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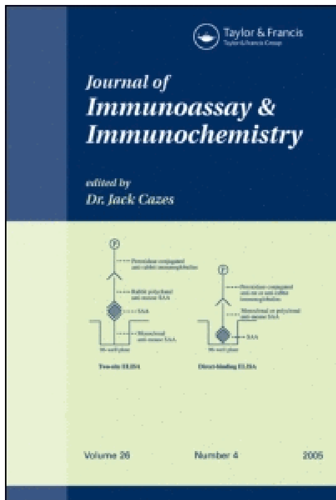

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QUANTIFICATION OF CELL-ASSOCIATED AND FREE ANTIGENS IN *BORDETELLA PERTUSSIS* SUSPENSIONS BY ANTIGEN BINDING ELISA

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ABSTRACT

In order to achieve batch-to-batch consistency of whole-cell pertussis vaccines, properties relevant for protection and safety should be characterised. Therefore, ELISAs to quantify pertussis toxin (PT), filamentous haemagglutinin (FHA), 92 kD outer membrane protein (92 kD-OMP) and pertactin (PRN) in *Bordetella pertussis* (*B. pertussis*) suspensions were developed. In this paper the influence of the bacterial growth stage on antigen production and antigen release into the supernatant was studied for pertussis strains 134, 509 and CS. The levels of cell-associated and free antigens during growth were strongly strain and antigen dependent. Because of this, the proportion of cell-associated antigens changed during cultivation for all three strains. Substantial amounts of PT and PRN were released into the supernatant, while little free FHA and 92 kD-OMP were found. The amount of cell-associated FHA declined rapidly during growth, whereas cell-associated 92 kD-OMP contents increased. These findings demonstrate that, although antigen exposure and release differ from strain to strain, the main factor that determines the antigen production and release is the growth phase.

(KEY WORDS: Antigen binding ELISA, whole-cell pertussis vaccine, antigen density, biomass, growth phase)

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INTRODUCTION

For decades whole-cell pertussis vaccines have proven to be efficacious in controlling whooping cough. In spite of the introduction of acellular vaccines, whole-cell vaccines will continue to be used for the forseeable future in most countries. For reliable efficacy, however, batch-to-batch consistency should be guaranteed.

Fine and Clarkson (1) reported significant differences in efficacy between whole-cell vaccines from several manufacturers as well as significant differences between several vaccine batches from a single manufacturer. Differences in efficacy may be attributed to differences in the production method and composition of various vaccines. Since it was demonstrated that the potency of whole-cell pertussis vaccines in the intracerebral mouse protection assay correlated with field protection (2), this test has been mandatory for controlling whole-cell pertussis vaccines (3). One of the disadvantages of this test is the great intra- and inter-laboratory variability (4). Moreover, the discriminative power of the MPT between potent and non-potent whole-cell vaccines is low (5). Recently Steinhoff et al. (6) compared the reactogenicity and immunogenicity of two licensed whole-cell pertussis vaccines. Although both vaccines passed the MPT, significant differences in immunogenicity in human infants were observed. The above-mentioned drawbacks of the MPT could be the underlying cause of these differences.

Consistent production of whole-cell vaccines implies that vaccine lots should be similar with regard to antigenic make-up. Since several pertussis

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antigens have proven to be protective (7), quantification of these antigens during the production process of whole-cell vaccine could be used as a means to characterise the vaccine. Few researchers have studied antigen contents in whole-cell vaccine, and results on antigen contents during the cultivation of the bacterium were not included (8-10).

The production process of whole-cell pertussis vaccine includes several distinct steps, such as cultivation, harvesting, centrifugation and inactivation. Analytical methods that monitor protective antigens during these process steps, provide insight into the influence of these steps on the pertussis cells. However, the only parameter currently used during production is biomass, determined as optical density.

Therefore, ELISAs for the quantification of cell-associated and free antigens in whole-cell suspensions were developed. Since Pertussis Toxin (PT), Filamentous Haemagglutinin (FHA), Pertactin (PRN) and 92 kD-Outer Membrane Protein (92 kD-OMP) are all known to be protective antigens in pertussis vaccines (11-14), these antigens were chosen to be quantified. In the present study the ELISAs were applied to measure antigen production and antigen release into the supernatant during cultivation. Furthermore, three pertussis strains were compared with respect to antigen exposure.

MATERIALS AND METHODS

Strains and growth conditions

Bordetella pertussis strains 134 and 509 (Dutch vaccine production strains) and strain CS (Chinese vaccine production strain) were used. The strains were

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stored in 10% glycerol, 90 % Verwey medium at -70°C. Ten ml of these -70°C cultures were used for the inoculation of 0.5-L shake flasks with 200 ml Verwey medium containing (g/L): 14, casamino acids (Difco, Detroit, USA); 0.2, KCl; 1, starch; 0.5, KH_2PO_4; 0.1, $MgCl_2.H_2O$; 0.02, nicotinic acid; 0.01, glutathione. After cultivation for 18 h at 35°C, these seed suspensions were used to inoculate 200 ml B2-medium (15) at an initial optical density (OD) at 590 nm of 0.05 (OD of 0.05 ≈ 1 x 10^9 organisms ml^{-1}).

Optical density

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Each strain was cultivated in duplicate shake flasks. One flask was used to monitor growth by measuring the OD of 1-ml samples. From the corresponding duplicate, 25-ml samples were taken as test suspension. Apparent biomass concentrations were estimated in 1 ml of the duplicate cultures by measuring OD at 590 nm (Vitalab 10 spectrophotometer, Vital Scientific, Dieren, The Netherlands) and expressed as International Opacity Units (IOU; 1 IOU corresponds to 1 x 10^9 organisms ml^{-1}). For each cultivation, the ODs of the duplicate flasks were essentially the same.

Sample treatment

During cultivation 25-ml samples were taken at three points in the growth phase: one sample halfway the exponential phase (stage 1), the second sample just before the early stationary phase (stage 2) and the last sample at the late stationary phase (stage 3). Each sample was divided in whole-cell suspension (WCS), saline suspended cells (SSC) and supernatant (SUP) as follows. Half of a 25-ml sample was directly distributed into six 5-ml screw-cap polypropylene vials (2 ml per

vial). The other half was centrifuged at 6,500 x *g* for 10 min at 4°C. The resulting supernatant was distributed into six vials (2 ml per vial). The cell pellet was resuspended in 12.5 ml saline and distributed into six vials (2 ml per vial). The OD of each sixth vial was measured. All samples were stored at -70°C until tested in ELISA.

Antigens

PT and PRN were a generous gift from Dr Rino Rappuoli (Biocine Research Centre, Sienna, Italy). FHA was purified by hydroxyapatite chromatography as described by Sato et al. (16). 92 kD-OMP was purified by $CaCl_2$-extraction of bacteria, followed by several centrifugation steps, ion exchange chromatography and gel filtration (17). A heat-inactivated cell suspension in saline from strain CS (184 IOU) was used as a reference cell suspension, containing antigen concentrations (determined against the purified antigens) in μg/ml of PT: 10.4, FHA: 94, 92 kD-OMP: 138, PRN: 116.

Monoclonal antibodies (mabs)

The following murine mabs were used: anti-PT (IgG_1); anti-FHA (IgG_1); anti-92 kD-OMP (IgG_1) and anti-PRN (IgM). All mabs against *B. pertussis* were described previously (18).

Antigen binding enzyme-linked immunosorbent assay (AnBi-ELISA)

The AnBi-ELISA was carried out as follows: Flat-bottomed polystyrene microtitre plates (Greiner, serial no. 655191, Frickenhausen, Germany) were blocked for 1 h at 37°C with phosphate buffered saline (PBS, pH 7.2) containing

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0.5 % (w/v) bovine serum albumin (Organon Teknika, Boxtel, The Netherlands), 260 µl per well.

After blocking, twofold dilution series of WCS, SSC and SUP were made in 100 µl volumes, starting with the undiluted sample. PBS containing 0.1 % Tween 80 (PBST) was used to dilute the samples. On each plate, the reference cell suspension was titrated for comparison. The dilution series of the samples were pre-incubated with 100 µl/well mab anti-PT, anti-FHA, anti-92 kD-OMP and anti-PRN, respectively, diluted 1:100,000 (anti-PT, anti-FHA and anti-92 kD-OMP) or 1:50,000 (anti-PRN) in PBST. Incubation of the sample-mab mixtures was done for 1.5 h at 37°C and then overnight at room temperature.

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Polystyrene, high binding capacity, microtitre plates (Greiner, serial no. 655092, Frickenhausen, Germany) were coated with 100 µl/well PT, FHA, 92 kD-OMP and PRN, respectively, at a concentration of 0.5 µg/ml (92 kD-OMP, PRN) or 1µg/ml (PT, FHA) in PBS. The plates were incubated for 1.5 h at 37°C and then left overnight at room temperature. Next day, the plates were washed twice with tap water containing 0.05 % Tween 80 (TWT).

The antibody excess in the sample-mab mixtures was determined by transferring 100 µl of the mixtures to the microtitre plates coated with corresponding antigen and subsequent incubation for 2 h at 37°C. After washing with TWT, peroxidase labelled goat-anti-mouse IgG F_c (Cappel, Organon Teknika, West Chester, USA) diluted 1:5,000 (anti-PT, anti-FHA, anti-92 kD-OMP) or peroxidase labelled goat-anti-mouse IgG, IgA, IgM (Cappel, Organon Teknika, West Chester, USA) diluted 1:10,000 (anti-PRN) in PBST containing

0.5% (w/v) Protifar milk protein concentrate (Nutricia, Zoetermeer, The Netherlands), was added to each well in 100 µl volumes. The plates were incubated for 1.5 h at 37°C and washed with TWT as before.

Next, 100 µl tetramethylbenzidine (TMB) substrate was added to each well. This substrate consisted of 54 ml distilled water, 1 ml TMB (Sigma, st. Louis, USA) in ethanol 96% (6 mg/ml), 6 ml 0.11 M sodium acetate buffer, pH 5.5, and 25 µl 30% H_2O_2 (Merck, Darmstadt, Germany). After 10 min, the reaction was stopped by adding 100 µl/well 2 M H_2SO_4. The absorbance was measured at 450 nm using an automatic platereader (Biotek Instruments, Winooski, USA). Antigen concentrations were calculated by means of a 4-parameter fitting analysis (Kineticalc software, Biotek instruments, Winooski, USA) and expressed as µg/ml. Subsequently concentrations were converted to ng/IOU. Antigen concentrations in SUP were converted to ng per IOU of the matching WCS. Single way Analysis of Variance (ANOVA) was used to evaluate the significance of differences ($p < 0.05$) in antigen contents during bacterial growth on the one hand and between strains on the other hand.

RESULTS

Reproducibility of bacterial growth

Bordetella pertussis strain 134, 509 and CS were cultivated three times at short intervals of two to three weeks. All three strains showed reproducible growth (Fig. 1). Maximal biomass concentrations of 68 ± 7 IOU (geometric mean +/- 1 SD), 26 ± 2 IOU and 28 ± 3 IOU were reached with strains 134, 509 and CS, respectively. Probably because of lysis or cell aggregation, the apparent biomass

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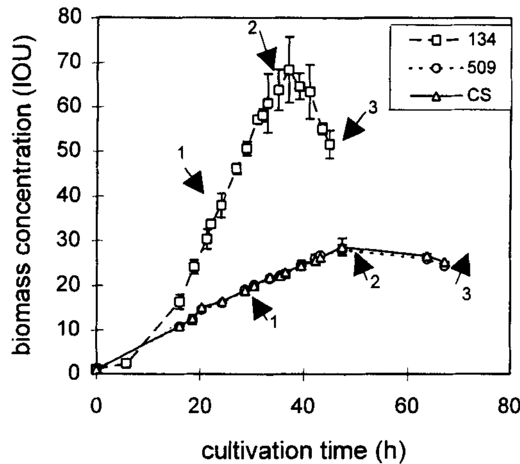

FIGURE 1. Cultivation of *B. pertussis* strains 134, 509 and CS in B2-medium. Error bars represent the standard deviation of three separate cultivations. Arrows indicate the sampling points halfway the exponential phase (stage 1), the early stationary phase (stage 2) and the late stationary phase (stage 3)

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concentration of strain 134 was reduced to about 80 % of its maximum within eight hours after reaching the maximum IOU. Twenty hours after reaching the maximum IOU, biomass concentration of strain 509 and CS was still 96 % and 97 %, respectively, of the maximum.

Antigen contents during growth

As described in Materials and Methods, samples taken during three growth stages of each cultivation (see in Fig. 1: arrows 1, 2 and 3) were divided in whole cell suspension (WCS), saline suspended cells (SSC) and supernatant (SUP), and investigated in ELISA. Statistical analysis of the raw data of each individual strain at each sampling point showed that differences in antigen contents among a triplicate cultivation were within experimental error. Therefore, we decided to

merge the raw ELISA data (per strain and per growth stage) of each triplicate cultivation for further analysis. The results of ELISA are summarised in Fig. 2A-D.

During cultivation the FHA concentration in both WCS and SSC strongly decreased for all three strains (Fig. 2A). In stage 1, there was no significant difference in FHA content between strain 509 and CS (p=0.58), but significantly less FHA was measured in strain 134. Between stage 1 and stage 2, the decrease of the FHA content was less in strain CS as compared to both other strains (cf. WCS1 and WCS2 in Fig. 2A). The amount of cell-associated FHA was much lower than the FHA content measured in whole cell suspensions, irrespective of strain or growth stage. Although the difference could be due to release of FHA into the supernatant, the measured amount of free FHA in supernatant was negligible for all three strains (Fig. 2A).

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In contrast with the FHA content, the 92 kD-OMP content increased during cultivation in both WCS and SSC as shown in Fig. 2B. This was true for all three strains. Significantly higher levels of 92 kD-OMP were reached in the corresponding SSC samples. In stage 3, the cell-associated 92 kD-OMP content of strain 509 and CS did not differ significantly (p=0.84), but significantly less 92 kD-OMP was measured for strain 134. During cultivation, little 92 kD-OMP was released into the supernatant for all three strains.

A substantial amount of the total PT content was present as free PT as is shown in Fig. 2C. Both strain 509 and CS released significantly less PT into the supernatant than strain 134. During cultivation the amount of PT released by

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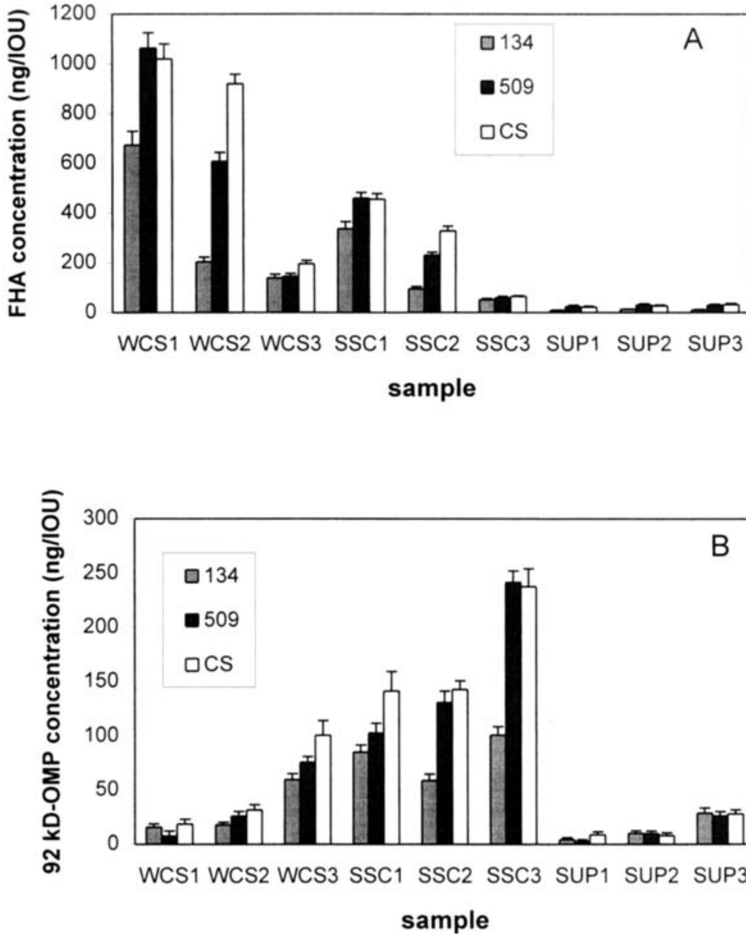
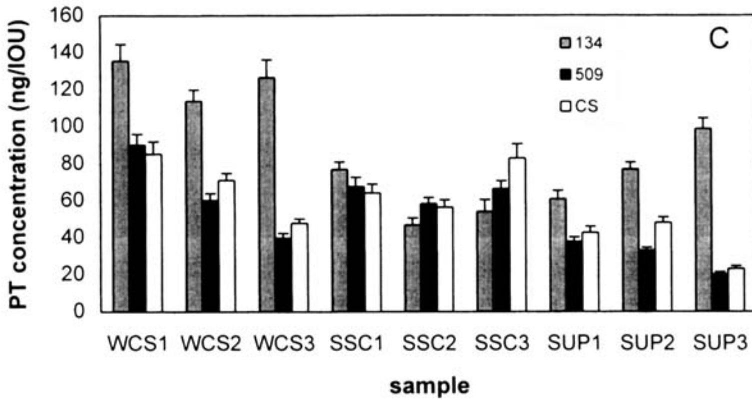


FIGURE 2. Results of FHA (A), 92 kD-OMP (B), PT (C) and PRN (D) contents in cultivation samples of *B. pertussis* strains 134, 509 and CS. Samples were divided in whole-cell suspension (WCS), saline-suspended cells (SSC) and supernatant (SUP) at growth stage 1, 2 and 3. The values are presented as mean + standard error of mean (error bars) of at least 29 (A), 17 (B), 27 (C), 12 (D) separate measurements.



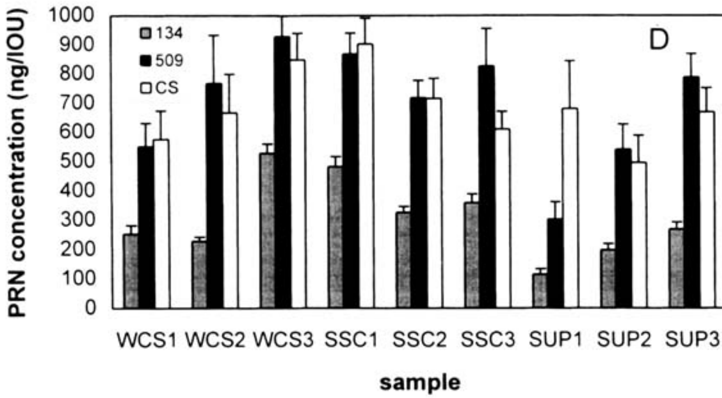


FIGURE 2. Continued

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strain 134 increased, whereas the free PT content for both other strains decreased. Surprisingly, the cell-associated PT contents did not differ significantly during the three growth stages and were more or less the same for all three strains.

Strain 134 contained significantly less cell-associated and free PRN than both other strains as shown in Fig. 2D. Moreover, between stage 1 and stage 2 the cell-associated PRN content decreased whereas the PRN content of both other strains did not significantly change during cultivation. The amount of free PRN increased for strain 134 and 509 during cultivation but did not significantly change for strain CS ($p=0.50$).

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DISCUSSION

The present study revealed that *B. pertussis* suspensions show marked differences in antigen exposure and release. These differences vary not only from strain to strain, but also from one growth stage to another. Antigen contents were normalised from μg/ml to ng/IOU in order to compare strains and growth stages. Thus, the cell-associated antigen contents are a direct measure for the antigen density per cell. Since a final lot of whole-cell pertussis vaccine consists of saline suspended cells, high cell surface densities of protective antigens are likely to augment the potency of a pertussis vaccine. Therefore, it is important to examine antigen densities per cell instead of antigen concentrations per volume.

Although strain 134 reached a higher biomass concentration and higher antigen concentrations per volume than strain 509 and CS, in general, the antigen densities, i.e. the antigen contents per cell of strain 134 were lower than those of the latter strains. Moreover, whereas the level of cell-associated 92 kD-OMP

increased during cultivation, that of FHA declined for all three strains. Thus, biomass concentration is not necessarily proportional to antigen density and is therefore a poor criterium to achieve consistent vaccine lots.

For all three strains, antigen exposure as a function of growth phase was strongly antigen dependent. As a consequence, the relation of the cell-associated antigens during growth varied substantially. During stage 1, the ratios (on weight basis) of cell-associated FHA, 92 kD-OMP, PT, and PRN were, respectively, about 4:1:1:6 for strain 134 and 7:2:1:14 for both strain 509 and CS. During stage 2, these ratios were, respectively, 2:1:1:7 for strain 134, 4:2:1:12 for strain 509, and 6:3:1:13 for strain CS. During stage 3 the ratios of antigens had changed in, respectively, 1:2:1:7 for strain 134, 1:4:1:14 for strain 509 and 1:4:1:10 for strain CS. In a production process, the bacteria will usually be harvested in stage 2. Based on a human vaccine dose (16 IOU) the antigen levels during this stage were in the ranges 1.5-5.2 μg FHA, 0.9-2.3 μg 92 kD-OMP, 0.7-0.9 μg PT, and 5.2-11.5 μg PRN per dose, respectively. Very little is known about which components of whole-cell pertussis vaccines are important for protective immunity (19). Nevertheless, a variation in the antigen contents between vaccine lots would mean a varying vaccine composition, which may influence the vaccine quality. These data show that monitoring protective antigens during the production process is of utmost importance to achieve consistent vaccine lots.

As for the distribution between free and cell-associated antigens during cultivation, especially PT and PRN were released in substantial amounts into the medium. The sum of the amount of free and cell-associated PT on the average

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equalled the amount of PT in the whole-cell suspension. This, however, was not true for 92 kD-OMP, FHA and PRN (Fig. 2), indicating that cells cannot be regarded as rigid spheres with the antigens fixed on the cell surface. Antigen exposure and antigen release by the cells may be a continuous process which can easily be affected by centrifuging and resuspending the cells. This means that a centrifugation step as encountered in the production process may influence the antigen density of the cell suspension. Moreover, antigens released into the medium are discarded during the centrifugation step and will therefore not contribute to the potency of the suspension. Therefore, in a production process the choice of harvest time of the whole-cell suspension should be a compromise between high surface densities of the antigens and high biomass concentration.

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Despite the fact that the FHA amount in the whole-cell suspension decreased drastically during cultivation, only little free FHA was found in the supernatant. Possibly, shear forces generated by shaking the culture flasks caused chain scission of FHA. This explanation is supported by earlier published findings about the loss of haemagglutinin activity of FHA due to hydromechanical forces (20, 21). Rodriguez et al. (21) revealed by SDS-PAGE a partial disappearance of 180-220 kD FHA components in a FHA-solution under stirred conditions, which was not seen after static incubation. This loss was accompanied by an increase of components of molecular weight lower than 97 kD. The substantial differences in FHA amount between WCS and corresponding SSC samples (Fig. 2A) indicate that shear forces generated by centrifugation and resuspension may also have caused chain scission of FHA.

It is interesting that the cell-associated 92 kD-OMP content increased while FHA disappeared during cultivation. Both FHA and 92 kD-OMP are surface-associated proteins (14, 22). Therefore, a possible explanation for this finding is that FHA and 92 kD-OMP may be closely situated on the cell surface. In that case, the loss of FHA during cultivation and treatment of the samples could cause a better access of the anti-92 kD-OMP mab to 92 kD-OMP.

In conclusion, the results of this study clearly show that biomass concentration alone is a poor criterium to characterise *B. pertussis* suspensions used for whole-cell vaccine. Established inter-strain variations with regard to antigen densities enable a manufacturer to make a rational choice of strains to be used in a whole-cell vaccine. The large differences in antigen densities per cell and release of antigens during the different culture stages show that the time of harvest should not be based on cessation of growth. Ideally, pertussis cultivations would show fast growing bacteria that express high antigen densities per cell and release little or no antigens into the growth medium. Therefore, further research on growth conditions of the bacteria is needed to establish, for example, the influence of medium components on antigen production and release. Finally, it can be assumed that the potency of whole-cell pertussis vaccines is enhanced by high levels of protective antigens. Therefore, the AnBi-ELISAs presented here are attractive not only as a tool to select strains, harvest time and growth conditions, but also to predict the quality of *B. pertussis* suspensions.

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