Vaccine 34 (2016) 4429–4436

Contents lists available at ScienceDirect

Vaccine

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

Adaptive immune response to whole cell pertussis vaccine reflects vaccine quality: A possible complementation to the Pertussis Serological Potency test

M.E. Hoonakker [a,b,*,1], L.M. Verhagen [a,c], L. van der Maas [a], B. Metz [a], J.P. Uittenbogaard [a], B. van de Waterbeemd [a], C.A.C.M. van Els [c], W. van Eden [d], C.F.M. Hendriksen [a,b], A. Sloots [a], W.G.H. Han [c]

[a] Institute for Translational Vaccinology (Intravacc), Bilthoven, The Netherlands
[b] Utrecht University, Faculty of Veterinary Medicine, Department Animals in Science and Society, Utrecht, The Netherlands
[c] Centre for Immunology of Infectious Diseases and Vaccines, National Institute for Public Health and the Environment, Bilthoven, The Netherlands
[d] Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form 17 June 2016
Accepted 6 July 2016
Available online 21 July 2016

Keywords:
Whole cell *Bordetella pertussis* vaccines
Alternatives to animal experiments
T helper cells
Antibodies

ABSTRACT

Whole cell *Bordetella pertussis* (wP) vaccines are still used in many countries to protect against the respiratory disease pertussis. The potency of whole-cell pertussis vaccine lots is determined by an intracerebral challenge test (the Kendrick test). This test is criticized due to lack of immunological relevance of the read-out after an intracerebral challenge with *B. pertussis*. The alternative *in vivo* test, which assesses specific antibody levels in serum after wP vaccination, is the Pertussis Serological Potency test (PSPT). Although the PSPT focuses on a parameter that contributes to protection, the protective immune mechanisms after wP vaccination includes more elements than specific antibody responses only. In this study, additional parameters were investigated, i.e. circulating pro-inflammatory cytokines, antibody specificity and T helper cell responses and it was evaluated whether they can be used as complementary readout parameters in the PSPT to assess wP lot quality. By deliberate manipulation of the vaccine preparation procedure, a panel of high, intermediate and low quality wP vaccines were made. The results revealed that these vaccines induced similar IL-6 and IP10 levels in serum 4 h after vaccination (innate responses) and similar antibody levels directed against the entire bacterium. In contrast, the induced antibody specificity to distinct wP antigens differed after vaccination with high, intermediate and low quality wP vaccines. In addition, the magnitude of wP-induced Th cell responses (Th17, Th1 and Th2) was reduced after vaccination with a wP vaccine of low quality. T cell responses and antibody specificity are therefore correlates of qualitative differences in the investigated vaccines, while the current parameter of the PSPT alone was not sensitive enough to distinguish between vaccines of different qualities. This study demonstrates that assessment of the magnitude of Th cell responses and the antigen specificity of antibodies induced by wP vaccination could form valuable complementary parameters to the PSPT.

1. Introduction

Whole cell pertussis (wP) vaccines are used since the '50 and are still widely applied as an effective strategy in Latin America, Africa and Asia [1] to induce protection against whooping cough, caused by the Gram-negative bacterium *Bordetella pertussis*. Due to occasional side effects associated with wP vaccination, wP vaccines have been replaced by safer acellular pertussis (aP) vaccines in the 1990's in most industrialized countries. Though both aP vaccines and wP vaccines are effective in pertussis prevention campaigns, recent studies indicate that wP vaccination provides superior protection and durability of immunity [2,3]. It is therefore likely that wP vaccines will stay the vaccine of choice in many regions of the world in the near future.

Vaccine production is a biological process and is therefore associated with inherent variability in vaccine lot potency and safety, characteristics that are measured by regulatory required animal tests. The relevance of many of these *in vivo* models is disputable because they are not always susceptible to the pathogen of interest

* Corresponding author at: Institute for Translational Vaccinology (Intravacc), Bilthoven, The Netherlands.
E-mail address: Marieke.Hoonakker@Intravacc.nl (M.E. Hoonakker).
[1] Tel.: +31 307920451.

http://dx.doi.org/10.1016/j.vaccine.2016.07.011
0264-410X/

[4] and they do not always accurately mimic the human immune system [5]. Since mice are normally not susceptible for respiratory *B. pertussis* infection, the protective properties of wP vaccines are assessed by an intracerebral challenge of vaccinated mice, with lethality as end parameter [4]. Because it is unlikely that the pathology and immunology of a cerebral infection accurately reflects the responses induced by a natural respiratory infection in humans, and because of animal welfare reasons, there is a urgent need for alternative *in vivo* models and *in vitro* models that can provide a more accurate reflection of clinically relevant aspects of wP vaccine quality.

One of the alternatives under development is the Pertussis Serological Potency test (PSPT) [6–8], a test based on the detection of wP vaccine-induced *B. pertussis*-specific antibodies in mouse serum, a parameter that contribute to protection. However, the protective immune mechanisms after wP vaccination also depend on the vaccine's capacity to induce appropriate activation of innate immune responses and subsequent vaccine-specific T- and B cell responses. It has been shown that in humans *B. pertussis* infection as well as wP vaccination induce the formation of *B. pertussis*-specific antibodies [9,10], T helper (Th) 1 cells and/or Th17 directed cellular responses [11–13]. Similar adaptive immune mechanisms contribute to clearance of respiratory *B. pertussis* infection in mice [14,15]. In this study, we investigated whether other immune parameters can complement the current readout parameter of the PSPT, being *B. pertussis*-specific antibody responses, using wP vaccines of various protective properties [16]. We studied the type of immune response these wP vaccines induce in two murine outbred strains (RIVM:NIH and CD1; strains used for wP potency testing) and evaluated whether the novel measured immune parameters reflect vaccine quality.

2. Materials and methods

2.1. Preparation of wP vaccines

In this study, experimental wP vaccines were prepared as described in detail elsewhere [16]. Briefly, vaccines were generated from the *B. pertussis* strain 509 (Intravacc) cultured in a 3L bioreactor (2L working volume). The bioreactor was equipped with a six-bladed Rushton stirrer (Applikon, Schiedam, The Netherlands). After a pre-culture of the bacterium, the reactor was seeded at 5% (v/v), grown at 35 °C and after 3–5 reactor volumes steady state was assumed. Subsequently, deliberate downregulation of virulence genes ($t = 0$) was induced by addition of medium containing 50 mM $MgSO_4$. Meanwhile, $MgSO_4$ was added directly to the culture to instantly change the concentration to 50 mM $MgSO_4$. Samples were taken just before addition of $MgSO_4$ (0 h) and after 6 and 24 h. To generate vaccines, the samples were inactivated by adding formaldehyde (16 mM) followed by heating at 56 °C (for 10 min). Three individual runs were performed and corresponding vaccine preparations were pooled and referred to as vaccine A ($t = 0$), vaccine C ($t = 6$) and vaccine E ($t = 24$). The vaccine were diluted to 8 IOU/mL and 0.5 IOU/mL based on the OD_{590} of the vaccines ($1 * OD_{590} = 20$ IOU). The *in vivo* challenge test [16] revealed that the potency of vaccine A was the highest (7.0 IU/mL), the potency of vaccine C was intermediate (4.8 IU/mL) and the potency of vaccine E was the lowest (0.8 IU/mL) (Table 1). Vaccine A and C fulfilled the regulatory requirements with respect to potency, while vaccine E did not. In contrast to the behaviour of the vaccines in the intracerebral challenge test, differences in protective properties were not consistently found in respiratory infection experiments (data not shown). This discrepancy indicates that the intracerebral infection model has a higher sensitivity for subtle difference in wP vaccine quality, confirmed by earlier studies using similar vaccines [17,18]. If a respiratory challenge model would be used to detect qualitative differences between wP vaccines, adaptation and optimisation of this *in vivo* method will be required. In contrast, *in vitro* and immunochemical evaluation [19] showed that hTLR4 and moDC activating as well as the virulence protein content corresponds well with *in vivo* potencies (Table 1).

2.2. Ethical statement and immunisation of mice

This study was approved by the local Committee on Animal Experimentation of PD-Alt under permit numbers 201300053 and 201400128. Groups of adult (20–24 g) mice (n = 6 mice/group) of the outbred RIVM:NIH strain were vaccinated once i.p. with wP vaccine A, C or E at a dose of 0.25 IOU/mouse or 4 IOU/mouse, or were not vaccinated. Adult (20–24 g) mice of the outbred CD1 strain (Harlan, the Netherlands) were injected once i.p. with wP vaccine A, C or E at a dose of 4 IOU/mouse wP vaccine ($n = 12$ mice/group) or PBS ($n = 8$ mice). Similar to the conventional PSPT, all groups consisted of an equal number of females and males. CD1 and NIH mice both originate from Swiss mice, but their lineages were separated in 1930 [20]. These mouse strains are used for quality control testing of wP vaccines, i.e. for the Kendrick test [21] and the PSPT [7].

From the CD1 mice, blood samples were taken by orbital puncture 4 h after vaccination. 28 days after vaccination, CD1 and RIVM:NIH mice were bled under anaesthesia (isoflurane in O_2) and sacrificed (cervical dislocation) and sera and spleens were isolated. Whole blood was collected in blood collection tubes (Z Serum Sep GOLD, Greiner Bio-One), centrifuged (15 min, 3500 r.p.m.) and serum was stored at −80 °C until further use.

2.3. Analysis of B. pertussis-specific antibodies

IgG1 and IgG2A antibodies were measured using ELISA plates coated with inactivated whole *B. pertussis* bacteria (Kh96/01 or BP18323 as indicated). Sera were diluted 1:100 followed by a dilution series of 1:3. *B. pertussis*-specific antibodies were detected by HRP-conjugated goat-anti-mouse IgG1 and IgG2A antibodies (Southern Biotech) and subsequent incubation with peroxidase substrate for 10 min, and 2 M H_2SO_4. The presented values are the detected ODs at 450 nm (ELISA reader, Bio-Tek) × dilution of the serum. The limit of detection for both antibody subclasses was at

Table 1
Properties of vaccines used within this study.

	Vaccine A	Vaccine C	Vaccine E	Reference
Virulence proteins content	High	Intermediate	Low	Hoonakker et al. (submitted)
Protective properties	7.0[a]	4.8[a]	0.8[a]	Metz et al. [16]
Activation of moDC	High	High/intermediate	Low	Hoonakker et al. [19] Biologicals
Activation of hTLR4	High	Intermediate	Low	Hoonakker et al. (submitted)
Activation of mTLR4	High	High	High	Hoonakker et al. (submitted)

[a] Sufficient is a potency of at least 4 IU/mL and 95% interval with a lower limit of 2 IU/mL.

an OD of 0.1. The sera of vaccinated but non-responding mice (OD < 0.1) were excluded from the 2D electrophoresis analysis.

The antigen specificity of the IgG antibodies was analysed as described in detail by Raeven et al. [22]. Briefly, *B. pertussis* (strain 509) lysate was separated by 2D electrophoreses and transferred to a nitro cellulose membrane. For both mouse strains, blots were incubated with pooled sera of vaccinated or naïve mice (diluted 1:1000), followed by incubation with a goat-anti-mouse IgG_{total} antibody labelled with IR800 (diluted 1:5000) and spots were analysed using an Odyssey infrared imager and Delta2D software (Version 4.5). Spots were manually excised from the Coomassie stained gels and digested with trypsine in 50 mM triethylammonium bicarbonate buffer (pH 8.5). After vacuum drying and dissolving in formic acid/dimethyl sulfoxide/water (0.1/5/94.9%), peptides were analysed by LC-MS on a Orbitrap Fusion Lumos mass spectrometer (MS) according to the procedure described in detail by Raeven et al. [22]. Before 2D electrophoresis, the identity and relative content of the proteins in the heat inactivated vaccines was analysed by MS by Metz et al. [16].

2.4. wP specific in vitro stimulation of splenocytes

Homogenized spleens were treated with erythrocyte lysis buffer. The splenocytes were cultured in 24-well plates (9×10^6 cells/well (Casy TTC Roche)) in complete medium (IMDM medium (Gibco), supplemented with 8% FCS, 1% L-Glutamine–Penicillin–Streptomycin solution (Sigma), and 20 μM β-mercaptoethanol (Sigma)). The cells were stimulated with wP vaccine (3 μg/mL) or medium and culture supernatant was collected after 3 or 6 days (as indicated).

2.5. Cytokine profiling using multiplex technology and ELISA

Cytokine levels in culture supernatant of the splenocytes and sera 4 h after immunisation were measured using a customized Milliplex mouse cytokine 4-plex Luminex kit (IL-5, IL-10, IL-17 and TNFα) and 5-plex Luminex kit (IL-12p40, IL-12p70, IP-10, IL-6 and IL-1α) (Millipore) respectively, according to the manufacturer's protocol. Measurements and data analysis were performed with Bio-Plex 200, using Bio-Plex Manager software (version 5.0, Bio-Rad Laboratories). IFN-γ in culture supernatant was detected using a commercial ELISA kit (R&D systems), according to the manufacturer's protocol.

2.6. Intracellular cytokine staining of in vitro wP vaccine stimulated splenocytes

Intracellular cytokine staining (ICS) was performed on *in vitro* cultured splenocytes, isolated and stimulated as described in Section 2.4. Before intracellular cytokine staining, the splenocytes of the CD1 mice were re-cultured in U-bottom 96-well plates and restimulated overnight (ON) with wP vaccine (3 μg/mL) or medium for intracellular cytokine analysis using flowcytometry. A pilot experiment showed that a 3 days culture (9×10^6 cells/well) followed by ON restimulation resulted in the highest sensitivity and percentage of viable cells (data not shown). After culture and stimulation, splenocytes were treated with 10 μg/ mL Golgi-plug (BD Biosciences), 1 μg/ mL αCD28 (BD Pharmingen), and 1 μg/ mL αCD49d (BD Pharmingen) for 5 h. Splenocytes were then stained with Pacific blue-conjugated anti-CD4 (Biolegend), FITC-conjugated anti-CD44 (BD Biosciences), and with LIVE/DEAD Aqua (Invitrogen). Subsequently, splenocytes were fixed, permeabilised using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences), according to the manufacturer's protocol, followed by staining with PE-conjugated anti-IFNγ (BD Biosciences), APC-conjugated anti-IL-5 (Biolegend), and PerCP-Cy5.5-conjugated anti-IL-17a (eBioscience). Data were acquired using a FACS Canto II (BD Biosciences), gated (Supplementary Fig. S1) and analysed using FlowJo software (Tree Star).

2.7. Statistical analysis

After log-transformation (Figs. 1, 3 and 4 and Table 2), significant differences were analysed using a Student's *t*-test. The Benjamini-Hochberg method was used for correction of multiple comparisons [23].

3. Results

3.1. wP vaccine quality does not affect early innate cytokine levels measured in serum

Because of the pivotal role of innate immune responses for protective immunity, cytokine/chemokine levels in sera of CD1 mice were studied shortly (4 h) after vaccination. Immunisation with all wP vaccines induced elevated levels of IP-10 and IL-6 (Fig. 1), while none of the vaccines induced detectable levels of IL-12p40 or IL-12p70 in serum (data not shown). Vaccine A (high quality) and E (low quality) slightly reduced the level of IL-1α in serum compared to naïve mice, while there was no effect of vaccine C (intermediate quality) on the level of this cytokine. The levels of the assessed cytokines were not significantly different between the vaccine groups, indicating that wP vaccine quality did not affect the detected innate immune cell response in these mice.

3.2. B. pertussis-specific IgG levels are not related to wP vaccine quality

Evaluation of the conventional readout parameter of the PSPT, revealed that immunisation with all of the wP vaccines resulted

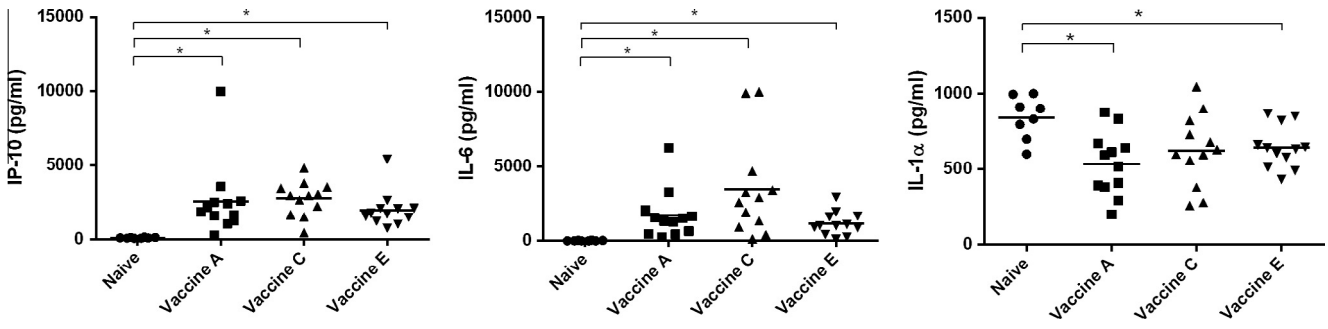
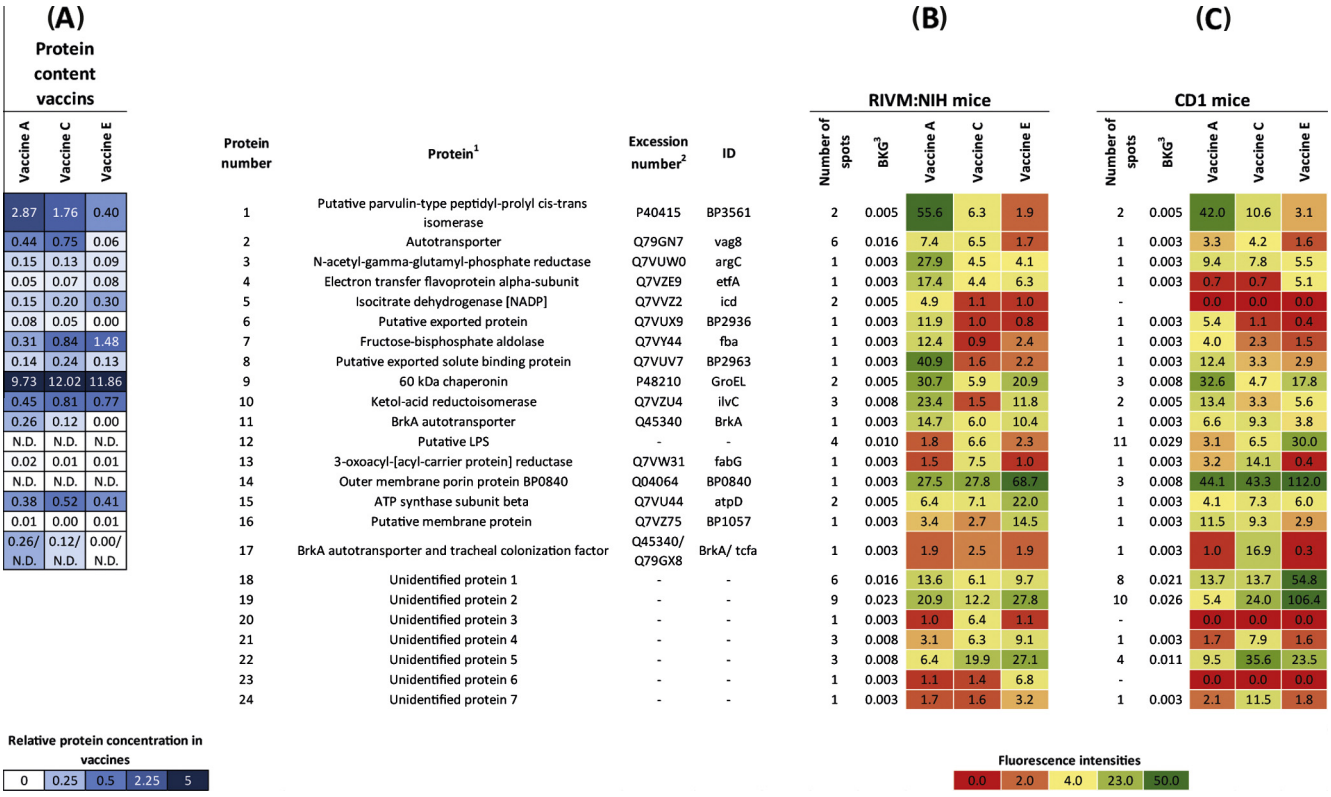


Fig. 1. Innate cytokine levels in sera shortly after wP vaccination. CD1 mice were immunised i.p. with wP vaccine A, C, E (4 IOU/mouse, 12 mice per group) or were injected i.p. with PBS (8 mice). After 4 h, blood samples were collected and cytokines levels in sera were determined using a multiplex assay. The measured cytokine levels (mean per group) are shown. Each dot represents one mouse. *Significant difference between indicated groups for $p < 0.05$.



Vaccine A	Vaccine C	Vaccine E	Protein number	Protein[1]	Excession number[2]	ID	RIVM:NIH mice: Number of spots	RIVM:NIH mice: BKG[3]	RIVM:NIH mice: Vaccine A	RIVM:NIH mice: Vaccine C	RIVM:NIH mice: Vaccine E	CD1 mice: Number of spots	CD1 mice: BKG[3]	CD1 mice: Vaccine A	CD1 mice: Vaccine C	CD1 mice: Vaccine E
2.87	1.76	0.40	1	Putative parvulin-type peptidyl-prolyl cis-trans isomerase	P40415	BP3561	2	0.005	55.6	6.3	1.9	2	0.005	42.0	10.6	3.1
0.44	0.75	0.06	2	Autotransporter	Q79GN7	vag8	6	0.016	7.4	6.5	1.7	1	0.003	3.3	4.2	1.6
0.15	0.13	0.09	3	N-acetyl-gamma-glutamyl-phosphate reductase	Q7VUW0	argC	1	0.003	27.9	4.5	4.1	1	0.003	9.4	7.8	5.5
0.05	0.07	0.08	4	Electron transfer flavoprotein alpha-subunit	Q7VZE9	etfA	1	0.003	17.4	4.4	6.3	1	0.003	0.7	0.7	5.1
0.15	0.20	0.30	5	Isocitrate dehydrogenase [NADP]	Q7VVZ2	icd	2	0.005	4.9	1.1	1.0	-		0.0	0.0	0.0
0.08	0.05	0.00	6	Putative exported protein	Q7VUX9	BP2936	1	0.003	11.9	1.0	0.8	1	0.003	5.4	1.1	0.4
0.31	0.84	1.48	7	Fructose-bisphosphate aldolase	Q7VY44	fba	1	0.003	12.4	0.9	2.4	1	0.003	4.0	2.3	1.5
0.14	0.24	0.13	8	Putative exported solute binding protein	Q7VUV7	BP2963	1	0.003	40.9	1.6	2.2	1	0.003	12.4	3.3	2.9
9.73	12.02	11.86	9	60 kDa chaperonin	P48210	GroEL	2	0.005	30.7	5.9	20.9	3	0.008	32.6	4.7	17.8
0.45	0.81	0.77	10	Ketol-acid reductoisomerase	Q7VZU4	ilvC	3	0.008	23.4	1.5	11.8	2	0.005	13.4	3.3	5.6
0.26	0.12	0.00	11	BrkA autotransporter	Q45340	BrkA	1	0.003	14.7	6.0	10.4	1	0.003	6.6	9.3	3.8
N.D.	N.D.	N.D.	12	Putative LPS	-	-	4	0.010	1.8	6.6	2.3	11	0.029	3.1	6.5	30.0
0.02	0.01	0.01	13	3-oxoacyl-[acyl-carrier protein] reductase	Q7VW31	fabG	1	0.003	1.5	7.5	1.0	1	0.003	3.2	14.1	0.4
N.D.	N.D.	N.D.	14	Outer membrane porin protein BP0840	Q04064	BP0840	1	0.003	27.5	27.8	68.7	3	0.008	44.1	43.3	112.0
0.38	0.52	0.41	15	ATP synthase subunit beta	Q7VU44	atpD	2	0.005	6.4	7.1	22.0	1	0.003	4.1	7.3	6.0
0.01	0.00	0.01	16	Putative membrane protein	Q7VZ75	BP1057	1	0.003	3.4	2.7	14.5	1	0.003	11.5	9.3	2.9
0.26/ N.D.	0.12/ N.D.	0.00/ N.D.	17	BrkA autotransporter and tracheal colonization factor	Q45340/ Q79GX8	BrkA/ tcfa	1	0.003	1.9	2.5	1.9	1	0.003	1.0	16.9	0.3
			18	Unidentified protein 1	-	-	6	0.016	13.6	6.1	9.7	8	0.021	13.7	13.7	54.8
			19	Unidentified protein 2	-	-	9	0.023	20.9	12.2	27.8	10	0.026	5.4	24.0	106.4
			20	Unidentified protein 3	-	-	1	0.003	1.0	6.4	1.1	-		0.0	0.0	0.0
			21	Unidentified protein 4	-	-	3	0.008	3.1	6.3	9.1	1	0.003	1.7	7.9	1.6
			22	Unidentified protein 5	-	-	3	0.008	6.4	19.9	27.1	4	0.011	9.5	35.6	23.5
			23	Unidentified protein 6	-	-	1	0.003	1.1	1.4	6.8	-		0.0	0.0	0.0
			24	Unidentified protein 7	-	-	1	0.003	1.7	1.6	3.2	1	0.003	2.1	11.5	1.8

Fig. 2. Antigen-specificity of IgG antibodies in sera of wP vaccinated mice. Lysate of *B. pertussis* strain 509 was separated by 2D electrophoresis and incubated with pooled sera of wP vaccinated (4 IOU/mouse) or naïve RIVM:NIH or CD1 mice. Non-responders were not included (OD < 0,1), being one mouse in the vaccine A group (RIVM:NIH), one mouse in the vaccine C group (CD1) and one mouse in the vaccine E group (CD1). Spots were manually excised from the Coomassie stained blots and analysed by LC-MS (Supplementary Fig. S1). Antibody specificity to 23 *B. pertussis* proteins were identified in sera of wP vaccinated mice. Based on experiment by Raeven et al. [22] one spot (number 12) was considered to correspond to antibody bound LPS. Proteins were ranked based on the responses of the RIVM:NIH mice according to the following order: vaccine A > C ⩾ E, vaccine A > C < E, vaccine A < C > E, vaccine A ⩽ C < E. The protein numbers in this figure and the spot numbers in Fig. S1 correspond. (A) The relative content of the proteins identified after immunoblotting was analysed by Mass Spectrometry (adapted from Metz et al. [16]) and is shown for vaccine A, C and E as a heat map. (B) and (C) show the intensity of the spots after immunoblotting analysed for RIVM:NIH and CD1 mice separately. The intensities of the antibody bound spots were analysed in three individual blots in gray values using an Odyssey infrared imager. The intensities of multiple spots for one protein were enumerated and corrected for the background of this protein. A two fold increase above background was considered a significant induction of specific antibodie.

in IgG1 and IgG2 antibodies directed against the *B. pertussis* bacterium in both outbred mouse strains (Table 2). In general, there were no significant differences between the vaccine groups, except that vaccine C induced a higher IgG1 antibody response in RIVM:NIH mice compared to those induced by vaccine A (Table 2). No significant differences in total IgG levels were observed (data not shown). These data indicate that the experimental wP vaccines used within this study induced potent *B. pertussis*-specific IgG responses, but that in general these responses were not affected by vaccine quality.

3.3. Antigen specificities of antibodies after wP vaccination are affected by vaccine quality

A combination of 2D electrophoresis and Western blotting was used to identify the specificity of wP vaccine-induced antibodies directed against particular *B. pertussis* proteins [22]. The analysis revealed much overlap between the specificities of antibodies produced by both mouse strains (RIVM:NIH and CD1). In sera of mice immunised with vaccine A, C and E, we identified significant antibodies levels directed against 18, 17 and 17 (RIVM:NIH), and 17, 19 and 14 antigens (CD1 mice), respectively (Fig. 2B and C, Supplementary Figs. S2 and S3). In agreement with the study by Raeven et al. using wP vaccine established from BP1917 in combination with 2D electrophoresis [22], we could not detect antibodies directed against PRN, FHA and PTx. In contrast, an earlier study by Stenger et al. [24] demonstrated antibodies against FHA and PRN were detected upon immunization with a multivalent vaccine containing BP509 and BP134, using mouse multiplex immunoassay. The discrepancy might be the result of the used vaccine strain, other vaccine components, the sensitivity of the detection methods or a combination of these factors, though antibodies against aP proteins were detected in sera of aP vaccinated mice using 2D electrophoresis [22]. The identified proteins are involved in various processes including pathogenesis, cell adhesion, glycolysis, fatty acid synthesis and (ion) transport (Uniprot taxonomic identifier 257313A).

For many of the identified proteins, antibodies were detected in the sera after immunization with all of the wP vaccines, though levels were frequently higher after immunisation with vaccine A compared to vaccine C and vaccine E. Vaccine A immunized mice of both strains produced higher antibody levels directed against 7 proteins (Fig. 2B and C, numbers 1, 3, 6–10) than immunized with vaccine C or E. In addition, vaccine A immunized RIVM:NIH mice produced higher levels against 5 additional proteins (numbers 2, 4, 5, 11 and 18) and CD1 mice against 1 additional protein (number 16) compared mice that were administered with vaccine C or E.

Interestingly, there were a couple of proteins for which - after immunisation with vaccine C - antibody levels were lower or higher compared to vaccine A and E immunized mice. Lower amounts of antibodies induced by vaccine C were directed against protein number 9, 10, 11 and 19 (RIVM:NIH) and protein number 9 and 10 (CD1), while levels induced by vaccine C and directed against protein number 12, 13, 17 and 20 (RIVM:NIH) and protein number 11, 13, 21, 22 and 24 (CD1) were higher than induced by

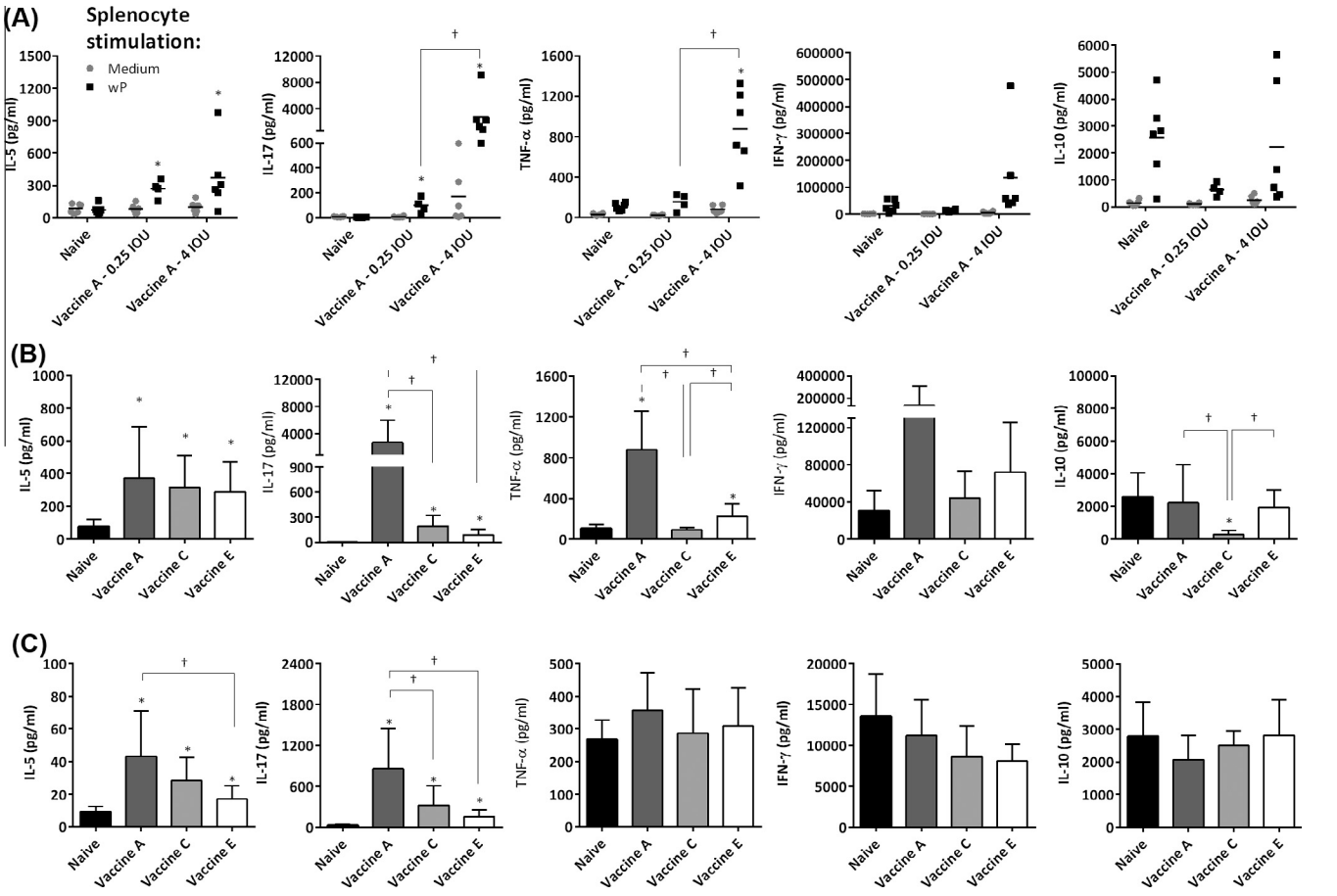


Fig. 3. Th cell cytokine responses after immunisation with wP vaccines. Mice were injected i.p. with wP vaccine A, C, E (4 IOU/mouse), vaccine A (0.25 IOU/mouse) or PBS. The splenocytes were collected 28 days after vaccination and cultured in medium or medium supplemented with 3 μg/mL wP vaccine (wP). (A) Cytokine levels in culture supernatant after 6 days of cultivation of splenocytes obtained from untreated and immunised RIVM:NIH mice, receiving an indicated dose of vaccine A (n = 4–6 mice/group). (B) Cytokine levels in the supernatant of splenocytes of RIVM:NIH mice, immunised with 4 IOU/mouse of the indicated wP vaccines or left untreated (n = 4–6 mice/group), after 6 days of *in vitro* culture with wP vaccine. (C) Cytokine levels in the supernatant of splenocytes of CD1 mice, immunised with a 4 IOU/mouse of the indicated wP vaccines or left untreated (n = 8–12 mice/per group), after 3 days (optimal for additional ICS analysis) of *in vitro* culture with wP vaccine. The measured cytokines levels are presented as mean ± SD. *significantly different from naïve mice with $p < 0.05$. †significant difference between indicated groups for $p < 0.05$.

vaccine A en E. Moreover, antibody levels against specific proteins were dominant in sera of vaccine E immunized mice, being number 14, 15, 16, 22–24 (RIVM:NIH) and 4, 12, 14 and 18 and 19 (CD1).

Since the protein composition of the wP vaccines differs, availability of antigens could cause the distinct pattern of antibody responses. The relative protein concentrations and spot intensities for other proteins did not always follow the same order (e.g. protein number 7). Remarkably, antibody levels against protein number 14 were relatively high after immunization with all wP vaccines, while the level of this protein in the vaccines was below the level of detection suggesting that it has strong immunogenic properties. Nevertheless, for a number of proteins (number 1 and 6) antibody levels corresponded with the relative protein concentrations in the respective vaccines (Fig. 2A). Collectively, these data show that the antigen-specificity of IgG antibodies varied between the groups immunised with the three wP vaccines under study. Based on this evaluation, proteins could be selected for an assay that enables detection of vaccine quality dependent antibody levels or specificities.

3.4. Magnitude of wP vaccine-specific Th cell cytokine responses is associated with vaccine quality

Analysis of the produced cytokines in culture supernatant of splenocytes indicative for the presence of several Th cell subsets (IL-5 for Th2 cells, IL-17 for Th17 cells, TNF-α and IFN-γ for Th1 cells and IL-10 for Treg cells), revealed that immunisation of RIVM:NIH mice with vaccine A resulted in wP specific and dose-dependent secretion of IL-5, IL-17 and TNF-α by splenocytes, but had no significant effect on IFN-γ and IL-10 production (Fig. 3A and B). Similarly, immunisation of CD1 mice with wP vaccine A resulted in vaccine-specific secretion of IL-5 and IL-17 (Fig. 3C). For both mouse strains, the magnitude of the IL-17 response induced by vaccine C and E was significantly lower than induced by vaccine A (Fig. 3B and C). Compared to vaccine A, the level of TNF-α produced by splenocytes was significantly lower when RIVM:NIH mice were immunised with vaccine E and IL-5 levels were significantly lower when CD1 mice were immunised with vaccine C or E. In addition, immunisation of RIVM:NIH mice with vaccine C resulted in significantly reduced *B. pertussis*-specific IL-10 and TNF-α responses compared to vaccines A and E. However, IL-10 levels did not exceed the IL-10 levels produced by naïve mice, indicating that the IL-10 production was not vaccine specific. In both mouse strains, no significant reduction in vaccine induced IFN-γ secretion was detected in culture supernatants. Stimulation of the splenocytes with a cocktail of the aP vaccine antigens FHA, PTx and PRN resulted in a similar pattern, but generally lower levels of these cytokines (Fig. S4).

Analysis of intracellular cytokine secretion at a single cell level revealed that the percentage of IFNγ-secreting Th cells

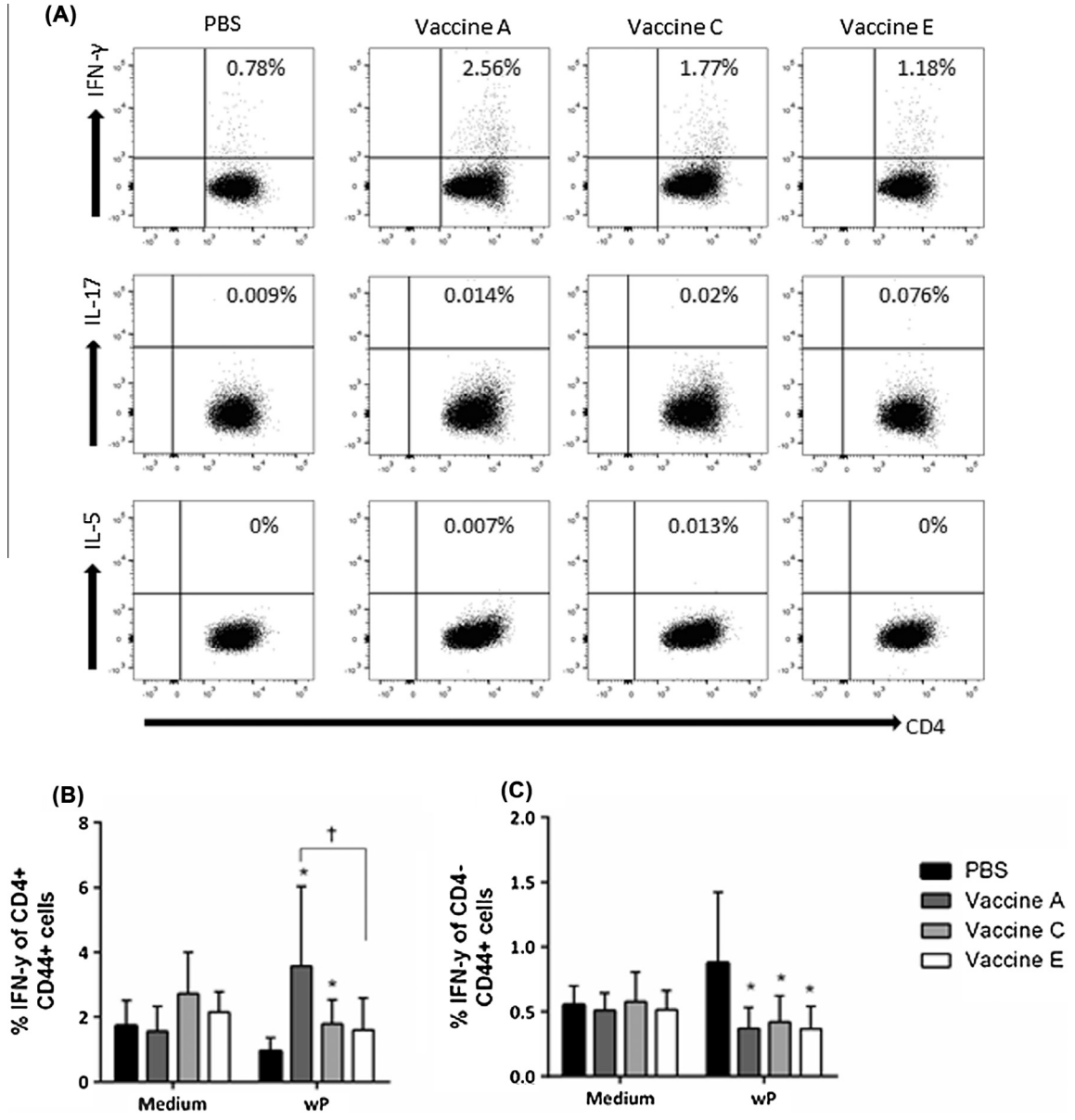


Fig. 4. Intracellular cytokine staining of splenocytes of wP vaccinated mice. Groups of 12 CD1 mice were immunised i.p. with wP vaccine A, C, E (4 IOU/mouse). A group of 8 mice were injected i.p. with PBS (naïve mice). Splenocytes of mice were collected 28 days after vaccination and cultured for 3 days in medium or medium supplemented with 3 μg/mL wP vaccine. Then, cells were analysed by flowcytometry after an additional ON restimulation. (A) Dotplots of $CD4^+CD44^+$ T cells from one representative mice vaccinated as indicated and stimulated *in vitro* with wP vaccine. (B and C) Summary of the percentage IFN-γ positive cells within the $CD4^+CD44^+$ T cells or $CD4^-CD44^+$ cells, respectively, of mice vaccinated as indicated and stimulated *in vitro* with medium or wP vaccine. *significantly different from corresponding naïve mice with $p < 0.05$. †significant difference between indicated groups for $p < 0.05$.

($CD4^+CD44^+$) was significantly lower when CD1 mice were immunised with vaccine E compared to vaccine A (Fig. 4A and B), while there was no vaccine specific effect on the percentage of IL-17 and IL-5 positive $CD4^+CD44^+$ T cells (Fig. 4A). This suggests that the kinetics of the analysed cytokines may differ. In addition, IFN-γ producing and activated non-CD4 cells ($CD4^-CD44^+$ cells) were identified, possibly $CD8^+$ T cells or NK cells, irrespective of the presence of wP stimulation in both naïve and vaccinated CD1 mice (Fig. 4C). This constitutive production of IFN-γ may have masked the contribution of wP vaccine specific IFN-γ-producing $CD4^+$ T cells in supernatant analyses. Taken together, these data indicate that wP vaccine quality is reflected in the magnitude of pertussis cytokine responses indicative for specific Th cell subsets, i.e. Th1 and Th17 responses (both strains) and Th2 responses (CD1).

4. Discussion and conclusions

We studied whether serological properties in combination with additional biomarkers of the immune response to wP vaccination may reflect vaccine quality and can be used as a complementation to the current readout parameters in the Pertussis Serological Potency test (PSPT). Three major observations were made. Firstly, wP vaccination increased the levels of circulating pro-inflammatory cytokines and chemokines shortly after vaccination, and it induced IgG responses directed against the entire bacterium. However, these parameters did not discriminate between vaccines of varying qualities. Secondly, the wP vaccine of high quality (vaccine A) was a potent inducer of especially Th17 and Th1 cytokines, but also of Th2 responses, and induced a larger number of antibodies with distinct antigen specificities compared to the

Table 2
B. pertussis specific IgG levels[a] in sera 28 days after vaccination.

Vaccination	RIVM/NIH[b]		CD1[c]	
	IgG1	IgG2A	IgG1	IgG2A
Unvaccinated	n.d.	n.d.	n.d.	n.d.
Vaccine A - 0.25 IOU	55 ± 23[d]	17 ± 15		
Vaccine C - 0.25 IOU	47 ± 20[d]	9 ± 2		
Vaccine E - 0.25 IOU	37 ± 37[d]	18 ± 12		
Vaccine A - 4 IOU	772 ± 519[d]	921 ± 590[d]	2283 ± 934[d]	1100 ± 953[d]
Vaccine C - 4 IOU	1904 ± 457[d,e]	1436 ± 382[d]	2862 ± 1250[d]	1769 ± 1206[d]
Vaccine E - 4 IOU	658 ± 485[d]	760 ± 340[d]	1558 ± 759[d]	1319 ± 892[d]

n.d. not detected.
[a] OD measured at 450 nm × dilution of sera.
[b] Antibodies against *B. pertussis* strain 18323.
[c] Antibodies against *B. pertussis* strain Kh96/01.
[d] $p < 0.05$ vaccination vs naïve mice.
[e] $p < 0.05$ vaccination vs vaccine A.

vaccines of lower quality. Thirdly, the effects were found in two outbred mouse strains. These outbred mouse strains (NIH and CD1) are commonly used for quality control testing (e.g. for the Kendrick test [21] and the PSPT [7]), since they provide a more relevant reflection of the variation within a human population than inbred mouse strains [20,25,26]. The data obtained provide evidence that determining the antigen specificity of wP vaccine-induced antibodies and the magnitude of wP vaccine-induced Th cell responses can provide useful and immunologically relevant additional parameters for assessing vaccine quality in the PSPT. Nevertheless, validation studies are needed before these parameters can be implemented.

Clearance of a respiratory *B pertussis* infection has been shown to depend on induction of appropriate Th cell responses, particularly Th1 [15,27] and Th17-type responses [28,29], in combination with B cell and antibody responses. Here, we showed that a low wP vaccine quality (vaccine E) is associated with a reduced magnitude of such pertussis Th17 responses, and also Th1 and Th2 responses, and with a distinct antibody specificities compared to the responses induced by a vaccine of optimal quality (vaccine A). The observed differences in Th cell responses and antibody specificity are most likely not the result of variations in vaccine-induced innate immune responses, since we could not detect differences in the levels of cytokines in sera shortly after vaccination (Fig. 1). However, since there are indications that lymphocytes contribute to protection against intracerebral infection [30,31], the Th1 and Th17 responses induced by the wP vaccines might have played a role in protective properties of the vaccines against intracerebral infection observed by Metz et al. [16]. A study by Raeven et al. has recently demonstrated that antibodies induced by an experimental wP vaccine derived from *B. pertussis* strain BP1917 are primarily directed against BrkA, GroEL and Vag8 [22]. In our study, both GroEL and Vag8 (protein number 9 and 2 respectively) as well as several other proteins were dominant in the humoral response of vaccine A compared to vaccine C and E immunized RIVM:NIH and CD1 mice. Remarkably, distinct antibody profiles were induced with different antigen-specificities after immunization with vaccine C or E. These characteristic antibody patterns could partly be explained by the distinct amounts of specific antigens, such as lower levels of Vag8, BP3561 and argC (protein number 2, 1 and 3 respectively), in these vaccines and might have influenced vaccines potency. Of these proteins, Vag8 is described as a virulence protein [32] that contributes to resistance to complement mediated killing [33]. Furthermore, this protein has a role in vaccine-induced immunity [34]. To elucidate the exact contribution of Vag8 and other proteins to vaccine potency, further studied will be needed. Although these results show that vaccine quality can be recognised based on the pattern of antibody-specificity, 2D electrophoresis might be too complicated for routine testing. The analysis, however, might enable the selection of one of several candidate proteins, which can be used to establish a routine quality control test.

There are certain virulence pertussis antigens, e.g. filamentous hemagglutinin, pertussis toxin and pertactin described that exhibit Th cell immune-modulatory properties [35]. Antibodies directed against these proteins were not detected, but the different quantities of these proteins in the vaccines might have narrowed or changed the antigen-specificity of Th or Treg cell response and thereby affected the magnitude of the induced *B. pertussis*-specific T cell response. Further identification of the antigen specificity of wP-induced T cells will be needed to reveal the contribution of antigen-specific immunomodulation of the wP vaccine-induced T cell responses.

The PSPT was developed as an alternative to the current regulatory required intracerebral challenge test for wP vaccines [6–8]. The official medicines control laboratories network estimated that in Europe approximately 100.000 mice are required annually for wP potency testing (personal communication). The PSPT not only reduces distress levels inflicted on the animals, but also reduces animal number with approximately 25% [7]. Since B and T cell responses both contribute to whole cell vaccine induced protection, this study demonstrates the relevance of measuring T cell responses as well as antibody specificity and thereby contributes to the improvement of the current PSPT, though thorough and manufacturer specific evaluation, optimisation and validation studies will be required. Based on the results of this study, we hypothesise that Th17 cells and antibodies directed against Vag8, BP3561 and argC might contribute to wP vaccine induced protection, though further studies into the role of these immune components are required. In the scope of this study, application of parameters such as the magnitude of wP vaccine specific IL-17 responses detected in the supernatant and levels of antibodies directed against specific proteins (e.g. Vag8), might increase the probability of the implementation of the PSPT for wP vaccine lot release testing.

Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

Acknowledgements

This study was part of the programme Dutch ministry of Health, Welfare and Sports and the Dutch ministry of Economic Affairs that aims to develop alternatives to animal experiments. We are grateful to Mervin Vriezen, Nicole Ruiterkamp, Johan van der

Gun (Bilthoven Biologicals), Rob Vandebriel, Arnoud Akkermans, Jolanda Brummelman (RIVM), René Raeven and Gideon Kersten (Intravacc) for helpful advice during the study. We thank Michel Weyts (Intravacc) for preparing the experimental wP vaccines, and Jose Ferreira (RIVM) for advice on the statistical analyses.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.07.011.

References

[1] Libster R, Edwards KM. Re-emergence of pertussis: what are the solutions? Expert Rev Vaccines 2012;11:1331–46.
[2] Witt MA, Katz PH, Witt DJ. Unexpectedly limited durability of immunity following acellular pertussis vaccination in preadolescents in a North American outbreak. Clin Infect Dis: Off Publ Infect Dis Soc Am 2012;54:1730–5.
[3] Gambhir M, Clark TA, Cauchemez S, Tartof SY, Swerdlow DL, Ferguson NM. A change in vaccine efficacy and duration of protection explains recent rises in pertussis incidence in the United States. PLoS Comput Biol 2015;11:e1004138.
[4] van der Ark AA, Hozbor DF, Boog CJ, Metz B, van den Dobbelsteen GP, van Els CA. Resurgence of pertussis calls for re-evaluation of pertussis animal models. Expert Rev Vaccines 2012;11:1121–37.
[5] Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci USA 2013;110(9):3507–12.
[6] van der Ark A, van Straaten-van de Kappelle I, Akkermans A, Hendriksen C, van de Donk H. Development of pertussis serological potency test. Serological assessment of antibody response induced by whole cell vaccine as an alternative to mouse protection in an intracerebral challenge model. Biol: J Int Assoc Biol Standard 1994;22:233–42.
[7] van der Ark A, van Straaten-van de Kappelle I, Olander RM, Enssle K, Jadhav S, van de Donk H, et al. The pertussis serological potency test. Collaborative study to evaluatereplacement of the mouse protection test. Biol: J Int Assoc Biol Stand 2000;28:105–18.
[8] von Hunolstein C, Gomez Miguel MJ, Pezzella C, Scopetti F, Behr-Gross ME, Halder M, et al. Evaluation of two serological methods for potency testing of whole cell pertussis vaccines. Pharmeuropa bio/Biol Standard Program EDQM 2008;2008:7–18.
[9] Cherry JD, Gornbein J, Heininger U, Stehr K. A search for serologic correlates of immunity to Bordetella pertussis cough illnesses. Vaccine 1998;16:1901–6.
[10] Mahon BP, Sheahan BJ, Griffin F, Murphy G, Mills KH. Atypical disease after Bordetella pertussis respiratory infection of mice with targeted disruptions of interferon-gamma receptor or immunoglobulin mu chain genes. J Exp Med 1997;186:1843–51.
[11] Ausiello CM, Urbani F, la Sala A, Lande R, Cassone A. Vaccine- and antigen-dependent type 1 and type 2 cytokine induction after primary vaccination of infants with whole-cell or acellular pertussis vaccines. Infect Immun 1997;65:2168–74.
[12] Higgs R, Higgins SC, Ross PJ, Mills KH. Immunity to the respiratory pathogen Bordetella pertussis. Mucosal Immunol 2012;5:485–500.
[13] Ryan M, Murphy G, Ryan E, Nilsson L, Shackley F, Gothefors L, et al. Distinct T-cell subtypes induced with whole cell and acellular pertussis vaccines in children. Immunology 1998;93:1–10.
[14] Mills KH, Ryan M, Ryan E, Mahon BP. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against Bordetella pertussis. Infect Immun 1998;66:594–602.
[15] Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog 2013;9:e1003264.
[16] Metz B, Hoonakker M, Uittenbogaard JP, Weyts M, Mommen GPM, Meiring HD, et al. Proteome analysis is a powerful tool to evaluate antigen expression during upstream processing of whole-cell pertussis vaccines. J. Proteome Res. (submitted for publication).
[17] Bellalou J, Relyveld EH. Studies on culture conditions of Bordetella pertussis and relationship to immunogenicity of vaccines. Ann Microbiol 1984;135B:101–10.
[18] Dolby JM, Ackers JP, Dolby DE. The effect of the antigen which elicits the bactericidal antibody and of the mouse-protective antigen on the growth of Bordetella pertussis in the mouse brain. J Hyg 1975;74:71–83.
[19] Hoonakker ME, Verhagen LM, Hendriksen CF, van Els CA, Vandebriel RJ, Sloots A, et al. In vitro innate immune cell based models to assess whole cell Bordetella pertussis vaccine quality: a proof of principle. Biol: J Int Assoc Biol Standard 2015;43:100–9.
[20] Chia R, Achilli F, Festing MF, Fisher EM. The origins and uses of mouse outbred stocks. Nat Genet 2005;37:1181–6.
[21] van Straaten-van de Kappelle I, van der Gun JW, Marsman FR, Hendriksen CF, van de Donk HJ. Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. Biol: J Int Assoc Biol Standard 1997;25:41–57.
[22] Raeven RH, van der Maas L, Tilstra W, Uittenbogaard JP, Bindels TH, Kuipers B, et al. Immunoproteomic profiling of Bordetella pertussis outer membrane vesicle vaccine reveals broad and balanced humoral immunogenicity. J Proteome Res 2015;14(7):2929–42.
[23] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc: Ser B (Methodol) 1995;57:289–300.
[24] Stenger RM, Smits M, Kuipers B, van Gaans-van den Brink J, Poelen M, Boog CJ, et al. Impaired long-term maintenance and function of Bordetella pertussis specific B cell memory. Vaccine 2010;28:6637–46.
[25] WHO. Manual for quality control of diphtheria, tetanus and pertussis vaccines; 2013. p. 12. <http://appswhoint/iris/bitstream/10665/80681/1/WHO_IVB_1111_engpdf>.
[26] Aldinger KA, Sokoloff G, Rosenberg DM, Palmer AA, Millen KJ. Genetic variation and population substructure in outbred CD-1 mice: implications for genome-wide association studies. PLoS ONE 2009;4:e4729.
[27] Mills KH, Barnard A, Watkins J, Redhead K. Cell-mediated immunity to Bordetella pertussis: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun 1993;61:399–410.
[28] Dunne A, Ross PJ, Pospisilova E, Masin J, Meaney A, Sutton CE, et al. Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against Bordetella pertussis. J Immunol 2010;185:1711–9.
[29] Andreasen C, Powell DA, Carbonetti NH. Pertussis toxin stimulates IL-17 production in response to Bordetella pertussis infection in mice. PLoS ONE 2009;4:e7079.
[30] Adams GJ, Hopewell JW. Enhancement of intracerebral infection of mice with Bordetella pertussis. J Med Microbiol 1970;3:15–27.
[31] Dolby JM, Dolby DE, Bronne-Shanbury CJ. The effects of humoral, cellular and non-specific immunity on intracerebral Bordetella pertussis infections in mice. J Hyg 1975;74:85–102.
[32] Finn TM, Amsbaugh DF. Vag8, a Bordetella pertussis bvg-regulated protein. Infect Immun 1998;66:3985–9.
[33] Marr N, Shah NR, Lee R, Kim EJ, Fernandez RC. Bordetella pertussis autotransporter Vag8 binds human C1 esterase inhibitor and confers serum resistance. PLoS ONE 2011;6:e20585.
[34] de Gouw D, de Jonge MI, Hermans PW, Wessels HJ, Zomer A, Berends A, et al. Proteomics-identified Bvg-activated autotransporters protect against bordetella pertussis in a mouse model. PLoS ONE 2014;9:e105011.
[35] McGuirk P, McCann C, Mills KH. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. J Exp Med 2002;195:221–31.