune responses is, to our knowledge, unknown, but increased levels of Ptx expression, such as relating to the *ptxP3* mutation [41], will likely have an impact.

On the other hand, antigenic mismatches between vaccine-induced immune responses and the antigenic profile of circulating strains, accumulating through ongoing strain adaptation, will compromise long-term effectiveness of protective immune mechanisms in the vaccinated population. Antibody or T-cell cross reactivity between allelic variants of Ptx S1, Prn and fimbriae may still ensure protection, depending on the absolute level of the memory response, but loss of entire expression of important vaccine antigens such as Ptx and/or Prn in circulating strains will unambiguously narrow the effective breadth of the recall response [45].

Hence, suboptimal long-term maintenance of protective immunity against whooping cough relates to complex interplay between *B. pertussis* components and the host immune system at various time points of their interaction. More fundamental understanding of the essential levels and mechanisms of immunity correlating with protection against disease, as well as of the major obstruction(s) for their long-term persistence, is needed to help design improved vaccines with a longer duration of protection. Animal research is instrumental in developing such knowledge, as well as in investigating novel vaccine candidates.

Expert commentary

Various animal models are available for pertussis research (Table 1) [95,96]; however, these should be reviewed and optimized

in the context of the emergence of novel *B. pertussis* strain variants in the vaccinated host, and the need for innovation of vaccines or schedules to improve the duration of protection. Ideally, new vaccination strategies for pertussis should be studied in a reasonably accessible animal model that can sense differences in clinical manifestations (more or less resembling human pathophysiology) after infection with *B. pertussis* strain variants, and can assess protection and duration of immune mechanisms relevant to human, preferably against acceptable costs. However, it is unlikely that one animal species or one model will answer all research questions. Also, all experimental animal species will have lifespan restrictions when testing durability of protection. Moreover, no model, not even the human, has disclosed a defined correlate or surrogate of protection for pertussis (Table 1) [12,18,22,96–113]. Although some animal models (ic-MPT and in-MCM) showed an indirect relationship between vaccine-induced short-term protection in humans and animals [18,19,24], this correlation may fade in time due to the continuous genetic and antigenic evolution of *B. pertussis*. It is our view that the ongoing pathogen adaptation of *B. pertussis* asks for innovation of presently available animal models throughout the chain of vaccine research, development and postmarketing surveillance, introducing the use of circulating *B. pertussis* strains. Notably, excluded from this innovation are mandatory animal tests for the quality control of vaccine batches – that is, so-called toxicity and potency tests – since these are used to demonstrate safety and consistency in production and do not relate to the efficacy of vaccines.

Introducing currently circulating pertussis strains in various animal models will have consequences for their design, as the models are generally optimized for the use of laboratory-attenuated infectious strains [114–116]. Pertussis animal models imply various animal species, each allowing the assessment of one or more experimental parameters, such as strain pathogenicity, host response to infection, or immunogenicity and effectiveness of candidate or registered vaccines, but also having their limitations (Table 1) [95,96]. For instance, nonhuman primates are the most suitable experimental animals for pathogenicity research since these animals can display classical whooping cough or persistent coughing [97,106,109–111]. When compared with rodents their immune responses are likely more similar to humans, and these animals live longer, allowing duration of protection to be studied over time. However, these models are expensive and ethically not acceptable for wide-scale use. A more accessible and the most commonly used animal model in pertussis vaccine research is the in-MCM (Table 1) [22,95]. This model was shown to predict efficacy of ACV, as well as WCV, in a relatively reproducible manner [22,23], but in fact measures prevention of *B. pertussis* infections by host defense mechanisms of the upper rather than the lower respiratory tract [99]. Using this model, Mills and coworkers demonstrated similarities to human immune parameters such as the requirement of specific T and B cells for protection [23], and, in accordance with clinical studies, no clear correlation between specific antibody responses and protection [11,24]. However, the initially found similar ranking of the ACV under test in terms of estimated efficacy in children and potency in the in-MCM could not formally be confirmed in an international collaborative study [117]. As an exception, the coughing rat model displays symptoms reminiscent of human disease and could therefore serve as a more relevant infection model in a small experimental animal. This model has only been used to a limited extent due to the intrabronchial infection procedure, involving agarose-embedded bacteria [101–104,108]. However, recently we demonstrated the feasibility of the coughing rat model to study the pathogenesis of different circulating strains and that the route of infection, intrabronchial instead of intranasal, determines the level of disease and immunity against *B. pertussis* [118]. Until now however, reasons of convenience, such as availability of species-specific immunochemical reagents and in-house animal husbandry experience, often guide the choice of the pertussis animal species in pertussis models.

Depending on the phase in pertussis vaccine research and development, and for each topic to be studied an experimental animal species should be chosen (e.g., mouse, rat, monkey and others). Furthermore, the animal model of choice should be optimized with respect to: covariates for infection (e.g., clinical isolate, laboratory strain, recombinant strain, inoculum and route); covariates for vaccination (e.g., vaccine or immunogen, dose, route and schedule); and end points of disease, protection and/or immunity (e.g., disease features, reduction of disease features, clearance, magnitude, quality and duration of immunological features), as is summarized in Table 2. In the following section, we discuss improvements of basic infection, vaccination and challenge models and their use to substantiate the protectiveness of current pertussis vaccines and novel vaccine candidates against newly circulating *B. pertussis* strain variants.

Infection models

Infection models are needed in basic pertussis research and also form the basis for challenge models to test effectiveness of vaccine candidates or regimens in the preclinical phase. The choice of covariates and end points are highly decisive for whether differences in pathogenicity between *B. pertussis* isolates can be demonstrated or not, and should be made carefully (Table 2). The most commonly used *B. pertussis* strains in animal models of infection, 18323, Wellcome 28 and Tohama I, are laboratory subcultured and attenuated strains, and hence genetically different from clinical isolates [37,114,119]. Since differences in genome sequences may affect the pathogenicity of *B. pertussis* in animals and humans, these laboratory strains are not representative for current *B. pertussis* strains. In fact, the same is true for clinical isolates and vaccine strains descending from the prevaccination era, from which currently circulating *B. pertussis* strains also differ genetically through loss, rearrangement and adaptation of genes during 50 years of mass vaccination [120,121]. Infection models should therefore be optimized for the use of relevant clinical and recombinant *B. pertussis* strains in addition to laboratory strains. Recently data from Van Gent *et al.* suggested that an allelic shift from *prn1* to *prn*2 had a trade-off in the ability of *ptxP1* clinical isolates to colonize naive hosts in a rodent animal model, *prn2* strains being less capable of colonizing the mouse lung [122]. Earlier, using isogenic mutants of

Table 1. Brief summary of features of pertussis animal models compared with human infection and immunity.

Animal species	Model	*B. pertussis* strain	Infection and shedding	Clinical signs postinfection	Surrogate of protection	Whole cell vaccines	Acellular vaccines	Vaccine candidates	Remarks
Mouse	Kendrick test (ic-MPT) [17]	Only strain 18323 is infectious and virulent clinical isolates are not†	Intracerebral (10^7–10^8 CFU)	Lethal due to meningitis and no signs of classical disease	Concentration of antibody against outer membrane proteins at day 14 is an indication for survival [102,103]	Mandatory release test. Relationship between WCV efficacy and potency but poor in reproducibility [104]	Not protective, only in suckling mouse test [18]	OMV are protective. 92 kDa/38 kDa OMP ratio is indicative for protectiveness of vaccine under test [95]	In use to demonstrate consistency in production and stability of WCV
Mouse	Respiratory challenge model (RMCM and in-MCM) [20,21]	Laboratory strains [102] and clinical isolates occasionally [34]. 18323 more rapidly cleared than other strains†	Intranasal or aerosol (10^3–10^7 CFU); shedding in ±6 weeks	No signs of classical pertussis, only weight retardation	No direct correlation of protection between antibody and/or cellular immune responses and clearing of the lungs	PtxA1/Prn2 WCV are more protective than Ptx2/Prn1 WCV [97]	ACV protectiveness in mice related to ACV efficacy in children [22] but could not be confirmed in an international collaborative study	OMV [122–124] and BPZE1 [118–121] are protective. Relationship between anti-Ptx and FHA antibody responses and protection [90]	Mostly used as research model and suggested for preclinical studies (WHO)
Mouse	Immunogenicity test	Not applicable	Not applicable	Not applicable	Antibody responses are indicative for protection	Low in anti-Ptx, FHA and Prn titers and high in anti-OMP titers [102–104]	Release test for ACV in Europe and America but no direct correlation with protection in man [19–22]	No reports	In use to demonstrate consistency in production and stability of ACV
Rat	CRM [91–94,98]	Mainly 18323 and Tohama 1 embedded in agarose	Intrabronchial (10^5–10^8 CFU); shedding in ±3 weeks	Resembles human disease including paroxysmal coughing	No direct correlation of protection between antibody and/or cellular immune responses and clinical disease	Protects against disease and not infection as in humans	Protects against disease and not infection as in humans	No reports	Interesting as preclinical model
Rabbit	Respiratory infection model [101]	Multiple clinical isolates	Intranasal and intratracheal, (10^6–10^{10} CFU); shedding in 2–10 months	Fever but no classical sign of disease. Interference by *B. bronchiseptica* infections†	Not established	No reports of vaccination studies			Switch of serotypes during infection observed

†Nonpublished observation of authors or personal comments of other scientist.
ACV: Acellular vaccine; CRM: Coughing rat model; FHA: Filamentous hemagglutinin; ic-MPT: Intracerebral mouse protection test; in-MCM: Intranasal mouse challenge model; OMP: Outer membrane protein; OMV: Outer membrane vesicle; RMCM: Respiratory mouse challenge model; WCV: Whole cell vaccine.

Table 1. Brief summary of features of pertussis animal models compared with human infection and immunity (cont.).

Animal species	Model	*B. pertussis* strain	Infection and shedding	Clinical signs postinfection	Surrogate of protection	Whole cell vaccines	Acellular vaccines	Vaccine candidates	Remarks
Piglet	Respiratory infection and challenge model [88,89]	Tohama 1 embedded in agarose	Intrapulmonary (10^9 CFU); shedding in 3–7 weeks	Resembles classical disease but no paroxysmal coughing or whoops	Not established	Not a suitable model to study parenteral vaccine efficacy. Only newborn piglets younger than 4 weeks are sensitive to pertussis infection. Piglets older than 4 weeks produce host defense proteins that protect against pertussis but not parapertussis infections			Interesting to study maternal immunity and neonatal immunization
Nonhuman primates	Respiratory infection models [96,99–101,108]	18323 and multiple clinical isolates	Oral, intranasal and intratrachial (up to 10^{11} CFU); shedding in 3–7weeks	Paroxysmal coughing or classical whooping cough; interference by bronchiseptica infections†	Not established	No reports of vaccination studies			Mostly used to study transmission and infection
Human	Respiratory infection and immunity	Circulating *B. pertussis* strains	Respiratory (10^2 CFU); shedding in 8–12 weeks in infants and 4–8 weeks in adults	Classical whooping cough (infants) or moderate respiratory disease (adults)	Both acquired humoral and cellular immune mechanisms are implied, but correlates of protection are unknown; anti-Ptx IgG titer is indicative for recent infection	Efficacy highly dependent on quality of WCV in use and estimated between 36 and 96%; efficacy related to potency in Kendrick test	Efficacy depends on formulation of ACV and is estimated between 59 and 85%. None of the clinical trials yielded a direct correlation between antibody titers and protection	Not yet tested	Correlates or surrogates of protection are not established (in humans nor in animals) due to involvement of multiple immune effector mechanisms against multiple antigens

†Nonpublished observation of authors or personal comments of other scientist.
ACV: Acellular vaccine; CRM: Coughing rat model; FHA: Filamentous hemagglutinin; ic-MPT: Intracerebral mouse protection test; in-MCM: Intranasal mouse challenge model; OMP: Outer membrane protein; OMV: Outer membrane vesicle; RMCM: Respiratory mouse challenge model; WCV: Whole cell vaccine.

the *B. pertussis* strain Tohama I expressing the alleles *ptxA1* or *ptxA2* and *prn1* or *prn2*, Komatsu *et al*. found that the emerged nonvaccine-type mutant *ptxA1/prn2* displayed a prolonged survival in naive mice compared with the other mutants investigated [107]. These results illustrate the feasibility of carefully designed experimental animal infection to unravel the role of allele shifts in *B. pertussis*.

The severity and/or course of infection are generally demonstrated by measuring the reduction in colonization of *B. pertussis* in the lungs in the animal model of infection. The clearance of *B. pertussis* in the nasopharynx and lungs, however, depends highly on the infectious dose, route of infection and the age of the animal of choice. The incubation period is often shorter and natural clearance of the bacteria is faster in animals compared with humans [96]. In particular, mice and rats are highly effective in eliminating bacteria in an aspecific manner from the nasopharynx, by contrast to, for example, monkeys having a more human-like clearance. High infectious doses and artificial routes of infection are therefore inevitable in mice and rats. However, these high pathogen loads will trigger inflammatory responses due to high levels of endotoxins and the bacteria may already have been cleared aspecifically from the respiratory tract before a genuine infection can develop. As an alternative, immobilization and inoculation of the bacterium deep in the lower airways allows the bacteria to colonize in a rat as well as a piglet model (Table 1) [98,101]. Therefore, animals infected with *B. pertussis* embedded in agarose to prevent aspecific clearance of *B. pertussis* can develop pertussis-specific clinical symptoms. Consequently, multiple disease features, such as lung pathology, lung clearance and leukocytosis, and even coughing (in rats), can be monitored and these clinical animal models may therefore underpin similarity or differences in the course or severity of infection between *B. pertussis* strains [van der Ark AAJ. Manuscript in preparation]. To a limited scale, results could be refined and confirmed in nonhuman primate studies [97,106,109–111]. Recently, Warfel *et al*. developed a novel pertussis infection model in Olive baboons (*Papio anubis*) [123]. They demonstrated that all pertussis-infected baboons developed clinical symptoms of pertussis as opposed to only 25% of rhesus macaques (*Macaca mulatta*). Although the exact mechanism remains unclear, it was postulated that the growth of virulent *B. pertussis* is temperature-dependently modulated. The lower normal body temperature of baboons (37–39°C) allows *B. pertussis* to grow in a virulent phase while in rhesus macaques, having a higher normal body temperature (38.7–39.8°C), the virulence of *B. pertussis* is reduced due to inactivation of *CyaA* at temperatures higher than 39°C.

Vaccination & challenge models

Vaccination models are used in the discovery phase of vaccine development to assess the immunogenicity of vaccine leads and in the preclinical phase of vaccine development to characterize the quality, quantity and duration of innate and/or adaptive immune responses induced by promising vaccine candidates. Challenge models, combining vaccination and infection, are generally used to assess the quality and level of vaccine-induced protection against a particular *B. pertussis* strain and often use high vaccine doses, up to one-quarter human dose (HD), to immunize the animals. This vaccine dose should be carefully reconsidered. Although high doses of vaccine induce higher immune responses that are easier to measure, they bear little or no relevant information about the effectiveness of the underlying immune effector mechanisms. When immunized with one-fourth HD, mice receive 12.5 HD per kilogram of bodyweight, while infants and adults are immunized with approximately 0.3 and <0.02 HD per kilogram of bodyweight, respectively.It is like that immune responses induced in mice by one-quarter HD do not wane rapidly, and are still maximal at the time of challenge [58]. This makes challenge models in their current form unsuitable to study the phenomenon of waning immunity, especially, if the sensitivity of the animal to the *B. pertussis* challenge inoculum may alter with increasing age. All of this complicates long-term follow-up studies (>1 year) in rodents after vaccination. Hence, the most-used pertussis research model, the in-MCM, in its current form may not be an appropriate model to test vaccine potency at prolonged term. It should be optimized per vaccine candidate with respect to vaccine dose and strain, and dose and time point of the bacterial challenge inoculum. Another animal species with promising perspectives for long-term protection studies is the baboon [123]. This experimental animal shows features of waning pertussis specific immune responses, has a longer lifespan than rodents, and seems to remain susceptible to pertussis infection with increasing age.

Finally, optimized challenge models should be introduced in the so-called 'postmarketing surveillance phase'. Despite being a suboptimal animal species for *B. pertussis* infection, mice are often used to assess vaccine-induced protection against *B. pertussis* after administration of an infectious challenge inoculum to vaccinated animals [95]. In view of the increasing antigenic mismatch between vaccine strains and clinical isolates and other evaluating features observed, we recommend that the potency of the current pertussis vaccines be periodically tested against emerging *B. pertussis* mutants in an optimized pertussis challenge model. This could reveal the risk of vaccine failure, especially in the face of new strains with functionally altered or inactivated genes, and serve as a feedback loop into the discovery phase of the pertussis vaccine research and development chain.

Scientific substantiation of mechanisms of protection

As stated earlier, direct correlates of protection are still not established for whooping cough, probably due to the fact that multiple target antigens or mechanisms together play a role. Correlates of protection are badly missed in pertussis animal models throughout the various phases of vaccine development (Table 2). The results of animal experiments are often decisive for the continuation of a vaccine candidate and for the release of vaccines. Also valuable would be the identification of early biomarkers predicting the outcome of adaptive immune mechanisms, such as their magnitude, multispecificity, type and long-term maintenance. An integrated strategy to study the

Table 2. Re-evaluation of pertussis animal models in various phases of vaccine research and development.

Discovery phase		
Basic animal models	Infection model to study pathogenicity of *Bordetella pertussis* strain variants and component of challenge model	Covariates • Animal species: monkey, rat or mouse† and age of animal • *B. pertussis* strain variants: old and recent clinical isolates • Route of infection: intranasal, aerosol, intratracheal or intrabronchial • Dose of infection: lethal or nonlethal dose that induces disease features End points • Disease features: lung colonization, lung pathology, coughing, leukocytosis and/or weight retardation, mortality • Time points of sampling to monitor course and severity of infection
	Vaccination model to screen immunogenicity of vaccine preparations and component of challenge model	Covariates • Animal species: mouse, rat, guinea pig, rabbit and/or monkey† • Vaccine preparations (laboratory scale) derived from *B. pertussis* strain variants • Route of immunization: intramuscular, subcutaneous, intraperitoneal, intranasal or alternative • Schedule of immunizations: dose (0.3 human dose/kg) and number of and time intervals between vaccinations End points • Antigen-specific antibody, T- and B-cell responses • Level of immunogenicity
	Challenge model combining vaccination and infection model to screen for protectiveness of vaccine leads	Covariates • Animal species: monkey, rat or mouse† and age of animal • Vaccination: route depending on animal species and vaccine type; serial dilution of vaccine dose and schedule to be optimized • Infection: route depending on animal species; dose of challenge inoculum and time interval between vaccination and challenge to be optimized End points • Survival • Single or multiple disease features (see basic infection model)
Host immune responses	Innate immunity to (components of) *B. pertussis* strain variant(s)	Basic model • Infection or vaccination model optimized for *B. pertussis* strain variants, source and time of host specimen sampling End points • (Early) gene signatures of lymphoid, lung and other tissues • Proteome signatures (e.g., chemokines, cytokines, receptor signaling or marker expression) • Cellular signatures (shifts in immune cell subsets) • Immunomodulation (activation or suppression of particular immune functions)
	Adaptive immunity to (components of) *B. pertussis* strain variant(s)	Basic model • Infection or vaccination model optimized for *B. pertussis* strain variants, source and time of host specimen sampling End points • Specificity, level and functionality of antibody responses • Specificity, magnitude and differentiation of B-cell responses (e.g., memory B cells vs plasma cells) • Magnitude, breadth and differentiation of T-cell effector and memory immune responses

Covariates and end points: choices depend on the aim of the study and take into account *B. pertussis* strain or allelic variants.
†Depending on availability of immunoreagents, knowledge of and experience with the model, and/or ethical considerations.

Table 2. Re-evaluation of pertussis animal models in various phases of vaccine research and development (cont.).

Preclinical phase		
Selection of vaccine candidates	Cross protection of vaccine candidates against *B. pertussis* strain variants	Basic model • Challenge model optimized for type of vaccine candidate and *B. pertussis* strain variants for infection, time interval between vaccination and infection End points • Level of protection against one or multiple disease features • Level of cross protectiveness against *B. pertussis* strain variants
Scientific substantiation of mechanisms of protection	Characterization of immunity after vaccination	Basic model • At least two vaccination models (animal species) adjusted to the type of preclinical (up-scalable) vaccine candidate and its delivery vehicle, with short- and long-term follow-up of immune responses End points • Features of antigen-specific host (innate and) adaptive immune responses and effector mechanisms • Duration of immune responses (or waning of immune responses)
	Optimization of vaccine formulation for long-term duration of protection against *B. pertussis* strain variants	Basic model • Challenge model reflecting waning of vaccine-induced immunity using variable vaccine formulations (i.e., adjuvants, solvents, preservatives or stabilizers) End points • Type and concentration of adjuvant to sustain protection • Identification, quality and quantity of innate and adaptive immune parameters that correlate with short- and/or long-term protection
Postmarketing surveillance		
Monitoring vaccine failure	Periodical control of effectiveness of licensed pertussis vaccines against circulating *B. pertussis* strains	Basic model • Challenge model suitable for use of licensed pertussis vaccine, for use of emerging *B. pertussis* strain(s) as challenge inoculum, and to sense (rapid) loss of vaccine-induced protection End points • Monitoring of short- and long-term vaccine-induced protection against one or multiple disease features • Duration of survival of *B. pertussis* strain(s) in the immune host

Covariates and end points: choices depend on the aim of the study and take into account *B. pertussis* strain or allelic variants.
†Depending on availability of immunoreagents, knowledge of and experience with the model, and/or ethical considerations.

mechanisms that form and sustain a highly protective immune response against pertussis could be 'systems biology'. This new field targets complex interactions in biological systems, such as the entire immune system, and uses, visualizes and interprets extensive data sets obtained from various technology platforms, such as genomics, transcriptomics and (immuno)proteomics. Currently, a few examples are described in the literature illustrating the advances of systems biology in vaccine research and development [124–126]. After vaccination or infection, the immune system reacts with the up- or down-regulation of genes associated with host defense. The corresponding proteins are involved in the elimination of the pathogen or the vaccine antigens – that is, associated with cytokine expression, cell differentiation, isotype switching or complement activation. Protection against pertussis is not based on one single feature, such as a minimal concentration of neutralizing antibodies, but on a detailed network of many, yet still hardly known, factors. Information obtained from initial clinical data sets using the technology platforms could be integrated, and predictive gene signatures could be further validated and optimized in animal models of protective immune responses against pertussis. Pulendran *et al.* suggested the development of a vaccination chip consisting of a minimal set of host genes which can elucidate the protective activity induced by a specific vaccine [127]. We suggest that such a vaccination chip should be developed for the most-used and informative animal species in pertussis vaccine research, the mouse, the rat and, eventually, monkeys. The systems biology approach would then allow an in depth evaluation of early gene signatures of vaccine leads, adjuvants and others, and the effect on the quality and duration of a protective immune response. Moreover, the systems biology approach in animal models should be linked to early human signatures related to durable specific immune responses in Phase I and II clinical studies of improved vaccines against pertussis.

Five-year view

The continuous need for animal models in pertussis vaccine research, development and release is unambiguous. However, a re-evaluation of the current pertussis animal models is necessary to find a solution for the resurgence of pertussis in well-vaccinated populations by innovating pertussis vaccines and vaccination. Depending on the phase of vaccine research and development, we suggest a focus on particular points for optimization or renewal of these models for the next 5 years (Table 2). The focus in the discovery phase is especially on improving infection models and studying immunogenicity of new vaccine leads. In preclinical development, an optimized challenge model with a relevant dose of vaccine candidate and choice of challenge strain is most important to predict efficacy. In this phase, we recommend to study not only the short-term but also the long-term features of immunity, protection and level of cross protection to various isolates. For some candidates, this can eventually also be performed in more sophisticated animal models in which more extended evaluation of protective immunity is feasible. Mandatory release and consistency testing of clinical batches of vaccines can still be performed in the available standard testing procedures including animal tests. Finally, in the postmarketing surveillance phase, alongside clinical 'Phase IV' studies, the use of animal models should be introduced to predict and measure loss of protection against currently circulating strains. Antigenic drift of *B. pertussis* vaccine antigens has so far been observed in Ptx, Prn and fimbriae, but more virulence factors of *B. pertussis* are evolving. These may not be direct targets of vaccine-induced immunity, but could rather influence the effectiveness of the memory host response or affect colonization or survival [90]. Hence, it is important in this phase to continuously collect clinical isolates in the vaccinated population, not only for assessment of virulence and immunomodulation properties in basic animal models, but also for periodical testing of effectiveness of licensed and used ACV or WCV in an optimized animal challenge model using the newest strains. In our view, a suitable animal model for such 'surveillance of protection', should sense changing biological properties of genomically drifting *B. pertussis* species and would preferably display clinical symptoms reminiscent of human disease.

Given the limited duration of protection of current WCVs as well as ACVs, new vaccination strategies should be developed avoiding a too narrow basis of protective antigens, as well as early exhaustion of host immune mechanisms. Insight into the induction of long-term protection could lead to a novel generation of optimized pertussis vaccines and vaccine components (antigens, adjuvants), immunization routes and vaccination schedules, possibly with the use of the systems biology approach linking early gene signatures to protective immune responses. Candidates include new vaccine concepts such as the live attenuated vaccine strain BPZE1 [100,128–131] and outer membrane vesicle vaccines [105,132–134], which all can be administered intranasally and may induce a wider, more effective and tissue-targeted long-term protective response. Alternatively, new adjuvants could be introduced that direct the vaccine-induced immune responses more effectively towards the preferred Th1 responses without evoking side effects. However, each new vaccination strategy will lead to further ongoing pathogen adaptation and it is therefore of great importance that animal testing and *in vitro* immunoassays are optimized and re-evaluated for strain usage on a regular basis.

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Key issues

- Pertussis resurged in well-vaccinated populations worldwide, largely due to rapid loss of protective immunity and pathogen adaptation. *Bordetella pertussis* remained endemic because available pertussis vaccines mainly prevent disease and to a lesser extent infection and transmission.
- Adaptation of currently circulating *B. pertussis* strains to the immune population may cause immune evasion through altered features either of pathogenicity or of the interaction with the host immune system. These altered features may allow the bacterium to (more) successfully circumvent vaccine-induced immunity, compromising the efficacy of current pertussis vaccines, at the short and long term.
- Re-evaluation and innovation of current pertussis animal models, to imply the use of newly circulating *B. pertussis* strains, is highly necessary in pertussis vaccine research and development. Models should be optimized with regard to choice of animal, infection and immunization procedures (e.g., route, doses, schedule and strains), and end points of infection, immunity and protection, to be able to monitor alterations in pathogenicity, and evasion of immunity and vaccine effectiveness as *B. pertussis* evolves.
- Direct correlates of protection for pertussis are still not established but are urgently needed throughout the various phases of vaccine research and development. Therefore, the identification of early biomarkers predicting the magnitude, multispecificity, type and long-term maintenance of adaptive immune mechanisms against the continuously evolving *B. pertussis* is of great importance.
- Postmarketing 'surveillance of protection' of registered vaccines against emerging vaccine-evasive strains of *B. pertussis* in improved animal challenge models, should be an extended activity of continuous disease surveillance programs. For this, collecting isolates in vaccinated populations is essential.

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