Collaborative study on a guinea pig serological method for the Assay of Acellular Pertussis vaccines

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ABSTRACT

An international collaborative study (coded BSP083) was performed under the aegis of the Biological Standardisation Programme supported by the Council of Europe and the European Commission, with the aim of replacing the in vivo challenge assays for potency determination of combined acellular pertussis (aP) vaccines by a refined procedure also allowing reduction of animal use.

This study investigates whether the immunogenicity of aP vaccine components could be assayed in a guinea pig (gp) serology model, using the same vaccine immunising doses as for D and T components potency testing, instead of using separate animals as is currently done. The BSP83 project is a follow up of 3 former collaborative studies (coded BSP019, BSP034 and BSP035) on serological methods for the potency testing of tetanus (T) and diphtheria (D) vaccines for human use. The use of gp instead of mice serology has the advantage of providing a larger volume of good quality antiserum for the assay of several vaccine components in the same sample, hence providing the opportunity for animal sparing.

The results of Phase I of the study demonstrated that gp serology may be a useful method for the immunogenicity assay of acellular pertussis vaccines. This was confirmed in Phase II of the study, using 7 different combined aP vaccines in an international collaborative study involving 17 laboratories from both public and private sectors.

Clear dose-response relationships were observed for different vaccines by ELISA, for antibodies against aP antigens, i.e. pertussis toxin (PT), filamentous haemagglutinin (FHA), fimbrial agglutinogens-2/3 (Fim 2/3) and pertactin (PRN). Intra- and inter-laboratory variations of aP ELISA results were found to be within an acceptable range.

For some combined vaccines, however, the range of vaccine dilutions for immunisation confirmed to be optimal for D and T potency testing may not provide optimal dose-response for all aP components. Method adjustments may thus be required and suitability should therefore be demonstrated for each vaccine combination and product prior to the application of this assay.

The results of this study support the use of the gp serological method for the determination of the immunogenicity of aP vaccines. The application of the method for batch release testing of combined D, T and aP vaccines could significantly contribute to the implementation of the 3R principles through reduction of animal use and improved animal welfare, whilst reducing costs.

As an outcome of this study, the Group of Experts No. 15 on Sera and Vaccines of the European Pharmacopoeia (Ph. Eur.) decided in February 2009 to include the gp serological assay as an example in the Ph. Eur. General chapter 2.7.16. on acellular pertussis vaccine assay.

KEYWORDS

Acellular pertussis vaccine, combined vaccines, potency testing, serology, 3R, guinea pig, collaborative study, European pharmacopoeia.

AIM

The aim of the present study was to investigate whether the immunogenicity of aP vaccine components could be assayed in the guinea pig model as D and T components and if the same vaccine immunising doses as for D and T components potency testing could be used instead of using separate animals as currently done.

1. INTRODUCTION

This study is part of the efforts of the EDQM to promote the 3Rs: Replacement, Reduction and Refinement of animal assays as proposed by Russell and Burch in 1959 and endorsed by the Council of Europe and the European Union in 1986 [1, 2, 3]. In this study, the indirect ELISA method for quantification of antibodies to aP antigens (PT, FHA, PRN and Fim-2/3) was assessed as an assay of aP components in combined vaccines, using sera from the same gp as for potency testing of the D and T components.

A collaborative study on the evaluation of a gp serological method for routine batch release testing of combined aP vaccines was initiated in January 2005.

This study (BSP083) is a follow up of 3 former collaborative studies on guinea pig serological methods for testing of T and D vaccines for human use (BSP019, BSP035 and BSP034) [4, 5, 6]. The principal aim of these studies was to considerably reduce the number of animals used for potency determination of combined vaccines and to substitute the direct challenge assays by serological methods, in line with the 3Rs concept.

Before an alternative serological method for determination of multiple components can be introduced in the Ph. Eur., it is necessary to demonstrate that the dose-response curves provide a useful regression in the range of doses to be tested for each of the components simultaneously. This has been shown to be achievable for the D and T components in a wide range of vaccines on the European market in the studies BSP034 and BSP035 [4, 5, 6]. However, for each vaccine component, the condition of similarity of dose-response curves (test vs. standard) has to be fulfilled in terms of linearity and /or parallelism. Gps were chosen as the species for immunisation as they are used in Ph. Eur. procedures for potency testing of D and T vaccines [7, 8]. Use of gp instead

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of mice serology has *inter alia* the advantage of providing sufficient good quality antiserum for assay of several vaccine components, including D and T components, hence allowing significant reduction of animal use.

To allow interim evaluation of test results and to monitor study progress, the study BSP083 was divided into 2 phases. Pre-validation (Phase I) study was performed in 2 laboratories (NoMA and NIBSC) to develop standard operating procedures for detection of antibodies to PT, FHA, PRN and Fim-2/3 using ELISA methods, and to investigate whether the same gp sera used for serological assays of the D and T toxoid components would provide suitable dose-response curves for immunogenicity testing of various pertussis antigens by a multidose assay.

The results from the Phase I study indicated that the same sera used in the Phase III of the BSP034 study, representing different combined vaccines and immunisation schemes, could provide good dose response also for the pertussis antigens. Sufficient information was obtained to recommend the continuation to Phase II (collaborative study), in which the reliability of the acellular pertussis ELISA was studied by obtaining information on intra- and inter- laboratory variation. The results of the collaborative study are presented here.

2. PARTICIPANTS

Eighteen private and public sector medicines control laboratories (OMCLs), all familiar and experienced in the field of vaccine testing were invited to participate in the collaborative study and 17 laboratories returned results. Throughout this report, the laboratories are referred to by their code numbers (1 to 17), allocated at random and not necessarily corresponding to the order of appearance in the list of participants (see section 8).

3. MATERIALS AND METHODS

A detailed standard operating protocol, elaborated in the laboratories of the 2 project leaders during the Phase I of the study, covering necessary equipment, reagents, chemicals and ELISA procedures was provided to the participants.

3.1. Vaccines

Licensed lots of 7 different combined aP vaccines (coded A, B, C, D, E, F,G), passing specifications for all components as specified in the Ph. Eur. and marketing authorisation documents, obtained from 4 different manufacturers, were tested in this study. The vaccines were of the following composition: DTaP, DTaP-IPV, DTaP-IPV-Hib, DTaP-IPV-HepB, DTaP-HepB-Hib-IPV. The presence of the aP antigens in the respective vaccines are given in Table 1.

3.2. Animals and immunisation protocol

The anti-aP antiserum against the respective vaccines were produced in various laboratories, using gps maintained under standard animal housing conditions. Depending on the vaccine, 4-dose series with 2, 3 or 5-fold dilutions were prepared in 0.9 % sterile saline solution. For most of the vaccines, the starting dose was 1/5. Guinea pigs meeting the requirements of the Ph. Eur. for assay of D vaccine (adsorbed) [7] were immunised by subcutaneous injection of a vaccine dose. Animals were bled after 5 or 6 weeks. Only diluted doses of vaccine F were injected twice with an interval of 2 weeks and the gps bled 6 weeks after the 1st injection. Blood was collected on anaesthetised animals by heart puncture, in numbered tubes. The clotting procedure varied between laboratories. One procedure was to incubate the tubes at 37 °C for 1 hour and to store them overnight at + 4 °C. The tubes were centrifuged at 800 g for 20 minutes to separate the serum from the cells. Sera were aliquoted and stored below - 20 °C. Serum samples were shipped frozen, under temperature controlled conditions between laboratories, EDQM and participants to the collaborative study.

3.3. Sera

A total of 27 serum samples were provided to each participating laboratory. All test vaccine antisera were pools from at least 8-12 guinea pigs and were produced according to the details in section 3.2. above.

In addition to the test vaccine antisera, the panel included:

- a gp reference antiserum produced for EDQM by Sanofi Pasteur (EDQM code 30787); this antiserum was used as a positive control serum to assign a relative antibody titre expressed in arbitrary units (see section 4);
- 2 negative control sera from non-immune gps : Samples “Neg.” (also coded “17965”) and S68;
- 3 negative control sera from gps immunised against D and T but not aP components: Sample “DT” (coded 21005 or 98/572), S11 and S61.

Table 1 summarises all sera used.

Table 1 – *Vaccine composition and resulting serum samples*

Serum sample No.	Immunisation vaccine		aP components in vaccine			
	Code	Composition	PRN	PT	FHA	Fim-2/3
S11		DT	-	-	-	-
S44-46	A	DTaP-IPV + Hib	x	x	x	-
S61		DT	-	-	-	-
S64-66	B	DTaP-IPV-Hep B	x	x	x	-
S69-71	C	DTaP-IPV	-	x	-	-
S72-74	D	DTaP-IPV-Hib	x	x	x	x
S76-78	E	DTaP	-	x	-	-
S79-81	F	DTaP-IPV-Hib with booster	x	x	x	x
S87-89	G	DTaP-IPV	x	x	x	x
Reference (30787)	Ref.	DTaP-IPV-Hib with booster	x	x	x	x
Negative controls:						
Neg. (17965)		-	-	-	-	-
S68		-	-	-	-	-
DT (21005 or 98/572)		DT	-	-	-	-

Table 2 – *Proposed pre-dilution values of test sera*

Study sample	Immunisation (injection volume:1ml)		Assay predilution of sera			
	vaccine	Vaccine dilution	PRN	PT	FHA	Fim-2/3
S11	DT	1/6.25	1/5	1/5	1/5	1/5
S44	DTaP-IPV-Hib	1/5	1/200	1/800	1/800	1/800
S45	DTaP-IPV-Hib	1/45	1/10	1/400	1/400	1/400
S46	DTaP-IPV-Hib	1/15	1/50	1/400	1/800	1/800
S61	DT	1/15	1/10	1/10	1/10	1/10
S64	DTaP-IPV-HepB	1/5	1/50	1/400	1/800	1/800
S65	DTaP-IPV-HepB	1/15	1/40	1/800	1/800	1/800
S66	DTaP-IPV-HepB	1/45	1/10	1/800	1/400	1/800
S69	DTaP-IPV	1/5	1/40	1/40	1/40	1/40
S70	DTaP-IPV	1/15	1/20	1/20	1/20	1/20
S71	DTaP-IPV	1/45	1/5	1/5	1/5	1/5
S72	DTaP-IPV-Hib	1/1.67	1/50	1/400	1/800	1/400
S73	DTaP-IPV-Hib	1/5	1/5	1/400	1/800	1/200
S74	DTaP-IPV-Hib	1/15	1/5	1/200	1/400	1/50
S76	DTaP	1/5	1/50	1/50	1/50	1/50
S77	DTaP	1/15	1/10	1/10	1/10	1/10
S78	DTaP	1/45	1/5	1/5	1/5	1/5
S79	DTaP-IPV-Hib with booster	1/5	1/5	1/100	1/100	1/100
S80	DTaP-IPV-Hib with booster	1/25	1/5	1/50	1/50	1/50
S81	DTaP-IPV-Hib with booster	1/125	1/5	1/25	1/25	1/25
S87	DTaP-IPV	1/5	1/5	1/800	1/800	1/100
S88	DTaP-IPV	1/10	1/5	1/400	1/800	1/20
S89	DTaP-IPV	1/20	1/5	1/400	1/400	1/10
Ref.	DTaP-IPV-Hib with booster	1/2	1/1280	1/1600	1/1600	1/1600
Neg.	-	-	1/5	1/5	1/5	1/5
S68	-	-	1/5	1/5	1/5	1/5
DT	DT	-	1/5	1/5	1/5	1/5

Proposed pre-dilutions of antisera were provided (Table 2) to help in the ELISA assay, but participants were informed that optimum pre-dilution to achieve the best dose-response curve may need to be determined in-house.

3.4. Reagents

Antigens for coating of ELISA plates, PRN, PT, FHA and Fim-2/3 were generous donations from 2 manufacturers. Commercial reagents, such as Horse Radish Peroxidase (HRP) conjugated anti-gp antibody (eg. Sigma A-7289), 2,2 azino-di-3-ethylbenzothiazoline sulphonate (ABTS, eg. Sigma A-9941) substrate, dried skimmed milk and buffers were procured by each participant.

3.5. Acellular pertussis ELISA

The titres of antibodies directed against PRN, PT, FHA and Fim-2/3 in gp antiserum were determined by ELISA. Participants were advised to use samples that have not been freeze-thawed repeatedly for the ELISA.

Purified Bordetella pertussis antigens (PRN, PT, FHA or Fim 2/3, see 3.4.) were diluted in a carbonate buffer (pH 9.6) at their optimal concentration (4 µg/ml for PRN and 2 µg/ml for PT, FHA and Fim-2/3).

Flat-bottomed 96 well-microplates (eg. Nunc Maxisorp, cat. no. 442404) were coated with 100 µl/well of the appropriate antigen solution and incubated overnight at 4 °C in a humid atmosphere. The plates were washed 4 times with Washing Buffer (phosphate buffered saline pH 7.4 containing 0.05 % Tween 20). The plates were blocked by addition of 150 µl of Blocking buffer (Washing buffer containing 5 % dried skimmed milk) per well, followed by incubation for 1 hour at 37 °C in a humid atmosphere. After thorough washing with Washing Buffer and drying, the plates could be kept wrapped in foil for up to 1 week at 4 °C before use.

Two-fold dilution series of the serum samples prepared in Sample Buffer (Washing buffer containing 1 % skimmed milk) using appropriate pre-dilution values (see 3.3.) were added to the coated wells (200 µl/well). A reference serum ("Ref.") and several negative gp control sera were included in each plate to monitor the performance of the assay and for trend analysis (see item 3.3). Plates were incubated at 37 °C for 2 hours with the test sera, then washed before incubation for 1 hour at 37 °C in a humid atmosphere with HRP-conjugated goat anti-guinea pig antibody diluted in Sample Buffer (100 µl/well). After washing, the bound

antibodies were detected by incubation with an appropriate substrate (eg. ABTS, diluted in Sample buffer, 100 µl/well, for 30 minutes at room temperature and protected from light). Absorbance was read at 405 nm in the same order as during the addition of the substrate solution.

3.6. Study design

Participants were required to titrate each serum sample in 2 valid independent assays for detection of antibodies to each of the 4 aP antigens: PT, PRN, FHA and Fim-2/3, using the provided protocol and suggested pre-dilution of each serum. Experimental data were reported using the electronic data sheets provided. Participants were requested to report all modifications to the SOP in these datasheets.

4. STATISTICAL EVALUATION

The data were analysed at the EDQM using multi-parameter logistic curve analysis. Antibody titres were calculated in relation to the provided reference antiserum (EDQM code 30787, see Table 1). Intra-laboratory and inter-laboratory variations were assessed with standard methods for analysis of variance.

The data were analysed by fitting a 4-parameter logistic curve using the CombiStats software (version 4.0) [9]. The equation relating the responses y to the log-transformed doses x is:

$$y = d + \frac{a - d}{1 + e^{-b(x-c)}} + \epsilon$$

in which ϵ is an error term. Samples for which the first dilution (Row A of the plate) yielded an OD of less than $1/25^{\text{th}}$ above the minimum observed OD on the whole plate relative to the difference between the maximum and minimum observed OD on that plate were considered to be below the quantification limit and therefore not included in the model. So, only samples with responses satisfying the criterion:

$$\frac{y - y_{\min}}{y_{\max} - y_{\min}} \geq 1/25$$

were included. In 5 of the 413 assays there was not sufficient data available to estimate the upper asymptote in which cases a 3-parameter exponential curve was used instead:

$$y = d + e^{b(x-c)} + \epsilon$$

5. RESULTS

Seventeen laboratories submitted results from a total of 413 micro-titre plates, representing nearly 40,000 individual readings. Most laboratories carried out 2 assays for each of the 4 antigens as requested but additional assays were sometimes performed when assays were considered to be invalid. Laboratory 5 reported data for the PT antigen only. All datasets were analysed as described in section 4.

The quality of the fit for individual ELISA assays was in general excellent with correlation coefficients (*r)* above 0.99 in 98.3 per cent of the assays, above 0.995 in 94.2 per cent of the assays and above 0.999 in 49.9 per cent of the assays. The minimum observed correlation coefficient was 0.982.

Tables 3.1 to 3.3 show the estimated activity for each of the sera by antigen and by laboratory. The values shown are the geometric mean of the estimates from the 2 assays. In cases where more than 2 assays were performed, only the last 2 assays were included. In cases where only 1 result was available, that value is printed in bold and italic. When both results were below the quantification limit, the combined result was also considered to be below quantification (shown as <). When only one result was below quantification limit, the other result is printed. When the difference between both results was more than 3.36-fold (1.75 dilution step), the geometric mean is printed on a black background. When the difference was less than 3.36-fold but more than 2.38-fold (1.25 dilution step), the geometric mean is printed on a grey background. These thresholds were chosen for consistency with the tables published in BSP034 [6]. It can be seen that the majority of cases where more than 2.38-fold difference was found are for very low titre sera (below 1 AU/ml) where this difference is not of great importance. In the few cases where for higher titre sera a large difference between the 2 assays was found, the result also tends to differ from the other laboratories (see e.g. S72, Lab 16, FHA).

A graphical impression of the results is given in Figures 1 to 4. The results are shown as box-and-whisker plots per serum (see ANNEX I for explanations). They are grouped by vaccine and in order of descending activity per serum. Sera S45 and S46 are shown in reverse order because their labels were accidentally swapped prior to running the collaborative study.

6. DISCUSSION

Results of this study confirm the good specificity of the aP-ELISA tests used. On the whole, no antibody against components absent from the immunising vaccine was detected in the corresponding serum samples (Tables 3.1-3.3).

Control sera produced against DT vaccines (samples 11, 61 and DT negative control) were negative for all 4 aP components tested. Similarly, serum samples from animals immunised with combined aP vaccines lacking PRN (S69-71, S76-78), FHA (S69-71, S76-78) or Fim-2/3 (S44-46, S64-66, S69-71, S76-78) components did not contain detectable levels of antibodies against the corresponding antigens. Furthermore, none of the non-immune negative control sera (Neg. and S68) contained detectable levels of antibodies against the 4 aP antigens tested. In this study, no false positive antibody responses were seen for any of the aP antigens. Due to the sequence homology of surface antigens (such as of PRN) in different *Bordetella* species close to *B. pertussis*, sera from previously infected animals can cause cross reactions and high background values in aP ELISA. It is therefore important that the sanitary status of the gps is well monitored before serum sampling to ensure the absence of antibodies against species such as *B. bronchiseptica.* The negative control serum should preferably be taken from the same group of animals as used for the immunisation of test vaccines in the same animal facility and from the same suppliers to avoid bias if inadvertently a subclinical infection should have taken place (‘sentinel animals’).

The statistical analyses of the datasets showed that intra-laboratory and inter-laboratory variations of the aP ELISA methods used in this study are within acceptable range among the study participants (Tables 3.1-3.3 and Figures 1-4).

Reasonable median dose-response relationship was found in most cases, although the shape of the regression curve could vary considerably between vaccines. This supports the need for product- or product class- (vaccines of similar antigen and/or adjuvant composition) specific reference vaccines in routine testing.

This study used a single serum sample for each vaccine dilution group, which gave acceptable results. A modification of this method with titration of individual sera, as it may be expected during routine lot testing, may result in greater precision, accuracy, parallelism and linearity. The need of adjustments of the predilution values by each participating laboratory was limited. The proposed pre-dilution value (predicted during phase I study) of test sera in general provided acceptable titration curves in ELISA, with mainly

excellent fits. However, predilution values could have been adapted to obtain improved titration curves for some serum samples. For example, sera produced with vaccines A and B could have been more diluted for PT- and FHA-ELISA (i.e. 1/100 instead of 1/50). For Vaccine D, the predilution values could have been lower for PRN and higher for PT- and FHA-ELISA, but were satisfactory for Fim-2/3-ELISA.

It must however be noted that the sub-optimal predilutions of some serum samples did not impair the calculation of individual titres and did not affect the conclusions and outcome of this study.

In order to provide higher discriminative power between the vaccine batches, the guinea pigs were injected with a single immunising dose for the production of test serum samples, as recommended by the Ph. Eur. for D and T assays [7, 8]. In order to investigate if a common guinea pig reference serum could be used in this study, regression curves for the respective aP antigens of vaccine D and F were compared in the Phase I of this study. The results indicated that similar titration curves were obtained whether 1 or 2 immunising doses were given to the animals. It was further substantiated in the collaborative study that the booster dose did not impact on the shape of the regression curves of individual sera. Consequently the Reference serum, produced in guinea pigs given 2 doses of a vaccine containing 5 components was used in this study.

In the present study, a reference serum (Ref 30787) was used only to assign an arbitrary unitage to relative titres and was confirmed suitable for most of the tested vaccines. Reference vaccine was not used. The combined vaccines tested in the study contained 3, 4 or 5 vaccine components. In general, no obvious relationship between the degree of agreement and the number of vaccine components could be identified. For one 5-component vaccine tested (vaccine D), almost similar ELISA-titration curves were obtained for each aP component.

Using reference antiserum for calculation of potency of acellular Pertussis vaccines would be in contrast to the Ph. Eur. requirements of the monograph 1356 for the assay on Pertussis vaccine acellular, component, adsorbed performed in mice [10]. The capacity of the vaccine to induce the formation of specific antibodies is compared with the same capacity of a reference preparation examined in parallel. In line with this, the antibody titres in the sera of gps immunised with reference and test vaccines should be calculated. From the values obtained, the potency of the test vaccine in relation to the reference vaccine should be calculated by the usual statistical methods.

An unexpected lack of dose response was observed for vaccine B (DTaP-IPV-Hep B, serum samples 64-66), which showed very high antibody titres against PT and FHA. This might be due to the specific composition of this vaccine, as these 2 aP antigens are present in higher concentrations than PRN. Moreover, product-specific features cannot be excluded. It must be noted that vaccine A (S44-46) has a similar aP antigen composition to vaccine B but gave good dose-response curves for all aP antigens. These 2 vaccines however differed in the non- aP components: vaccine B includes HepB while vaccine A contains Hib component. It might be possible that the HepB component or the adjuvant used for this combined vaccine increases the immunogenicity of all aP antigens. The response against the PRN antigen was slightly lower than with other components in vaccine A (Figure 1, particularly at the 2nd and 3rd doses). The enhanced response against aP antigens, possibly due to the presence of the HepB component or to the composition of the adjuvant, could explain that the decreasing dose-response against PT and FHA components could not be observed in vaccine B at the immunising doses used in this study.

The immunising doses of the vaccines used in BSP083 were determined according to previous experiments in order to be optimal for D and T. It might be possible that these doses are not suitable for all aP components for all licensed vaccines. Further optimisation of the dilution range for the immunisation of the guinea pigs might thus be required for some products (such as vaccine B) to allow serology testing of all D, T and aP components in the same serum sample.

7. CONCLUSIONS

The current compendial (Ph. Eur.) assay of pertussis vaccine (acellular) [11] is an immunogenicity test in the mouse where the formation of specific antibodies induced by a test vaccine is compared with the same capacity of a reference vaccine preparation examined in parallel. The specific antibodies are determined using suitable immunochemical methods, e.g. ELISA.

The present results demonstrate that the aP antigens of combined vaccines can also be assayed in the guinea pig model, moreover using the same animal groups and with the same vaccine doses as for the potency testing of D and T components, for most of the vaccines. Where this is achievable, the number of animals can be considerably reduced during testing.

In order to avoid false positive results, negative control serum should preferably originate from sentinel individuals from the same group as used for the immunisation of the test vaccine and the sanitary status of the animals should be monitored, in particular for infection with *B. bronchiseptica*.

Because of the possible interaction between vaccine components, each laboratory will need to confirm if immunising doses are suitable and perform adjustments, if necessary, to validate the assay for the tested product.

As an outcome of this collaborative study, the Group of Experts for Sera and Vaccines (15) of the Ph. Eur. considered in February 2009 the addition of the serology method in guinea pigs as an example in the Ph. Eur. general chapter 2.7.16. on acellular pertussis vaccine assay.

This would allow to perform the serological assay of acellular pertussis vaccine in the same group of guinea pigs used for the assay of tetanus vaccine (adsorbed) and diphtheria vaccine (adsorbed), for combined vaccines containing acellular pertussis, diphtheria and tetanus components, when the common immunisation conditions (for example, doses, duration) have been demonstrated to be valid for these components.

8. PARTICIPANTS (IN ALPHABETICAL ORDER OF COUNTRY)

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9. ABBREVIATIONS

aP: acellular Pertussis; AU: Arbitrary Unit; BSP: Biological Standardisation Programme; D: Diphtheria; EDQM: European Directorate for the Quality of Medicines & HealthCare; FHA: Filamentous Haemagglutinin; Fim: Fimbriae; Gp(s): guinea pig(s); Hep B: Hepatitis B; Hib: Haemophilus influenza type b; IPV: Inactivated Polio Vaccine; IU: International Unit; NIBSC: National Institute for Biological Standards and Control; NoMA: Norwegian Medicines Agency; OD: Optical Density; OMCL: Official Medicines Control Laboratory; Ph. Eur.: European Pharmacopoeia; PRN: Pertactin; PT: Pertussis Toxin; SOP: Standard Operating Procedure; T: Tetanus; WHO: World Health Organization.

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Table 3.1 - *Estimated activities per serum and per laboratory (in AU/ml)*

		Plate 1									
Antigen	Lab	S68	S11	S44	S45	S46	S64	S65	S66	S69	DT
Pertactin (PRN)	1	0.0	0.0	169.2	6.6	47.0	258.9	89.5	33.9	<	0.0
	2	<	<	164.2	7.5	52.6	263.4	96.1	36.2	<	0.1
	3	0.2	0.1	205.3	9.8	51.1	247.8	101.4	35.0	<	0.3
	4	0.1	0.1	183.1	7.9	55.2	225.7	97.8	29.5	<	0.1
	6	0.1	0.1	178.2	7.9	55.5	236.7	92.7	35.3	<	0.1
	7	0.1	0.1	198.4	8.2	51.3	224.7	88.2	29.2	<	***0.1***
	8	0.1	0.1	177.1	7.7	52.7	218.0	86.7	22.5	<	0.1
	9	0.1	0.2	183.2	7.8	58.6	231.6	97.3	35.2	<	0.2
	10	0.0	0.0	174.9	5.0	48.3	244.4	84.8	23.8	<	<
	11	0.1	0.1	190.4	7.6	51.1	194.0	84.7	33.7	<	0.1
	12	0.1	0.1	217.0	7.6	49.6	224.3	93.4	33.1	<	0.1
	13	0.2	0.2	229.3	9.0	64.9	261.2	100.7	34.7	0.5	0.2
	14	0.2	0.2	228.9	11.7	78.7	286.9	124.1	48.6	**0.3**	0.2
	15	0.1	0.1	206.3	9.1	56.8	257.5	109.8	40.4	<	0.2
	16	0.5	0.4	250.6	12.3	88.3	314.0	146.5	58.2	1.0	0.6
	17	0.1	0.1	195.3	7.0	54.4	171.9	92.4	31.1	0.2	0.2
	Median	0.1	0.1	192.9	7.8	53.6	240.5	94.8	34.3	0.4	0.1
Pertussis toxin (PT)	1	<	<	730.8	265.3	758.2	714.5	1260.1	550.2	262.5	<
	2	<	<	660.0	234.3	528.4	675.5	803.3	452.3	187.7	<
	3	0.1	<	935.7	200.4	358.0	488.2	911.2	457.7	202.2	0.1
	4	<	<	787.8	256.4	605.9	679.3	989.1	493.6	275.6	<
	5	<	<	644.5	272.3	596.6	685.5	814.4	603.4	84.0	<
	6	<	<	733.3	238.9	552.9	665.3	846.1	450.3	224.6	<
	7	0.1	0.1	975.0	296.7	688.4	722.7	1343.5	498.5	254.0	0.1
	8	<	<	622.7	212.1	451.0	518.0	757.4	378.2	155.2	0.1
	9	0.1	0.1	722.1	264.9	562.4	671.4	1073.1	779.3	169.0	0.1
	10	<	0.0	734.6	211.1	559.6	557.1	846.6	388.9	104.9	<
	11	<	<	270.1	69.8	166.6	193.7	272.8	145.9	65.9	<
	12	0.0	<	900.5	250.2	430.6	658.7	970.5	508.7	219.5	0.0
	13	0.0	0.1	785.1	233.6	702.5	678.1	981.4	665.2	214.4	0.0
	14	0.2	0.2	837.2	260.6	685.7	783.1	1082.0	553.6	284.6	0.2
	15	0.0	0.1	715.0	215.8	450.1	583.2	791.3	392.3	163.8	0.0
	16	0.2	0.1	921.4	240.9	516.2	658.7	1039.7	661.1	292.5	0.2
	17	0.0	0.0	929.2	314.3	734.6	788.1	1523.5	628.6	195.2	0.0
	Median	0.1	0.1	759.8	239.9	556.2	668.4	975.9	496.0	208.3	0.1
Filamentous haemagglutinin (FHA)	1	<	<	477.1	133.2	299.0	403.8	400.4	144.0	<	<
	2	<	<	417.1	130.5	222.5	403.1	367.1	155.2	<	<
	3	0.1	<	526.8	122.5	293.9	441.7	461.1	177.7	<	0.1
	4	<	<	466.6	141.6	267.1	424.7	422.8	181.7	<	0.0
	6	<	<	447.4	136.2	252.6	390.8	370.4	154.6	<	<
	7	0.2	0.1	764.8	145.8	260.8	376.3	443.0	142.2	<	0.2
	8	<	<	497.6	154.7	291.4	436.9	416.6	170.2	<	0.1
	9	0.1	0.1	488.3	158.4	327.7	414.5	451.6	225.7	<	0.1
	10	0.1	0.0	341.3	80.0	184.5	275.9	258.7	96.8	<	0.1
	11	<	<	164.9	37.9	80.7	117.4	112.2	48.1	<	<
	12	0.0	<	585.9	139.2	263.8	438.5	434.4	179.3	<	<
	13	0.0	0.0	538.7	125.3	364.7	512.2	443.0	210.6	0.2	0.0
	14	0.1	0.1	539.8	155.2	285.3	413.5	409.6	168.3	0.1	0.1
	15	0.0	0.0	483.9	122.7	235.3	381.9	337.9	138.6	<	0.0
	16	0.1	0.0	619.7	206.5	488.3	246.2	397.1	269.0	0.1	0.1
	17	0.0	0.0	538.5	239.1	438.7	635.0	611.3	233.9	0.2	0.0
	Median	0.1	0.0	492.9	137.7	276.2	408.7	413.1	169.2	0.2	0.1
Fimbrial-2/3 (Fim-2/3)	1	<	<	<	<	<	<	<	<	<	<
	2	<	<	<	<	<	<	<	<	<	<
	3	0.1	<	<	<	<	<	<	<	<	0.1
	4	<	0.0	<	<	<	<	<	<	<	0.0
	6	<	<	<	<	<	<	<	<	<	<
	7	0.1	0.1	<	<	<	<	<	<	0.3	***0.0***
	8	<	<	<	<	<	0.1	<	0.1	<	0.1
	9	0.1	0.1	<	<	<	0.1	<	0.1	<	0.1
	10	0.0	0.0	<	<	<	<	<	<	<	0.0
	11	<	<	<	<	<	<	<	<	<	<
	12	<	<	<	<	<	<	<	<	<	<
	13	0.1	0.1	<	<	<	<	<	<	0.4	0.1
	14	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.1
	15	0.0	0.0	<	<	<	<	<	<	<	0.0
	16	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	**0.2**	**0.1**
	17	***0.0***	0.1	<	<	<	1.8	<	<	<	***0.0***
	Median	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.1

Explanations: Each value is the geometric mean of 2 assays. Values printed on a grey background are cases where the difference between both results is between 2.38-fold and 3.36-fold. Values printed on a black background are cases where the difference is more than 3.36-fold. When only 1 result was available it is printed in bold and italic. Values below quantification limit are printed as < . When no results are available this is indicated as - .

Table 3.2 - *Estimated activities per serum and per laboratory (in AU/ml)*

		Plate 2									
Antigen	Lab	S68	S70	S71	S72	S73	S74	S76	S77	S78	DT
Pertactin (PRN)	1	0.0	<	<	46.9	8.9	0.3	<	<	<	0.0
	2	0.1	<	<	56.9	11.4	0.6	<	<	<	0.1
	3	0.2	<	<	60.2	11.3	0.8	<	<	0.1	0.3
	4	0.1	<	0.1	53.0	11.0	0.6	<	<	<	0.1
	6	<	<	<	49.6	10.9	0.7	<	<	<	0.1
	7	0.1	<	***0.0***	53.0	9.9	0.7	<	0.1	***0.1***	***0.1***
	8	0.1	<	<	50.9	9.7	0.6	<	<	<	0.1
	9	**0.5**	0.4	***0.3***	52.6	11.4	1.1	<	0.4	***0.2***	0.4
	10	<	<	<	53.9	5.0	0.1	<	<	<	<
	11	0.1	0.1	0.1	43.8	8.0	0.7	0.4	0.1	0.0	0.1
	12	0.1	<	0.0	48.2	9.4	0.5	<	<	0.0	0.1
	13	0.2	0.3	0.1	57.6	10.3	1.0	0.8	0.2	0.1	0.2
	14	0.1	0.2	0.1	48.1	11.7	1.0	0.3	0.2	0.1	0.2
	15	0.1	<	0.1	49.1	11.9	0.8	<	0.1	0.1	0.2
	16	0.4	0.7	***0.4***	75.5	19.5	3.3	1.6	0.4	***0.4***	0.5
	17	0.1	0.1	0.1	36.7	7.6	0.7	0.2	0.1	0.1	0.1
	Median	0.1	0.3	0.1	51.8	10.6	0.7	0.4	0.2	0.1	0.1
Pertussis toxin (PT)	1	<	56.4	6.6	353.1	219.5	79.5	122.8	46.2	1.6	<
	2	<	52.0	5.7	284.6	213.8	75.9	109.9	36.1	1.5	<
	3	0.1	52.7	4.1	198.8	195.7	80.1	267.7	35.7	1.0	0.1
	4	<	47.9	7.1	284.3	247.8	82.0	118.0	35.8	2.3	<
	5	<	20.5	**2.5**	243.0	193.3	80.2	35.1	**10.1**	**0.8**	<
	6	<	50.8	5.7	282.9	235.4	79.8	129.2	33.3	1.6	<
	7	0.1	53.3	6.7	274.5	245.4	76.3	111.1	37.0	2.4	0.1
	8	<	33.3	3.1	258.2	230.9	79.3	65.9	21.3	1.1	0.1
	9	0.1	44.1	***4.9***	261.0	233.2	75.8	94.1	32.3	***1.6***	0.1
	10	<	40.2	2.3	283.9	219.1	68.8	60.2	17.2	0.9	<
	11	<	15.0	1.8	104.3	75.8	26.8	37.2	11.4	0.7	<
	12	<	51.3	7.0	297.8	235.4	80.7	109.3	40.4	2.1	0.0
	13	0.0	46.2	7.9	235.4	295.4	103.8	115.7	43.3	1.6	0.0
	14	0.1	57.5	9.2	277.5	229.2	70.4	131.0	38.6	2.2	0.1
	15	0.0	45.6	5.7	242.3	243.1	60.9	85.4	31.9	1.6	0.0
	16	0.1	61.3	14.7	332.9	233.4	86.2	189.1	50.3	3.4	0.2
	17	0.0	45.8	4.5	238.3	225.9	55.5	104.8	32.7	1.0	0.0
	Median	0.1	49.4	5.7	276.0	232.1	77.8	110.5	35.7	1.6	0.1
Filamentous haemagglutinin (FHA)	1	<	<	***0.1***	380.9	308.9	152.9	<	<	***0.0***	<
	2	<	<	***0.2***	379.6	261.5	117.3	<	<	***0.0***	<
	3	0.1	<	***0.2***	411.0	308.9	108.7	<	<	<	0.1
	4	<	<	0.2	397.4	303.4	126.2	<	<	<	<
	6	<	<	0.1	408.9	298.2	119.4	<	<	<	<
	7	0.2	0.2	***0.4***	412.9	293.7	133.2	<	0.2	***0.2***	0.2
	8	<	<	0.2	415.1	333.7	132.5	<	<	0.1	0.1
	9	0.1	<	0.2	405.0	306.2	122.8	<	0.1	0.1	0.1
	10	0.1	0.1	***0.4***	450.7	344.9	100.4	<	0.1	***0.0***	0.0
	11	<	<	***0.1***	140.6	89.6	37.8	<	<	***0.0***	<
	12	0.0	<	0.2	387.5	309.7	122.6	<	<	<	0.0
	13	0.0	0.1	***0.0***	459.0	361.5	174.0	0.2	0.0	0.0	0.0
	14	0.1	0.1	***0.1***	439.7	348.7	139.0	**0.1**	0.1	***0.1***	0.1
	15	0.0	<	***0.2***	445.6	355.8	141.4	<	<	***0.0***	0.0
	16	0.1	0.1	***0.4***	**758.2**	437.2	141.5	0.1	0.1	***0.0***	0.1
	17	***0.0***	0.1	***0.1***	365.9	302.9	92.4	0.1	0.0	***0.0***	***0.0***
	Median	0.1	0.1	0.2	409.9	308.9	124.5	0.1	0.1	0.0	0.1
Fimbrial-2/3 (Fim-2/3)	1	<	<	<	342.1	163.8	19.6	<	<	<	<
	2	<	<	-	319.3	143.0	23.0	<	<	-	<
	3	0.1	<	-	244.6	142.5	26.8	<	-	-	0.1
	4	<	***0.0***	<	325.5	156.6	23.7	<	<	***0.0***	<
	6	<	<	<	313.4	157.2	24.1	<	<	<	<
	7	0.2	0.4		***304.3***	163.0	20.3	1.0	0.2	-	***0.1***
	8	<	<	***0.0***	334.2	182.0	30.6	<	<	***0.0***	0.1
	9	0.1	<	***0.0***	266.6	125.3	21.4	<	<	***0.1***	0.1
	10	0.0	<	-	458.3	168.3	16.2	<	<		<
	11	<	<	***0.0***	108.3	43.6	7.3	<	<	***0.0***	<
	12	<	<	<	297.0	134.1	20.4	<	<	<	<
	13	0.1	0.2	0.1	292.3	152.9	24.5	0.5	0.1	-	0.1
	14	0.1	0.3	***0.1***	266.0	144.4	24.2	0.6	0.2	***0.1***	0.1
	15	0.0	<	***0.0***	254.3	125.1	17.3	<	<	***0.0***	0.0
	16	0.2	0.6	***0.2***	322.6	160.6	30.3	**0.5**	0.2	***0.1***	0.2
	17	***0.0***	0.2	***0.0***	218.2	103.8	11.9	<	0.1	***0.0***	***0.0***
	Median	0.1	0.3	0.0	300.7	148.7	22.2	0.6	0.2	0.0	0.1

Explanations: Each value is the geometric mean of 2 assays. Values printed on a grey background are cases where the difference between both results is between 2.38-fold and 3.36-fold. Values printed on a black background are cases where the difference is more than 3.36-fold. When only 1 result was available it is printed in bold and italic. Values below quantification limit are printed as < . When no results are available this is indicated as - .

Table 3.3 - *Estimated activities per serum and per laboratory (in AU/ml)*

		Plate 3									
Antigen	Lab	S68	S79	S80	S81	S87	S88	S89	S61	Neg	DT
Pertactin (PRN)	1	0.0	772.7	***215.2***	1.4	8.9	4.4	2.0	<	0.0	0.0
	2	***0.0***	751.2	174.6	1.7	8.4	4.2	2.1	<	***0.0***	***0.1***
	3	0.2	1572.1	516.1	1.9	8.4	4.3	2.5	<	0.3	0.2
	4	0.1	595.2	157.5	1.8	7.8	2.8	3.0	<	0.1	0.1
	6	<	681.7	169.4	1.8	8.2	4.3	2.4	<	0.1	0.1
	7	0.1	***554.0***	***123.0***	1.6	7.5	3.9	2.1	<	0.1	***0.1***
	8	0.1	***622.0***	***178.7***	1.7	5.0	4.0	1.7	<	0.1	0.1
	9	**0.2**	632.1	127.9	2.0	6.5	4.0	2.2	1.0	0.3	0.3
	10	0.0	***536.7***	***49.3***	0.7	2.7	2.0	0.9	0.1	<	<
	11	0.1	***678.2***	***174.2***	1.6	6.3	3.4	2.1	0.1	0.1	0.1
	12	0.1	***623.1***	***164.4***	1.7	7.6	3.8	2.1	<	0.0	0.1
	13	0.2	565.7	***164.8***	1.5	6.9	3.9	2.2	0.3	0.2	0.2
	14	0.2	746.4	204.6	2.1	9.2	4.7	2.7	0.2	0.2	0.2
	15	0.1	-	-	2.7	7.3	4.3	2.9	0.1	0.2	0.2
	16	0.4	-	-	2.8	12.2	5.7	3.4	0.5	0.3	0.4
	17	0.1	541.3	133.9	1.4	5.9	3.0	1.6	0.2	0.1	0.2
	Median	0.1	627.6	167.1	1.7	7.5	4.0	2.2	0.2	0.1	0.1
Pertussis toxin (PT)	1	<	555.9	239.7	0.3	356.8	244.3	135.3	<	<	<
	2	<	578.1	224.8	0.4	341.7	201.2	110.5	<	<	<
	3	0.1	510.5	225.2	0.5	535.3	261.2	170.3	<	0.1	0.1
	4	<	524.1	245.2	0.6	427.9	245.0	164.5	<	<	<
	5	<	459.9	257.4	**0.4**	338.8	200.3	146.0	<	<	<
	6	<	567.3	243.2	0.6	398.7	228.0	149.8	<	0.0	0.1
	7	0.0	712.4	269.6	0.6	384.3	214.4	139.7	0.1	0.1	0.1
	8	<	487.2	204.0	**0.4**	335.0	205.5	134.3	<	0.1	0.1
	9	**0.2**	597.4	280.2	1.0	373.7	263.8	143.9	0.9	0.2	0.2
	10	<	416.7	150.1	0.4	345.0	194.1	118.7	<	<	<
	11	0.0	205.5	84.5	0.3	120.3	76.7	58.5	<	0.0	0.0
	12	0.0	518.0	216.8	0.5	419.2	238.5	157.4	0.0	<	0.0
	13	0.1	890.0	290.0	0.6	432.9	209.3	156.1	0.1	0.1	0.1
	14	0.1	560.3	237.8	0.6	402.4	216.0	142.8	0.2	0.1	0.1
	15	0.0	493.2	224.8	0.4	336.3	209.8	115.8	0.1	0.1	0.1
	16	0.1	897.4	313.1	1.1	499.0	252.1	168.6	0.3	0.1	0.2
	17	0.0	601.1	272.5	0.5	399.2	249.1	141.3	0.1	0.0	0.0
	Median	0.1	558.1	238.7	0.5	391.5	222.0	142.1	0.1	0.1	0.1
Filamentous haemagglutinin (FHA)	1	<	1227.6	691.0	51.2	261.2	115.6	84.4	<	<	<
	2	<	1096.7	546.5	52.5	224.7	147.6	92.1	<	<	<
	3	0.1	1166.1	622.1	51.0	604.5	331.9	**84.1**	<	0.1	0.1
	4	<	1019.0	598.8	56.3	259.2	159.4	113.0	<	0.0	0.0
	6	<	1044.5	555.7	57.9	251.4	157.8	103.0	<	<	<
	7	0.2	1407.6	677.5	58.7	221.9	132.9	104.0	0.2	0.2	0.2
	8	<	1118.9	542.8	51.2	212.7	150.4	111.8	<	0.1	0.1
	9	0.1	1146.0	574.0	53.6	259.2	166.8	97.7	0.3	0.2	0.1
	10	0.0	885.9	413.4	31.2	201.9	114.5	41.8	<	<	<
	11	<	370.6	218.4	16.0	71.9	49.7	28.6	<	0.0	<
	12	<	975.5	496.0	53.8	262.3	165.9	107.4	<	0.0	<
	13	0.0	1187.9	641.8	54.5	300.4	237.3	141.4	0.1	0.1	0.0
	14	0.1	1074.3	606.8	59.2	296.6	183.0	121.3	0.2	0.1	0.1
	15	0.0	939.7	631.9	41.2	213.2	119.2	80.4	<	0.1	0.1
	16	0.1	1693.9	767.8	76.2	248.5	214.3	126.5	0.1	0.1	0.1
	17	***0.0***	1268.2	515.8	30.2	285.0	208.7	113.2	0.0	0.0	***0.0***
	Median	0.1	1107.8	586.4	53.1	255.3	158.6	103.5	0.1	0.1	0.1
Fimbrial-2/3 (Fim-2/3)	1	<	682.2	118.4	0.2	198.8	12.9	1.8	<	<	<
	2	<	770.2	134.0	0.3	192.7	18.5	2.8	<	<	<
	3	0.1	753.6	138.3	0.5	361.1	28.4	3.7	<	0.1	0.1
	4	<	582.9	141.6	0.3	223.3	15.9	2.7	<	<	<
	6	<	680.6	133.5	0.3	**72.2**	17.1	2.8	<	<	<
	7	0.2	821.2	155.7	0.7	230.4	21.6	4.5	0.4	0.2	***0.0***
	8	<	832.6	125.6	0.4	125.4	15.5	2.6	<	<	0.1
	9	0.1	764.5	144.2	0.4	193.4	14.8	2.8	0.4	0.1	0.1
	10	<	703.4	125.0	0.8	253.4	17.6	3.5	<	0.1	<
	11	<	267.5	51.8	0.1	61.7	5.7	1.1	<	<	<
	12	<	618.1	114.2	0.3	211.0	15.3	2.9	<	<	<
	13	0.1	676.8	174.8	0.7	178.4	17.0	3.2	0.2	0.1	0.1
	14	0.1	725.9	136.0	0.5	196.1	17.4	3.3	0.2	0.1	0.1
	15	0.1	631.9	132.8	0.3	197.1	**7.0**	**7.5**	<	0.1	0.1
	16	0.2	829.9	144.2	1.0	281.2	25.3	4.3	0.2	0.1	0.2
	17	***0.0***	536.0	110.8	0.4	114.0	9.9	1.9	0.1	0.0	***0.0***
	Median	0.1	692.8	133.7	0.4	196.6	16.4	2.8	0.2	0.1	0.1

Explanations: Each value is the geometric mean of 2 assays. Values printed on a grey background are cases where the difference between both results is between 2.38-fold and 3.36-fold. Values printed on a black background are cases where the difference is more than 3.36-fold. When only 1 result was available it is printed in bold and italic. Values below quantification limit are printed as < . When no results are available this is indicated as - .

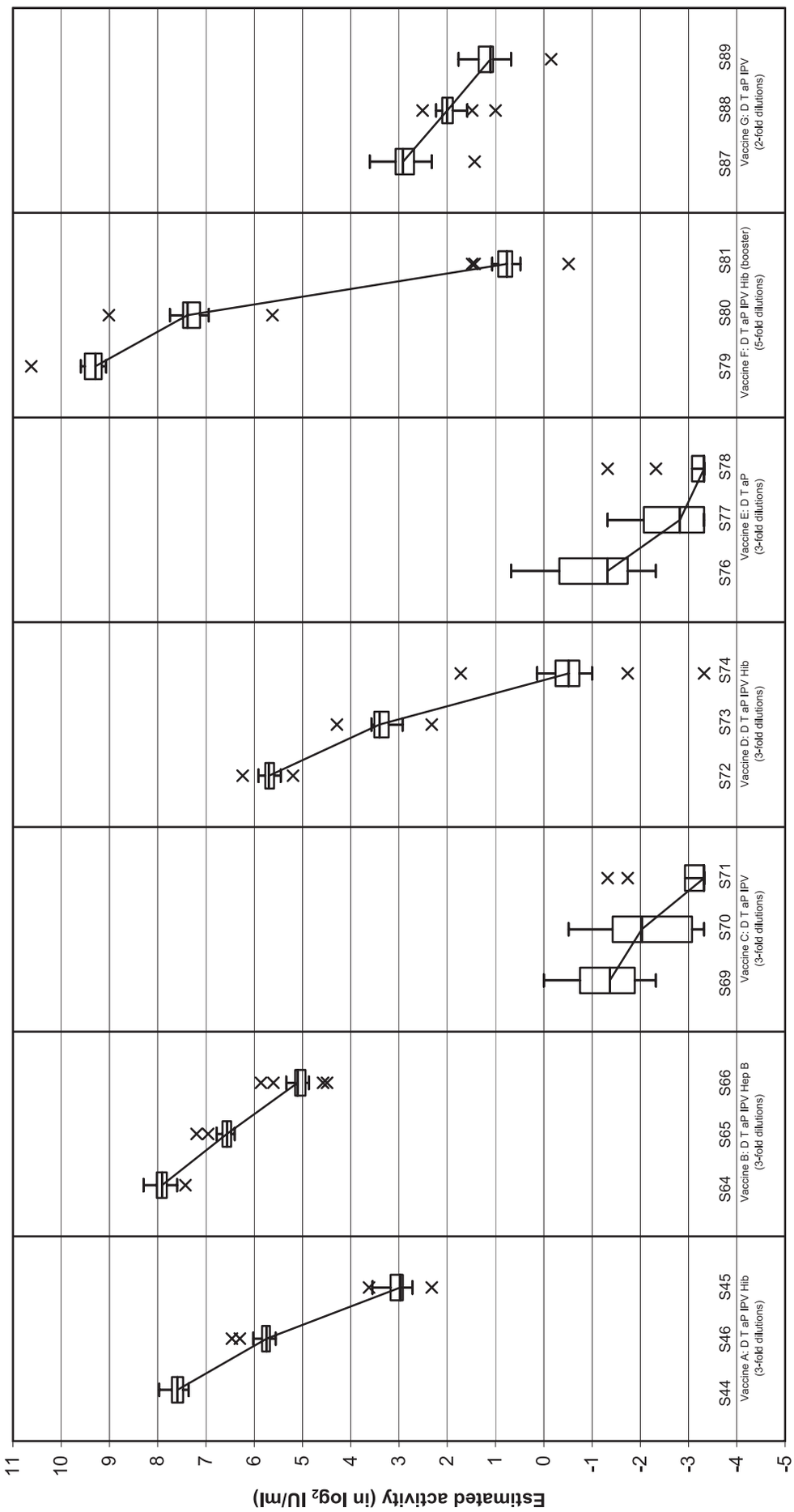


Figure 1 - *Box-and-whisker plots of results (Pertactin, PRN)*

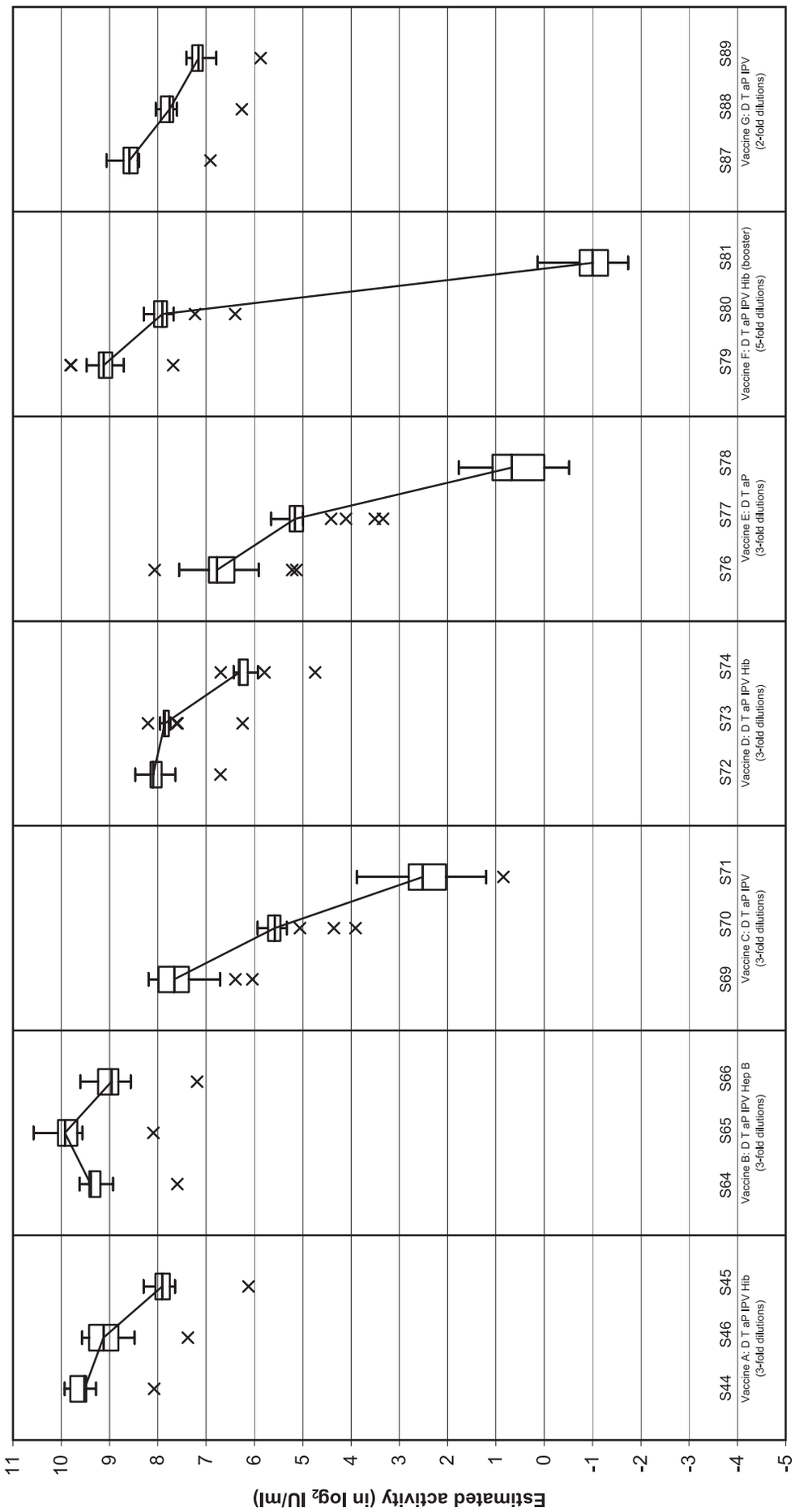


Figure 2 - *Box-and-whisker plots of results (Pertussis toxin, PT)*

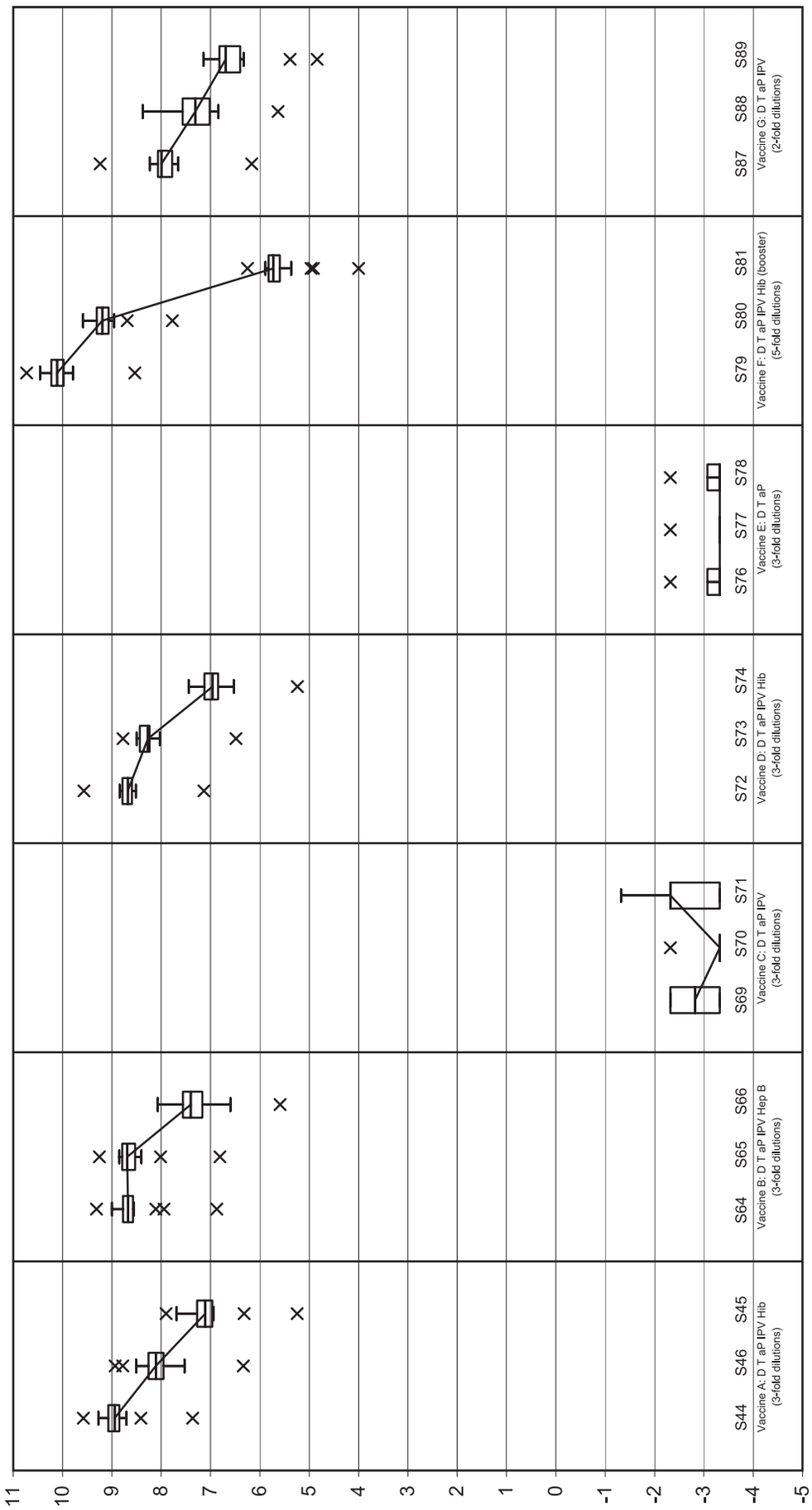


Figure 3 - *Box-and-whisker plots of results (Filamentous haemagglutinin, FHA)*

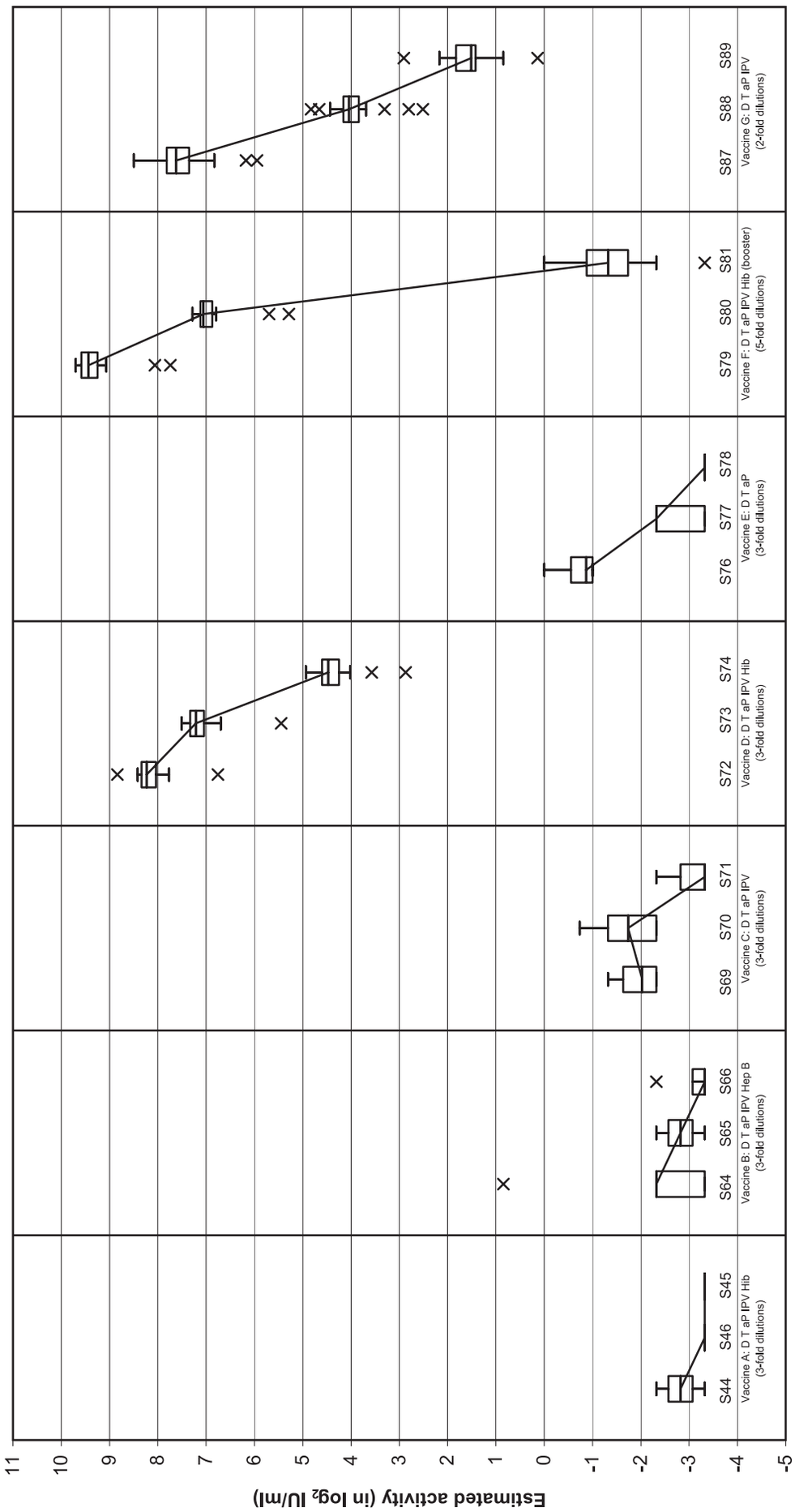
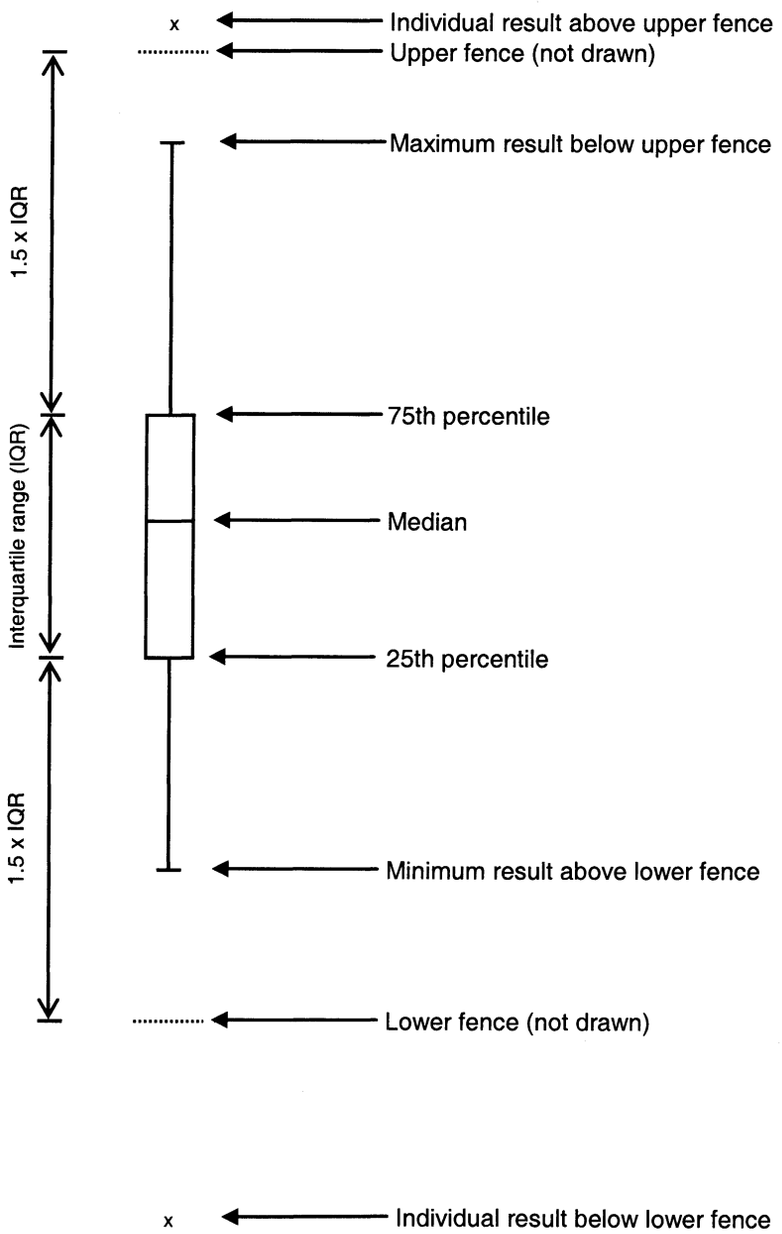


Figure 4 - *Box-and-whisker plots of results (Fimbrial-2/3, Fim-2/3)*



ANNEX 1: Explanations for the Box-and-whisker plots

The boxes show the interquartile range (the middle 50 per cent of the results) with the median as a horizontal bar. The medians are connected with straight lines. The whiskers represent the range of values within 1.5 times the interquartile range from the boxes. Values outside that range are symbolised by crossmarks.