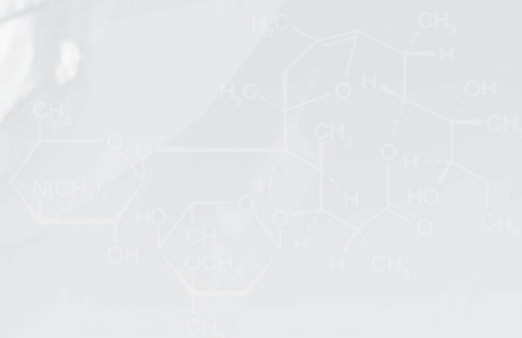


# *Serological Potency Tests for Diphtheria and Other Vaccines*

**Budapest, Hungary, 6-7 October 2004**

## **PROCEEDINGS**







# **SEROLOGICAL POTENCY TESTS FOR DIPHTHERIA AND OTHER VACCINES**

International Symposium  
organised by the  
European Directorate for the Quality of Medicines  
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## GENERAL INTRODUCTION

### Relevance of diphtheria vaccine potency testing by serological models.

#### General overview and experience from the clinics

Dr C. von Hunolstein, Istituto Superiore di Sanità, (I)

Dr C. von Hunolstein's slides are available on page 2 of this symposium-SessionI.pdf  
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The fact that today we meet to hear the results of the collaborative study conducted as part of the EDQM Biological Standardization Programme for the validation of serological methods for potency testing of diphtheria toxoid vaccines single or combined with tetanus toxoid and also other antigens, is another brilliant example and success of the big effort done by the scientific community in trying to meet some of the expectations of the 3Rs outlined by Russel and Burch in 1959 and adopted by the European Union in 1986: Replacements, reduction and refinement of animal assays.

Many studies conducted in the course of the recent years by some of you showed the feasibility to refine the Ph. Eur. diphtheria potency assay to eliminate animal suffering and to reduce the number of animals necessary for testing of production consistency of diphtheria vaccines. The details and results of the study will be presented later by the projects leaders.

I would like to update you about the epidemiology of diphtheria and the aetiologic agent, which recently, after the unexpected epidemic that occurred in the former Soviet Union has become again a subject of rather intense study and then I would like to show how serological testing in guinea pig can help in predicting the situation in clinical trials.

Diphtheria toxoid vaccines have now been successfully used since 50-60 years to fight this terrible disease that is diphtheria. The severity of the disease is dependent on the level of antitoxin antibodies present, the delay of diagnosis and thus proper case management. The understanding of a protective level of diphtheria antitoxin came initially from observation done by Ipsen 60-year ago [1]. He observed by the Schick test that the more antitoxin a patient had above 0.01 IU/ml, the better he was protected. Thus, WHO indicated a level of at least 0.1 IU/ml (measured by a functional assay) as desirable for individual protection and for epidemiological purposes a minimum protective level of 0.01 IU/ml. A very recent prospective study conducted by a Finnish group in collaboration with Russian colleagues during the last epidemic in Russia [2], showed that the level of antitoxin that gives significant protection is around 1 IU/ml. A titre lower than this represented a significant risk factor for having fever on admission, a membranous disease, extensive membrane formation, a toxic disease form, myocarditis, neuropathy and death.

As a result of the widespread use of immunization, diphtheria became increasingly uncommon in developed countries after the Second World War. In 1980 only 623 cases were reported in the European region of WHO. At that time the elimination of diphtheria in the European Region seemed imminent and the WHO Regional Committee for Europe endorsed the target of eliminating indigenous cases by the year 2000. The diseases became so rare that in 1995, in an important book of infectious diseases, it was reported that "Diphtheria has gone from a major health problem to a medical curiosity within recent memory and stands as a

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shining example of what can be accomplished with vigorous public health control measures, based on the results of solid scientific investigation” [3].

Thus, on the basis of these events we can consider that the strategy used to fight diphtheria, by this I mean vaccination has been appropriate. Retrospectively, it can be concluded that the vaccine used were of good quality and that the routine use of *in vivo* potency test has resulted in the release of very effective vaccines.

Vaccine potency is one relevant parameter that needs to be checked both by the manufacturer and by the OMCL. The aim of the potency assay is a) in preclinical phase to predict efficacy by immunogenicity and protection studies of the vaccine component in combination and particular formulation/ delivery, in a certain sense to predict the likely value for efficacy in humans; b) during the production to monitor lot to lot consistency.

The Ph. Eur. and WHO potency assays are both quantitative methods based on the principle of parallel line bioassay: both foresee the use of multi-dilution or single dilution assay [4,5]. The WHO already permits the use of antitoxin titration to evaluate the dose response instead of toxin challenge. Regarding the estimated potency, there are differences in the acceptable potencies between the two requirements. According to the Ph. Eur. the potency for primary immunization of infants and children must be, as 95% lower confidence limit (LCL) equal or higher than 30 IU per single human dose (SHD) while for WHO it can be half that value if using a multiple dilution potency assay system, since 30 IU per SHD is only required for the estimate.

In case of a vaccine for adolescent or adult the LCL has to be higher than 2 IU per SHD, even if this value is now accepted for booster dose starting from the age of 4 years.

In case of the single dilution assay the estimated potency of the SHD must be significantly higher than the minimum required.

US potency assay is more simple and more economical than the one foreseen by Ph. Eur. It is a qualitative assay in which a pool of sera obtained from guinea pigs (g.p.) at least 4 weeks after injection of 1/2 total human dose of undiluted vaccine is assayed by toxin neutralization in g.p. To be released the titre must be at least 2 AU/ml of antitoxin.

The potency of a vaccine is influenced by several factors such as content of diphtheria toxoid, presence and kind of adjuvant, presence of other antigens, route of administration and vaccination schedule.

From the unexpected diphtheria epidemic that struck the New Independent States in the 1990s and that did not allow to reach the proposed target to eliminate diphtheria in the European Region, “a posteriori” we have learnt so much and we have the proof that certain requirements set for vaccine potency and measures to control diphtheria are valid [6]. The epidemic started in the Russian Federation and therefrom affected all countries in the sub region by mid 1990. At the peak of the epidemic in 1995, 50,433 cases were reported in the NIS and 24 cases in other European countries (some of which related to NIS epidemic as was shown by ribotyping [7]), with the NIS accounting for 88% of case reported worldwide.

Only a massive, well-coordinated intervention of WHO, UNICEF, governmental and nongovernmental organisations and United Nations agencies brought under control the

epidemic. By the implementation of measures like children and adults mass immunization, strengthened surveillance, early diagnosis and high quality case management, rapid investigation and management of close contacts the total number declined and in 2003 there were 896 cases in the entire region. Thus, between 1990-2001 over 160,000 cases with a total of 4000 deaths were reported. The reason for the re-emergence of epidemic diphtheria in countries where immunization programs had nearly eliminated diphtheria in the 1970s are not fully understood but are thought to include the introduction of highly virulent clone of *C. diphtheriae* into the general population, the low coverage with diphtheria toxoid-containing vaccine among children in the 1980s and early 1990s, and a large gap of immunity among adults. The spread of the epidemic was facilitated by several factors: large-scale population movements, including the return to Russia and Ukraine of hundred of thousands of ethnic Slavs from Central Asian and Caucasian countries and the flight of refugees from fighting in Georgia, Armenia, Azerbaijan, etc; socioeconomic instability; partial deterioration of health infrastructure; delay in implementing aggressive measures to control epidemic; lack of adequate supplies for prevention and treatment in most countries [8].

The diphtheria epidemic alerted the scientific community and a new interest rose for diphtheria and *C. diphtheriae* in general. In 1993 a European Laboratory Working Group on Diphtheria (ELWGD) was established [9] and later, within the remit of the European Commission DGXII, BioMed 2 programme (1998–2001), a network of European Diphtheria Reference Centres was established as well as the European Sero-Epidemiological Network (ESEN), which is an active, integrated network of expert epidemiology and laboratory groups coordinated by the E. Miller, CDSC of United Kingdom (UK).

Serological survey conducted in seven European countries by the ESEN, documented the pattern of immunity to diphtheria in 1996 [10]. Although a variety of assays were used, the results were all standardized to an *in vitro* neutralization test to allow comparative analyses [11]. The data showed that there were large differences in the proportion of adults with insufficient levels of protection among different countries (35% of 50-60-years-old were found to be sero-negatives (titre <0.01 IU/ml) in Finland compared with 70 % in the UK); differences in the percentage of sero-negatives between female and males indicated the relevance of booster doses administered to adults; all countries have vaccination coverage >90%, but the accelerated schedule in the UK appears to result in lower anti-toxin titres than elsewhere; relevance of booster doses at school entry became evident. It was found that a large numbers of children had inadequate levels of protection in Sweden, where booster doses were not offered until 10 years of age [10,12].

Diphtheria toxoid vaccines are protective not only against diphtheria caused by *C. diphtheriae* but also against diphtheria caused by *Corynebacterium ulcerans*, which, in the last decade, has accounted for a number of cases of diphtheria [13,14].

Another aspect that deserves attention is the re-emergence of non-toxigenic *C. diphtheriae* strains in Europe, Australia, Canada and USA. Over the last 10 years severe pharyngitis and tonsillitis associated with the isolation of non-toxigenic strains have been described in UK and Italy [13,15]. Penicillin tolerance and *C. diphtheriae* ability to adhere to and entry cultured human respiratory epithelial cells is likely to contribute to bacterial eradication failures and asymptomatic carriage [16-18].

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Thus, I would say that diphtheria is again with us in Western countries. Mass immunisation used in the past to fight diphtheria gave very good results but the history of the NIS epidemic provides the proof and the message on the need for maintaining immunity in the population and this can be achieved only by using high quality vaccines and suitable immunisation schedules.

Now, I come back to the topic of this meeting and I would like to show how potency testing and in particular serological testing in guinea pig can be of great value both in checking the potency of diphtheria vaccine and in determining immunological interference between components of a combination vaccine.

Three kinds of vaccine containing the same amount of diphtheria toxoid and aluminium hydroxide were tested for potency according to the method of the Ph. Eur.. The protective antibodies levels were determined by Vero cell assay in the sera of the guinea pig after 28 day of vaccination. All vaccines passed the diphtheria potency test, but the diphtheria potency of the vaccine was enhanced by the presence of *B. pertussis* or reduced by the combinations with other antigens [19]. The level of antibodies measured in the animals vaccinated by the different vaccine gave exactly the same information on the potency of the vaccines. The responses to diphtheria and tetanus in clinical trials with comparable DTPw (diphtheria, tetanus, whole all pertussis) and DTaP (diphtheria, tetanus, acellular pertussis) vaccines showed that the response to diphtheria in infants is lower in the presence of aP (acellular pertussis), while tetanus response is not as much affected [20-22].

The level of antibodies induced in children boosted at the age of 5-6 years of age depends on the potency of the vaccine used: the paediatric preparation ( $\geq 30$  IU /HSD) induced twice the level of antibodies of the adult preparation ( $\geq 2$  IU/SHD) [23].

The effect of a combination of factors and how the potency assay predict the antibody levels induced in humans was also shown by different combinations of diphtheria vaccines used in the Swedish clinical trial of pertussis: all the vaccine elicited an antibody response for diphtheria greater than the minimum level required for protection. However, two vaccines that did not pass the Ph. Eur. specification for diphtheria potency, but the USP test, elicited a significant lower anti-diphtheria antibody response in infants [19].

These examples reported here as well as studies done by Gupta *et al.* [24], Shams and Heron [25] and other authors taking into consideration other vaccine antigens, suggest that serological potency testing in guinea pig may be able to predict the human response to diphtheria in various combination of vaccines helping us in keeping high the quality of the vaccines, thus the control of what can be reasonable be defined as a re-emergent infection.

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**SESSION I:  
ALTERNATIVE TO CHALLENGE ASSAYS IN ANIMALS  
FOR VACCINES CONTROL**

***Establishment of diphtheria potency assays based on serological methods  
Development and validation of *in vitro* methods***

Dr D. Sesardic, National Institute for Biological Standards and Control, (NIBSC), (UK)

Dr D. Sesardic's slides are available on page 14 of this symposium-SessionI.pdf  
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Diphtheria vaccine, manufactured by traditional formaldehyde inactivated toxin, is a key component in all current childhood combinations and is a backbone for combinations intended for boosting of adults and adolescent. More than ten different combinations, produced by several manufactures are currently licensed in Europe, containing either high of low dose of diphtheria toxoid. Traditionally, and in line with other toxoid vaccines, laboratory animals are used during production and for quality control to establish safety and potency [1],

Since the first meeting on Alternatives to Animals, held in Langen, 1994, [2] considerable progress was made on 3R activities in the European Pharmacopoeia (Ph. Eur.). Examples include reduction of number of animals by combining specific toxicity and toxicity reversal tests for toxoids, removal of abnormal toxicity test for some products and introduction of a single point potency test, as an option, for established products with consistent track record in multiple point assays. Refinement, by use of non-lethal or least severe end point was emphasized in general chapters and specifically introduced for tetanus by allowing potency by serology, as option. The latest version of the European Pharmacopoeia also includes replacement of *in vitro* with *in vitro* Vero cell test for specific toxicity of purified diphtheria toxoid [1] and relies on use of Biological Reference Preparation for Diphtheria Toxin to monitor assay sensitivity [3]. All of these achievements have been made possible by collaborative efforts and this presentation focused on studies which led to collaborative study, initiated by EDQM in 1991 and endorsed by the Council of Europe and Commission of European Communities in order to meet some of the 3Rs expectations. The principal aim of this collaborative study was to refine the Ph. Eur. diphtheria direct challenge assays for routine use and to explore the feasibility of using serum from the same animals for potency determination of other antigens in combinations, particularly tetanus.

Before it was possible to recommend the protocol for such ambitious validation study, considerable body of information was accumulated over the years ensuring that proposal protocol will be successful. As the proposed study aimed to replace direct challenge with serology, it was essential to establish importance of antibody response in protection. Existence of assays for detection of functional and neutralising antibodies to toxin made it possible to establish correlation with protection and with surrogate methods for antibodies such as ELISA, using relevant samples from suitable animal model [4].

There are a number of critical points that need to be considered in designing of serological assays, to replace challenge procedure. These could be summarized as: choice of species in terms of relevance and specificity, method for detection of antibodies in terms of their relevance, but also specificity and reliability as well as robustness and variability; range of immunizing doses and

## Serological Potency Tests for Diphtheria and other Vaccines

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choice of primary of booster regiment. As purpose of the assay is to provide maximum information on batch to batch variability, booster doses are usually not useful. Time post immunization is one of the critical factors as too early time after immunization will induce antibody response that may not correlate with functional response but any increase in time post immunization is costly and impractical for batch release purpose. Finally, choice of vaccine reference standards and run controls are critical for serological assays and essential for successful validation study.

Guinea pigs were chosen as the species for immunization because they provide the most suitable model for testing diphtheria and tetanus antigens. Guinea pigs are used in WHO/Ph. Eur. direct challenge methods and original WHO International Standards were calibrated in guinea pigs, therefore guinea pigs provide the only species in which cross-reference from serology to protection models can be made. In addition, previous studies have confirmed guinea pigs to provide most closely relevant response to humans for diphtheria [5] and can provide better response to functional epitopes [6] particularly at 5-6 weeks post immunization. It was previously reported in WHO Working group in 1999 that increasing time post immunization reduces differences between responses to diphtheria and tetanus as well as reduces differences between functional and total antibodies induced by these antigens, at least in currently licensed vaccines. Furthermore, guinea pigs provide antibody responses that are less prone to variability due to adjuvant and combination effect [7,8] and more importantly provide useful dose response for potency calculations for at least two antigens, with possibility of extending the studies to other antigens in combinations.

Whereas serology can indeed be considered as suitable surrogate potency assay for toxoid vaccines adjuvanted with aluminum salts, it must be recognized that new generation vaccines using same antigens given with different adjuvants or delivery systems will require evaluation in protection models. Serology model cannot be considered "ultimate" replacement model for all current and future diphtheria vaccines, but serology can be used to ensure that currently licensed vaccines entering the clinic are of consistent relevant activity, for batch release purpose.

In addition to studies focusing on refinement of animal procedures for potency, the key focus of the symposium, it is clear that other entirely *in vitro* methods can also be used to provide valuable information on diphtheria vaccines in relation to safety, consistency and stability. As diphtheria toxoid is known to be associated with high incidence of adverse reaction, particularly in booster schedules, new *in vitro* methods and requirements are desirable.

It is known that specific toxicity and LAL tests cannot detect or predict adverse reactions or pyrogenic substances in diphtheria vaccines. Recent studies in collaboration with Dr Stephen Poole, Division of Immunobiology and Endocrinology, NIBSC, confirmed that diphtheria toxoid and Hib, but not tetanus aP antigens or Inactivated Poliomyelitis Vaccine (IPV) can release high levels of IL-6 from human monocytes. Separate studies on booster diphtheria (dT) vaccine associated with adverse reaction in use also showed high levels of IL-6 release from human monocytes and high antigen content, as well as high degree of non-adsorbed antigens. Although it is not possible to say, at this stage, which factor is critical in high incidence of adverse reaction in use, it would be advisable to include maximum limit for amount of diphtheria toxoid, particularly in low dose vaccines intended for boosting of adults and adolescent.

We have developed a monoclonal antibody capture ELISA assay which in increasingly proving useful to provide information on product consistency. *In vitro* antigen assay can confirm presence and amount of relevant antigenic toxoid, can confirm degree of adsorption of antigen, and provide more discriminative product specific information than potency assays. However, the amount of

antigen detected will be highly dependent on the amount of antigen desorbed, method used in desorption process as well as on age of vaccines and specific reagents used for the assay. The information obtained may not always correlate with protection as antigenic toxoid is not the only component inducing protective immune response. Furthermore, information on antigenic interaction observed *in vitro*, for combined vaccines, may not correlate with interaction of observed *in vitro*.

However, we reported studies during meeting on Alternatives to Animals in Utrecht 2003 [9] which confirmed positive correlation between diphtheria and tetanus antigen content in vaccine with antibody response in guinea pigs, during accelerated temperature degradation studies. The assay has since been applied to testing of many different vaccine combinations, as part of batch release testing and shown to provide valuable information, particularly for vaccines with reduced antigen content for diphtheria and tetanus. Product-specific information is obtained which can confirm consistency and identify critical changes in product profile, such as degree of adsorption.

In addition to monitoring antigen content in final vaccines, the approach has shown highly useful in confirming differences in temperature stability between toxoids produced by the same manufacturer at different sites. Data generated to date suggest that *in vitro* stability testing of antigens prior to formulation, may provide valuable information that is relevant for potency of that antigen in final combination. More work and collaborative efforts will be required, but it is hoped that EDQM with support from Council of Europe and Commission of European Communities will recognize the role of *in vitro* antigen testing and support future studies in this field.

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**SESSION I:  
ALTERNATIVE TO CHALLENGE ASSAYS IN ANIMALS  
FOR VACCINES CONTROL**

***Establishment of diphtheria potency assays based on serological methods***  
**Evaluation of serological assays for diphtheria potency testing in combined  
vaccines: results from collaborative study BSP034**

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**Aim of the study**

The collaborative study, initiated in January 2001, is a contribution to EDQM's efforts to meet some of the expectations of the 3 Rs and specifically to validate alternative assays to replace, for batch release purposes, the Ph. Eur. *in vivo* direct challenge procedures for the potency determination of diphtheria toxoid vaccines. The principal aim was to explore the possibility of considerably reducing the number of animals used for potency determination of vaccines containing diphtheria and tetanus toxoid components and to refine the Ph. Eur. potency assays for routine use.

**Introduction**

The project is an extension of the collaborative study that led to refinement of Ph. Eur. *in vivo* direct challenge assays for potency testing of tetanus toxoid vaccines for human use, where both ELISA and toxin-binding inhibition assay (ToBI) were deemed valid methods for routine batch release testing of combined tetanus vaccines [1].

Guinea pigs were chosen as the species for immunisation as they are used in Ph. Eur. procedures for potency testing of diphtheria and tetanus vaccines [2,3,4] and have been previously used for validation of tetanus potency assays [1]. Mice, although used for potency testing of tetanus vaccines, are insensitive to diphtheria toxin and show great strain differences in the serological responses to tetanus toxoid [5], in particular when the whole cell pertussis [6] or Hib components [14] are present in combinations. Furthermore, previous studies [7] indicated that guinea pigs, in contrast to Balb/c and NIH strains of mice, have a similar response to fragment B of diphtheria toxin, harbouring the receptor binding domain, as man does, and could provide comparable information regarding immunogenicity of vaccines as in clinical trials [8]. Guinea pigs will provide more serum than mice possibly allowing potency determination of several components in combined vaccines.

The BSP034 study was divided into three consecutive phases. The pre-validation (Phase I) study was performed in two laboratories to verify protocols and select the optimal vaccine dilutions for immunisation of guinea pigs that would allow potency testing by challenge and serological methods. The results from the Phase I study [9] indicated that comparable diphtheria toxoid potency estimates were obtained in the Ph. Eur. intra-dermal challenge assay in guinea pigs, in Vero cell assay and in D-ELISA for vaccines of different potencies. The correlation between the challenge and the Vero cell assays corresponded to those between the challenge and D-ELISA, confirming that the antibodies play an important role in protection and that predominantly protective /neutralising antibodies are present in guinea

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pigs six weeks after immunisation. The study also provided preliminary information that sera from the same guinea pigs may be used for potency determination of both diphtheria and tetanus toxoid components of vaccines.

The Phase I study was extended in two laboratories to examine correlation of serology with *in vivo* toxin neutralisation test (TNT) using serum pools [16]. The data show that antitoxin potency obtained by Vero cell assay, D-ELISA and T-ELISA, using the guinea pig standard (GP), highly correlated with neutralising potency as determined in respective TNT assays.

In Phase II the suitability of the Vero cell and D-ELISA methods for potency testing of diphtheria toxoid containing vaccines were investigated in an additional five laboratories [10]. The correlation between diphtheria serological assays and lethal challenge assays were confirmed satisfactory as ca. 90 per cent of serum-estimates lead to correct prediction of mortality. All laboratories found identical ranking of the vaccines in all serological assays and in the valid challenge assays. The ranking order was identical to provided potency for the highest and the lowest vaccine.

In the Phase I/II studies a considerably smaller effect of Hib component on anti-tetanus response than previously reported for mouse challenge assays [14] and potential differences between T-ELISA and ToBI assays in detecting antibodies to carrier protein in Hib-containing combined vaccines [10] were observed.

The reliability of the serological assays was investigated in the Phase III study by obtaining information on repeatability and reproducibility.

### Phase III Study: Vaccines and sera

Table 1 provides a summary of serum codes and components of 13 vaccines from which they were produced. Manufacturers donating the vaccines and serum were identified with roman numerals I to IV. When sera were produced from animals bled after 5 instead of 6 weeks, the manufacturer code was followed by the letter a.

Table 1 - Overview of sera and the vaccines from which they originate.

Vaccine code	Components	Manufacturer code	Potency # (Diphtheria)	Potency # (Tetanus)	First vaccine dilution	Dilution step	Respective serum codes
A	D T	I	20	41	1/2	2.5	S01 S03 S02 S04
B	D T aP HepB IPV	II	73	163	1/10	2.5	S05 S06 S07 S08
C	d T	III	8	78	1/1	2.5	S09 S10 S11 S12
D	D T aP HepB IPV Hib*	II	102	206	1/10	2.5	S13 S14 S15 S16
E	D T	IV	63	126	1/10	2.5	S17 S18 S19 S20
F	D T aP HepB Hib* IPV	III	44	833	1/5.5	2.5	S21 S22 S23 S24
G	D T aP IPV Hib*	III	56.4	353	1/10	2.5	S25 S26 S27 S28
H	D T	IV	10	63	1/1.81	2.5	S29 S30 S31 S32
I	D T wP	IIa	50	337	1/14	2.5	S33 S34 S35
J	D T aP	IIa	84	120	1/5	3	S36 S37 S38 S39
K	D T aP IPV	IIa	68	98	1/5	3	S40 S41 S42 S43
L	D T aP IPV	IIIa	46	54	1/5	3	S48 S49 S50 S51
M	D T aP IPV Hib*	IIIa	41	179	1/5	3	S52 S53 S54 S55

# Potencies are in IU/0.5 ml. The dilution of the highest dose is listed together with the subsequent dilution steps.

\* Hib component corresponding to tetanus toxoid- polyribosylribose (PRP) conjugate

### Methods and study design

Participants were expected to perform:

- Vero cell toxin neutralisation assay (Vero cell assay)
- ELISA for diphtheria serology (D-ELISA)
- ELISA and/or ToBI for tetanus serology (T-ELISA and/or ToBI)

#### *Vero cell assay*

Two procedures of the Vero cell assay were described in the protocol. The limit of detection for guinea pig reference serum (NIBSC 98/572) was confirmed as 0.05 and 0.015 IU/ml for these methods, respectively [10]. Participants were requested to use one of the two provided procedures or, provided it had demonstrated suitable sensitivity, their in-house validated method.

#### *D-ELISA and T-ELISA*

The methods used for ELISA titration of diphtheria and tetanus antitoxin in guinea pig sera were essentially as described elsewhere [1, 14, 10]. Individual antitoxin titres were calculated with respect to GP reference and expressed in IU/ml.

#### *ToBI*

The method used for titration of tetanus antitoxin in guinea pig sera was as published elsewhere [1, 15].

### Results of the Phase III study

#### *Vero cell assay*

Although the results vary considerably from laboratory to laboratory, the data gives reasonable median dose response relationships. A clear regression can be observed for each vaccine although the shape of the curves can be very different from one another. Although there are quite a lot of “outlying” results, in general 50 per cent of the laboratories differ by not more than a factor 3 to 4 from each other. The method is able to discriminate exactly between 2-fold diluted serum samples.

Vaccine J (DTaP) and vaccine K (DTaP-IPV) were produced by the same manufacturer. Table 1 indicates that the Ph. Eur. challenge assay, as performed by the manufacturer, gave a lower potency for vaccine K than for J. Identical dilutions of immunising doses were used for these two vaccines for preparation of serum samples. No inhibitory effect of the IPV component on the diphtheria antitoxin/toxoid response is evident in the Vero cell assay or in D-ELISA.

#### *D-ELISA*

Quality of the fit was in general good with correlation coefficients ( $r$ ) above 0.99 in 95.7 per cent of the assays. The D-ELISA method is able to discriminate exactly between 2-fold diluted serum samples. A clear regression can be observed for each of the vaccines. Although the shape of the curves can be different from one another, the curves appear to have a rather similar shape and extension by both methods. Reproducibility is better with D-ELISA than with the Vero cell assay. The results of most laboratories do not differ by more than a factor of 2 to 3 from each other, whereas a factor of 3 to 4 was observed by the Vero cell assay.

### ***T-ELISA***

Quality of the fit between the standard curve and the respective samples was in general good, with correlation coefficients ( $r$ ) above 0.99 in 93.0 per cent of the assays. A clear regression is observed for each vaccine. This is an important observation because the vaccine doses were optimal for the diphtheria toxoid component and may not necessarily be optimal for the tetanus component. The results support the idea that the same sera can be used to determine the potency of both diphtheria and tetanus components. The results of most laboratories do not differ by more than a factor of 2 to 3 from each other.

### ***ToBI***

Quality of the fit was less good than with T-ELISA with correlation coefficients ( $r$ ) above 0.99 in 68.6 per cent of the assays. A clear regression can be observed for each of the vaccines. The results of most laboratories do not differ by more than a factor of 2 to 3 from each other.

### **Agreement between serological methods in Phase III study**

In order to investigate the agreement between the various methods two-way plots were generated. For each serum the median outcome of all laboratories were plotted.

*Vero cell assay and D-ELISA:* No obvious relationship between the degree of agreement and the number of vaccine components can be identified although vaccines containing only the diphtheria and tetanus toxoid components may show a better agreement than vaccines without the hepatitis B component but containing the polio and the haemophilus type b components. All sera with the highest degree of disagreement were produced from guinea pigs bled at 5, rather than 6 weeks.

*T-ELISA and ToBI:* All points of the T-ELISA assay are located above the diagonal line of agreement, indicating that a broader range of specific antibodies are detected by T-ELISA than by ToBI. In the present study it appears that vaccines with more components tend to give more disagreement than vaccines containing only tetanus and diphtheria toxoid. Vaccines containing Hib show significant ( $p < 0.02$ ) difference between the two methods to vaccines not containing this component, even when produced in different laboratories. This observation is in line with that of the previous study [1]. Unlike results with diphtheria serology no additional differences were noted for serum provided by one organising laboratory where the animals were bled at 5 instead of 6 weeks.

## **Conclusions**

### ***Diphtheria toxoid in combined vaccines***

Repeatability of D-ELISA is superior to that of the Vero cell assay, irrespective of the Vero cell method. In this study an average difference of a factor of 1.39 and 1.23 was observed between two Vero cell and two D-ELISA assays, respectively, within the same laboratory. Reproducibility is also markedly better with D-ELISA than with the Vero cell assay.

Earlier bleeding time may explain larger, up to a factor of 4, differences observed between the two methods. Previous studies have also shown that longer time post immunisation narrows differences between functional and non-functional antibody response to diphtheria toxoid [18].

In general, no obvious relationship between the degree of agreement and the number of vaccine components can be identified. However, in this study vaccines containing only the

diphtheria and tetanus toxoid components as well as seven-components vaccines, containing HepB and both the IPV and the Hib components, show a better agreement between the Vero cell assay and D-ELISA than DTaP vaccines containing IPV and/or Hib components.

### *Tetanus toxoid in combined vaccines*

Repeatability of T-ELISA appears to be somewhat better than that of ToBI. An average difference of a factor 1.20 between two T-ELISA assays within the same laboratory was found, compared to 1.34 between two ToBI assays. Because a small number of laboratories carried out the ToBI assays, it is not possible to make firm conclusions. However, the results were in line with results of the previous study [1]. Reproducibility is approximately the same with both methods, which is also consistent with previous results [1]. T-ELISA tended to give systematically higher results than the ToBI assay, indicating that a broader range of specific antibodies are detected by T-ELISA than by ToBI. This is not in line with the previous study, where T-ELISA tended to give higher values than ToBI in the low antitoxin range only, and the reverse was obtained in the high antitoxin range.

Vaccines containing Hib show significant difference between the two methods, indicating possible differences between T-ELISA and ToBI assays in detecting antibodies to carrier protein in Hib-containing vaccines, possibly resulting in different potency/ranking order by the two methods.

A good correlation between TNT in mice and T-ELISA was seen in the extended study [16]. The variability of estimates did not relate to vaccine type or vaccine immunising dose. It is also worth noting that a good comparison between TNT and T-ELISA was obtained for a vaccine containing DTaP-IPV-Hib.

### **For both D and T serological assays:**

Although antibody titres can be very different for the same vaccine type and dose, immunised in different laboratories by an identical method, vaccine potency will not be different as long as responses to the reference and test vaccine are equally affected.

The study results may be used in support of the replacement of the multi-dilution direct challenge procedures in different animal models, by a single dilution serology test, where appropriate, and to use sera from the same animals for potency testing of several components in combined vaccines.

### **Acknowledgement**

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## DISCUSSION

**Dr J. Arciniega:** I have a total of five questions, but I'm going to ask two of them because they are relevant to the study. The first one refers to the use of the word "relevance." I asked for a definition of the word relevance. I would like to ask you, what did you mean by that?

**Dr R. Winsnes:** We have taken note of your comments to the draft report and have substituted the word relevance with "repeatability and reproducibility."

**Dr J. Arciniega:** My other question refers to the use of the guinea pig reference serum. I understood from your presentations that no matter what reference serum you use, you get the same good prediction of potency by neutralisation using VERO cell assay. Even if you use the horse reference serum. So my question is, why do you need a guinea pig reference serum in the first place? Also I would like to know how it was prepared.

**Dr R. Winsnes:** I propose that Dr Sesardic answers these two questions.

**Dr D. Sesardic:** You clearly need two references to be able to show that you have consistency within your test and also consistency for the vaccine potency. There are two different references in use. One is to standardise the serological methods where you need a guinea pig reference. The other is the reference vaccine, to express the potency against it.

**Dr J. Arciniega:** That wasn't the question. The question is, from the presentations, it doesn't really seem to matter what serum reference you use. The prediction of potency by sero-neutralisation is similar. There is a difference in absolute anti-body value, depending on what reference you use - guinea pigs or horse. However, at the end it doesn't really matter.

**Dr R. Winsnes:** For determination of the potency of the vaccine I said that either reference serum could be used since the calculation is based on the antibody titres elicited by a reference vaccine. The antibody titre induced by a vaccine depends on which antiserum reference is used. It is worth noticing when comparing neutralising antibody titres in different species.

**Dr J. Arciniega:** What is the purpose of having a guinea pig reference then?

**Dr R. Winsnes:** The guinea pig reference serum is prepared by a similar method to the serum samples from the guinea pigs immunised with the test vaccines and is thus the most suitable reference preparation. The situation is different in your case since you do not use a reference vaccine but only direct calculation of antitoxin based on a reference antiserum.

**Dr J. Arciniega:** When you're discussing the relative potency, my understanding was that it doesn't matter what serum reference you use, so why prepare a guinea pig reference? You didn't mention how it was prepared. Was it a hyper-immune serum or was it obtained after a single immunisation?

**Dr D. Sesardic:** Guinea pig reference was prepared for the collaborative study and that was by immunising diphtheria and tetanus toxoids together – given once. The guinea pig reference was used in the collaborative study to standardise the serological assays and was described in

the report of study. WHO/Ph. Eur. international standards for diphtheria and tetanus vaccines were used. Animals were immunised by both preparations.

**Dr J. Arciniega:** Only once? Or were they hyper-immunised?

**Dr D. Sesardic:** Yes, only once. No, they were not hyper-immune. It was exactly at the same time that was used for test vaccines. This is the whole point. The reason why we were able to get a good relationship between functional anti-bodies and ELISA is precisely because the reference serum was bled at the same time point. As Dr Rajesh Gupta was saying for many years and the reason why he has always emphasised that there was a need for like-to-like reference standards of the serum in order to have a relationship between ELISA *in vitro*-toxin-neutralisation. That's why the guinea pig serum was made, but the guinea pig serum concept is not really used to calculate potency of vaccines. It is used only to standardise the assay.

**Dr J. Arciniega:** But did he do the comparison and did he find that there is no difference in the potency no matter what reference is used? Did you do that? Did you actually test that?

**Dr D. Sesardic:** Yes. Maybe our statistician can confirm that because it would make the reply more complete.

**Mr A. Daas:** I think this was the conclusion of phase two, that in fact you can include a reference serum, but you don't really need a well-calibrated reference serum. It's just to have a better inter-assay comparability. We didn't choose the horse serum, but I suppose it could be used as well.

**Dr J-M Spieser:** It should be known, Dr J. Arciniega, that for the equine standard, very little stock is available, so there would be a need to develop a new one. You should discuss that with your colleagues. Plus of course there is a difference between the reference standard for the expression of the potency and the run control for validating your system.

**Dr J. Arciniega:** Regarding the statistics, in your graphs the lines presented are not true regression lines? What are they?

**Mr A. Daas:** You mean the big figure that was shown? They are regression lines, it's just that the scale for the last set of vaccines is a little bit compressed. The classes are those that you have the highest first and then the decreasing doses.

**Dr J. Arciniega:** One of my comments was, why not use Altman and Bland instead of pure correlation? You can achieve pure correlation even if the prediction is pretty bad. In this case, the line passes through zero and has a slope of one, which means perfect correlation. But this is not true because all of the points are above that line.

**Mr A. Daas:** But the report doesn't mention correlation at all. There was no correlation coefficient calculated because exactly that is not appropriate to do. Neither is Altman-Bland in this case because the observations are not mutually independent. What you are talking about there is a diagonal line and then dots, which are mostly above that line. That is the line of agreement - the perfect line of agreement.

**Dr J-M Spieser:** Ok. Another question there.

**Dr R. Gupta:** I have a couple of questions. I can comment on the reference standards. I don't know the background because I was not involved in this project, but I can give my direction as to why we always speak about using similar references as the test samples. I think one of the reasons why we used guinea pig references is due to ELISA. In ELISA you have to use the same species specific reference and the second reason is that there was a lot of controversy about five or six years back about the dose of toxin in neutralization tests. How much toxin you should use in the toxin neutralisation test. The best solution was to bridge that gap by having a guinea pig reference, which can be calibrated by high toxin dose in animal neutralization test against horse antitoxin. For testing samples by the Vero method whatever toxin dose, high or low, you choose you will get the same results when you use this guinea pig reference serum - let's say you make a guinea pig reference at 4 weeks and calibrate by high toxin dose. The FDA made quite a big attempt to make that reference standard and calibrate it by the high toxin dose. So you will get units like three or four units. Then you go back and titrate test sera by low toxin or high toxin. You will always see more than two units for a passing vaccine, not fractions of units, as was the case with equine reference using low toxin. I think that was one of the issues with the FDA - they wanted always to see more than two units or whole units (1-4 units), not fractions (0.1 – 0.4 units) as with low toxin using high affinity reference serum. That is the background, I think, of the reference.

**Dr J-M. Spieser:** Are you saying that depending on the choice of your reference then you will make your system always passing?

**Dr R. Gupta:** Not always passing. It all depends on the reference serum. I think that in the FDA we are more used to seeing two units (high unitage), which is not true high affinity antibodies. The true high affinity anti-bodies are like 0.2, 0.3 units, but the FDA cannot pass the vaccines with such low units, though 0.2 or 0.3 units of high affinity antibodies is the same as 2 units of low affinity antibodies. They (FDA) would like to see two units. The solution therefore was to have two units, you put a very low avidity reference standard, similar to the test sera and calibrate by the high toxin neutralisation test. Then you have that standard calibrated by high toxin dose in low affinity antibodies, you go back to your test sera and your test will be similar in terms of avidity test and reference sera. Once you have a high unit standard - it doesn't matter if you test with high dose or low dose, you are going to see two units (high unitage). The vaccine will still be discriminated based on the quality of the toxoid. It's not that you will pass a bad vaccine.

About the other comments regarding Dr von Hunolstein's presentation, I think that was very nice, we should keep that in mind when we change the potency test. I just want to make some comments about the protective levels, like when Ipsen gave the levels of 0.01 international units per ml as protective levels, these were not the units of protective levels for the individuals. Ipsen's paper itself shows a lot of breakthrough cases in individuals with 0.01 IU/ml or higher. People even with 1 unit got the diphtheria disease. Protective level of 0.01 units/ml are for the population. When more than 80 % of the population will have that 0.01 international unit and then the bacteria cannot have a transmission from person to person. That's when you start protecting, but if you consider an individual protective level that's when we have a problem. This is what happened in the former Soviet Union or with Russia, when their immunisation rate went lower than 80% of population, we started seeing cases. They might have protective levels on those 55% of immunized population, but individual breakthrough cases will be there. Ipsen's paper actually shows that if you go and read that

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1950's or 60's year old paper. We were involved in that diphtheria project when they started seeing these diphtheria cases in former Soviet Union. I was in Massachusetts at that time and we were trying to prepare a diphtheria immune globulin. That's when we reviewed all the levels we wanted for a diphtheria immune globulin which can be protective.

Another remark is about the new guinea pig model. I'm very glad to see that ultimately the guinea pig is chosen over mice. We had been saying that for quite some time although there are some differences in the doses. Our model was based more on the US potency test which also, I think, can discriminate between good and bad vaccines without the need for a reference standard. Guinea pigs give you very consistent results and in the US for the last 50 years we have been passing vaccines using that test at 2 units. This means that it has been consistent over the 50 years without the use of a reference standard. I think that during the break we can go over some of the issues with the A and B sub units of diphtheria toxin and the mice not having the receptors for diphtheria toxin, so guinea pig is the appropriate model for the potency of diphtheria toxoid.

**Dr J-M. Spieser:** Thank you very much for your comments. Some remarks?

**Dr R. Winsnes:** I just want to comment that Japanese vaccine controllers state that a mice strain exists which can respond better to the B subunit of diphtheria toxin than the others, but it is not in common use.

**Dr N. Chirmule:** I had a couple of questions regarding the study and also a comment from the previous speaker. Obviously we are gathered here to discuss what assays could replace the challenge model. So you are looking at, let's say the neutralising anti-body and the virus assay which was the binding assay. The variability of these two assays by nature is going to be very different, the neutralising anti-body being much more than the ELISA assay. My question relating to that is in this plot that is seen in the study how many times did each person /each lab test the samples?

**Dr R. Winsnes:** At least two times.

**Dr N. Chirmule:** Two times? But the variability of the assays I presume is somewhere around 25 – 35 %? Each assay? The ELISA versus the neutralising anti-body for diphtheria?

**Dr R. Winsnes:** Repeatability of D-ELISA is superior to that of the Vero cell assay. In this study an average difference of a factor of 1.39 and 1.23 was observed between two Vero cell and two D-ELISA assays, respectively, within the same laboratory. Reproducibility is also markedly better with D-ELISA than with the Vero cell assay..

**Dr N. Chirmule:** So in the two runs that you do, the two times that you test you will see 23% variability within that on average?

**Dr R. Winsnes:** On average, yes.

**Dr N. Chirmule:** So one point to make in this concurrence report is probably to have standardisation bars across each point. That will also help.

Another question relating to that same study is, were these guinea pigs challenged themselves? I guess you are looking at immune responses in these guinea pigs or in the sera?

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**Dr R. Winsnes:** In these two plots, the antibody titres obtained by two serological methods are compared. In the Phase I and II studies the animals were bled two days before they were challenged by the diphtheria toxin, either by the intradermal assay or by the lethal assay.

**Dr N. Chirmule:** Thank you. The other question was merely a comment. I think SMM agrees that this is something that people should start thinking about, because that anti-body may not be the only mechanism by which protection may occur. Perhaps we can discuss this over the coffee break.

**Dr J-M Spieser:** We will come back tomorrow to discuss the position of such an alternative, whether gold standards still remain or whether it is just as it was mentioned for routine batch testing.

**Dr D. Wood:** A comment and a question. The comment first, WHO circulated with the agreement of the EDQM the study reports to some of the experts that we consult in this area and we have received no comments. Just to pass that message onto you. I think it's actually a very good study. Congratulations to the study organisers.

My question relates to the observation that you made of the increased variability with the Hib containing vaccines and the tetanus response. What do you think are the implications for that greater variability between the functional anti-body response and the binding anti-body response?

**Dr R. Winsnes:** If you are going to use one of these methods for batch release/consistency testing you should stick to only one, because it seems that the ELISA detects a broader range of antibodies than the TOBI assay. I believe there may be anti-bodies to the carrier of the PRP. Maybe it's not such a good thing with the toxin binding inhibition assay. You could use the TOBI but then you should use the TOBI that I use in trend analysis and for both releasing OMCLs and for the manufacturers.

**Dr M. Duchêne:** Just a very simple question. What will be the principle for interpreting the result regarding the final status of a lot? Would it be a quantity of anti-body or comparison with a vaccine?

**Dr R. Winsnes:** From the experience of this study we have proposed that you should immunise with both a reference vaccine and a test vaccine and bleed the animals, take the serum, titrate the serum samples with respect to antibody titre induced by the respective vaccine doses and then you will refer to the international units of the reference vaccine and not international units of the antiserum by the final calculation.

**Dr M. Duchêne:** So the reference vaccine will be used to have a final conclusion on the status of the lot? It will be by comparison?

**Dr R. Winsnes:** We have proposed that the potency of a test vaccine is calculated with reference to the international unit assignment of the standard vaccine. One should immunise some animals with a reference vaccine and some with a test vaccine and calculate the potency with reference to the standard vaccine.

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**Dr J-M. Spieser:** We have only two additional presentations left. We can still come back on the whole of the presentations after the two last presentations and have an additional longer debate at the end of the day. Thank you.

**SESSION I:  
ALTERNATIVE TO CHALLENGE ASSAYS IN ANIMALS  
FOR VACCINES CONTROL**

*Establishment of diphtheria potency assays based on serological methods  
Implementation of tetanus and diphtheria potency assays based on serological  
methods*

**Introducing serological methods: what is needed for a specific  
vaccine/combination?**

Dr R. Winsnes, NoMA (N)

Dr R. Winsnes' slides are available on page 29 of this symposium-SessionI.pdf  
[http://www.pheur.org/site/page\\_dynamique.php3?lien=M&lien\\_page=4&id=2](http://www.pheur.org/site/page_dynamique.php3?lien=M&lien_page=4&id=2)

**Summary**

A collaborative study for the validation of the functional Vero cell assay and the non-functional D-ELISA as alternatives to the Ph. Eur. *in vivo* challenge methods for routine batch release of diphtheria toxoid-containing vaccines is finalised. The study was performed under the aegis of the Biological Standardisation Programme and supported by the Council of Europe and the European Commission. Comparable potency estimates of the diphtheria component in various vaccine combinations from several manufacturers were obtained [1,2,3,4]. Parameters analysed included:

- correlation of vaccine potencies obtained by direct challenge test and by the serological assays
- prediction of protection/survival based on antibody concentrations obtained in Vero cell assay and D-ELISA, respectively, compared with actual protection/death
- correlation of antibody concentrations in D-ELISA, Vero cell assay and Toxin Neutralisation Test (TNT) in guinea-pigs.
- assay repeatability and reproducibility of both Vero cell assay and D-ELISA

The results were found acceptable for proposing inclusion of serological methods in Ph. Eur.

A similar collaborative study on serological alternatives to the Ph. Eur. *in vivo* challenge methods for potency determination of the tetanus toxoid component in vaccines was finalised in 2000 (5). That study resulted in an additional Ph. Eur. method and a guideline on assay of tetanus vaccine (adsorbed).

Introduction into Ph. Eur. of serological alternative methods for routine batch release of diphtheria toxoid-containing vaccines is proposed in this presentation. A similar text as used in Ph. Eur. chapter 2.7.8. Assay of tetanus vaccine (adsorbed), method C. Determination of antibodies in guinea-pigs may be used. In addition an encouragement to try to use the same guinea-pig antisera for assay of both the diphtheria and the tetanus component should be introduced.

It is proposed that after verification of the suitability of the method for the product, an alternative serological method is used wherever possible in the interest of animal welfare. For this purpose, a suitable number of batches (usually 3) are assayed by the serological method and the Method of intradermal challenge or the Method of lethal challenge. The methodology used in the collaborative study where the same animals were bled and challenged is recommended for the validation.

**For 2.7.6. Assay of diphtheria vaccine (adsorbed)** Relevant extracts of the Ph. Eur. chapter 2.7.8. Assay of tetanus vaccine (adsorbed) is used to propose a text for inclusion into :

### **2.7.6 Assay of diphtheria vaccine (adsorbed).**

“The potency of diphtheria vaccine is determined by administration of the vaccine to guinea-pigs followed either by challenge with diphtheria toxin (method A or B) or by determination of the titre of antibodies against tetanus toxin or toxoid in the serum of guinea-pigs (method C). For vaccines containing both diphtheria and tetanus toxoid, sera from the same immunized guinea-pigs should be used for potency calculation of both vaccine components, if applicable. By method A and B the potency of diphtheria vaccine (adsorbed) is determined comparing the dose of the vaccine required to protect guinea-pigs from the effects of either an erythrogenic dose of diphtheria toxin administered intradermally or a lethal dose of diphtheria toxin administered subcutaneously with the dose of a reference preparation, calibrated in International Units, needed to give the same protection. Also for method C the potency of the vaccine is calculated by comparison with a reference vaccine, calibrated in International Units. For methods A and B, in countries where the intradermal method is not obligatory, the LD<sub>50</sub> method may be used.

The International Unit is the activity contained in a stated amount of the International Standard for diphtheria toxoid (adsorbed). The equivalence in International Units of the International Standard is stated by the World Health Organisation.

*Diphtheria vaccine (adsorbed) BRP* is calibrated in International Units with reference to the International Standard.

The method chosen for assay of diphtheria vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;
2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for routine assay of batches of vaccine, but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B.

Where different vaccines (monovalent or combinations) are prepared from diphtheria toxoid of the same origin, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccine. For combinations with a whole-cell pertussis component, a separate demonstration of equivalence must be made for the highest combination.

The design of the assays described below follows a parallel-line model with 3 dilutions for the test and reference preparations. Based on the potency data obtained in multi-dilution assays, it may be possible to decrease the number of animals needed to obtain a statistically significant result by applying a simplified model using a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required but does not give information on the dose-response curves and their linearity, parallelism and significant slope. The simplified model may lead to a considerable reduction in the number of animals required and

## Serological Potency Tests for Diphtheria and other Vaccines

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its use must be considered in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

- mean and standard deviation of relative antitoxin/antibody titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation,
- antitoxin/antibody titres or scores of run controls (positive and negative serum samples),
- ratio of antitoxin/antibody titres or scores for the positive serum control and the serum samples corresponding to the reference vaccine.

The potency of tetanus vaccine is determined by administration of:

### METHOD A. INTRADERMAL CHALLENGE IN GUINEA-PIGS

Selection and distribution of the test animals, etc.

### METHOD B. LETHAL CHALLENGE IN GUINEA-PIGS

Selection and distribution of the test animals, etc.

### METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

Selection and distribution of the test animals

Reference preparation

Dilution of the test and reference preparations

Immunisation

Blood sampling

Preparation of serum samples

Determination of antibody titre

Calculation of potency

Requirements for a valid assay

A similar guideline as provided for potency assays of tetanus toxoid is proposed for the potency of diphtheria toxoid. An example with headings is given below:

### **Assay of diphtheria vaccine (adsorbed): guidelines**

#### METHOD A. INTRADERMAL CHALLENGE IN GUINEA-PIGS

Reading and interpretation of results

#### METHOD B. LETHAL CHALLENGE IN GUINEA-PIGS

Reading and interpretation of results

#### METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

Preparation of serum samples

Determination of antibody titre

### **Determination of antibody titre in guinea-pig serum by enzyme-linked immunosorbent assay (ELISA)**

Dilutions of test and reference sera are made on ELISA plates coated with diphtheria toxoid. A positive guinea-pig serum control and a negative guinea-pig serum control are included on each plate to monitor the assay performance.....

*Reagents and equipment*

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*Method*

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### **Determination of antibody titre in guinea-pig serum by Vero cell assay**

Introductory paragraph.

*Reagents and equipment*

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*Method*

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### **References**

- [1] Winsnes R., Sesardic D., Daas A., Rigsby P. A Vero cell method for potency testing of diphtheria vaccines. *Dev. Biol. Stand.* **111**, 141-8 (2002).
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**SESSION I:  
ALTERNATIVE TO CHALLENGE ASSAYS IN ANIMALS  
FOR VACCINES CONTROL**

*Establishment of diphtheria potency assays based on serological methods  
Implementation of tetanus and diphtheria potency assays based on serological  
methods*

**Statistical basis for introduction of one-dilution serological potency assays  
for combined vaccines**

Mr A. Daas, Biostatistician, EDQM, Council of Europe, (F)

Mr A. Daas' slides are available on page 34 of this symposium-SessionI.pdf  
[http://www.pheur.org/site/page\\_dynamique.php3?lien=M&lien\\_page=4&id=2](http://www.pheur.org/site/page_dynamique.php3?lien=M&lien_page=4&id=2)

The ultimate goal of the studies BSP034 (Diphtheria vaccines) and BSP035 (Tetanus vaccines) is the replacement of the current *in-vivo* multi-dilution assays for individual components by a single dose serological assay for multiple components. The many different types of composition of the currently marketed multivalent vaccines make it practically impossible to study this matter in a general way, but the inclusion of a wide variety of vaccines make it possible to demonstrate for a representative selection of products the feasibility of a single dose approach, or otherwise to acquire insight to which extent reduction of animals can be achieved. An example from the collaborative study will be discussed in more detail.

A first step could be to investigate the precision of the challenge assay versus the serological assay. The European Pharmacopoeia requires that the confidence limits fall between 50 and 200 per cent of the estimated potency. A typical challenge assay with 4 doses (2.5-fold dilution steps) and 12 animals per dose has in best case a confidence interval from 70 to 140 per cent of the estimate, and on average a confidence interval from 60 to 165 per cent of the estimate. An example from the collaborative study was shown (slides 2 and 3). When the mortality rates are replaced by the individual serum activities as titrated in the Vero-cell assay or ELISA, the precision improves considerably. The confidence interval will typically be comprised between 70 and 140 per cent of the estimate, a precision that could only be achieved in a best-case scenario using the challenge assay.

The increased precision would allow for an immediate reduction of the number of animals needed to meet the current requirements. Instead of 12 animals per dose, only 6 to 8 animals per dose would be needed to achieve the same degree of precision. Further reduction would be possible by using only 3 doses instead of 4 doses because of the reduced risk of having a dose with 'full' response. This example shows that a reduction of 50 per cent of the animals is statistically possible.

The approach discussed above replaced the multi-dose challenge assay by a multi-dose serological assay. Another approach to reduce the number of animals would be to replace the multi-dose challenge assay by a single-dose challenge assay. In practice we are only interested in the lower confidence limit of an estimate, but with a multi-dose assay we also

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obtain an upper confidence limit. This unnecessary information comes at a high cost (slide 4). However, even for a vaccine with an expected potency of 60 IU per human dose (Diphtheria) it is not easy to demonstrate that it contains more than 30 IU per human dose if only 12 animals are used. The example (slide 5) shows a critical situation where even a close to optimal choice of dilutions cannot discriminate the potency from the minimum requirement.

Before we look more closely to this example we will now discuss the recommended statistical test for single dose assays, namely the test of Wilcoxon-Mann-Whitney (WMW). This test has many advantages over parametric tests such as the more commonly known t-test:

- The t-test assumes that the underlying distributions are normal. WMW does not make this assumption. This is an important advantage, especially in the case of vero-cell assays where the results tend to follow a discrete distribution rather than a continuous distribution.
- Most parametric tests assume homoscedasticity of the responses. WMW does not make this assumption. This is also an important advantage because variability tends to increase at higher dose levels.
- The t-test is very sensitive to outliers. Methods to identify outliers with the purpose to remove them are highly undesirable from a statistical point of view. WMW is not sensitive to outliers because only the ranks of the activities are used.
- WMW is a very universal test because it can deal with all types of ordinal responses:
  - Quantal data (yes/no, as obtained with lethal challenge). For quantal data WMW is equivalent to Fischer's exact test.
  - Scored data (scores as obtained with intradermal challenge).
  - Quantitative data (e.g. absorbances as obtained with ELISA).
  - Mixed data (e.g. quantitative responses with a category "below detection limit").
- No transformation of responses is necessary. Parametric tests sometimes require a transformation of the data in order to satisfy specific criteria. No transformation is necessary with WMW because it is invariant under (monotonous) transformations.
- In many practical cases WMW is statistically more (or much more) efficient than the t-test.

At this point the increased precision of the serological assay gives a crucial contribution. When the mortality rates in the example are replaced by the serum activities, a clear discrimination from the minimum requirement can be made (slides 7 and 8). This example also demonstrates the advantage of the WMW-test that activities below the quantification limit can conveniently be classified as a group by assigning them an activity of 0 IU/dose. The activities obtained with Vero cells give a statistically significant result ( $p < 0.05$ ) and the activities obtained with ELISA give a highly significant result ( $p < 0.01$ ).

Since for most vaccines the potency of the Tetanus component is much more above the minimum requirement than the Diphtheria component it is easy to show that the serum activities from the same animals lead to a highly significant discrimination. For the vaccine in the example this would mean that 8 to 10 animals per batch would be sufficient to test for both components (slide 9).

An unexpected and very promising outcome of the study was the observation that all vaccines included in the study induced a useful regression of the activities for both components over the complete range of doses tested (slide 10). This is surprising because the doses were chosen to be optimal for the diphtheria component, which does not necessarily imply a useful

regression for the tetanus component. This observation is strong support for the possibility that for a wide range of products the same animals can be used in a single dose assay to test for both components simultaneously.

However, even in a single-dose assay the underlying assumption of similarity of dose-response curves of the standard and test vaccines need to be fulfilled. The example as shown in this presentation reveals no problems with linearity or parallelism if the scope is restricted to the laboratory which carried out the assay (slide 12). But when the results from all other participants are combined it can be shown that some vaccines have steeper slopes (T d) and some have a flatter slope (D T wP) than the diphtheria standard (slide 13). This may indicate that in some cases only like-to-like comparisons should be made, rather than comparing a multivalent vaccine with a monovalent standard.

In the like-to-like situation we do not expect fundamental problems with similarity of dose-response curves. Any dissimilarity must be due to experimental conditions or chance and cannot be attributed to fundamental different behaviour when diluted. Therefore, in a well controlled routine assay situation, no systematic checks for linearity or parallelism are needed. As a check for assay consistency it might be sufficient to include a vaccine control.

A question was raised from the audience if the problem with unequal slopes of different types of vaccines should really be regarded as a serious reason to abandon the idea of a monovalent (international) standard. There are 2 different answers to this question. The more fundamental answer is that unequivocal proof of systematic differences cannot be ignored for it means that the potency of the test vaccine cannot be expressed as a constant ratio of the standard. The more practical answer might be that in a fixed assay protocol the outcome would only be seen as a proof of consistency leading to acceptance/rejection of the batch, even though the quantification in terms of international units is, strictly taken, not valid.

The last question that was addressed in the presentation was how many animals we really need in a single dose assay. The answer depends on several key parameters:

- The true (but unknown) potency of the test vaccine (Pt)
- The minimum requirement that has to be met (Pr)
- The slope of the regression in the linear part of the dose-response curve (b)
- The standard deviation of the responses (s)
- The number of animals that die before the end of the experiment.
- The most critical component (i.e. whichever is closest to the limit).

In theory it should be possible to calculate the number of animals needed with the above parameters. However, in practice it will more likely be based on experience. An example was nonetheless shown with results from computer simulations using the above parameters. This gives a reasonable impression of what may be expected in practice.

The table (slide 16) shows that it may not for all products be possible to introduce a single-dose assay. For products with a diphtheria potency higher than 60 IU/dose a single-dose assay should be feasible with an acceptably low number of animals. For products with a potency closer to the minimum requirement of 30 IU/dose the number of animals needed in a single-dose assay increases rapidly. For these products it seems that the best achievable would be a 2-dose assay (2 doses of test and standard) or a 2+1-dose assay (2 doses of the standard and 1 dose for each of the test vaccines).

In conclusion, these studies have shown that the single dose serological assay is a promising alternative to the multi-dose *in-vivo* assay. There is compelling evidence that the same animals can be used to test for different components in a wide range of products. The question of similarity of dose-response curves will have to be addressed on a case by case basis.

### DISCUSSION

The discussion showed great interest in the single dilution model as a way to significantly reduce the number of animals per test whilst maintaining the assurance that the vaccines comply with the Ph. Eur. requirements.

It was emphasized that, for the single dilution approach to be workable, vaccines to be tested had to have a true potency that exceeded the minimum required quite significantly and that the approach would not be feasible for vaccines that had a potency close to the minimum required. Monitoring consistency of the potency would be more difficult with the single dilution model as compared to the multiple dilution model but e.g. the difference or ratio between the responses to test vaccine and reference vaccine could be used as a consistency monitoring parameter. It was also mentioned that a "point versus line" model (i.e. one test vaccine dilution vs. two or three dilutions of reference vaccine) would allow a more quantitative evaluation while still reducing the number of animals required importantly. In the single dilution model, assumptions of significant regression, linearity and parallelism of the dose-response lines would have to be backed-up by multiple-dilution assays during the validation of the model. It was also emphasized that the combination of the single dilution approach with the simultaneous assay of multiple vaccine components and possibly also with the exchange of sera to be assayed by the different laboratories involved in releasing the vaccines would result in the ultimate reduction of the number of animals required.

**SESSION II:**  
**PERSPECTIVES FOR COMBINED VACCINES CONTROL**

*Implementation of diphtheria and tetanus potency assays by serology on the same animals for routine batch release*

**OMCL's point of view**

Dr D. Garcia, Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS), (F)

Dr D. Garcia's slides are available on page 2 of this symposium-SessionII.pdf  
[http://www.pheur.org/site/page\\_dynamique.php3?lien=M&lien\\_page=4&id=2](http://www.pheur.org/site/page_dynamique.php3?lien=M&lien_page=4&id=2)

In the framework of the general discussion on Diphtheria and Tetanus potency testing, the OMCLs point of view on the feasibility of the new procedures has been asked. This is a difficult task since various situations do exist throughout Europe due to the diversity of regulatory structures and laboratory expertise. At Afssaps we have a routine batch release activity implying potency testing on animals on various combined vaccines and consequently a lot of exchanges with other OMCLs on this issue. From our example we will try to give some thoughts on the existing situation, the possible improvements that could be suggested and their added value for the OMCLs.

As an introduction some general information and figures are presented to summarize Afssaps control and batch release activities:

As an example, 1810 batch release certificates have been issued during the year 2003. Among them, 711 certificates were intended to the European market, 388 to non European countries and 711 for United Nations agencies.

These certificates correspond to the control of 41 different types of vaccines including single or combined viral vaccines and bacterial vaccines. The controls are carried out according to the relevant European OCABR guidelines and to WHO requirements.

More than 90 different types of controls are performed on purified components, on final bulks products or on final lots. In parallel to this batch release activity, Afssaps also performs expertises for WHO implying controls on products from worldwide vaccine manufacturers.

Most of the combined vaccines include the Diphtheria and Tetanus components. Diphtheria and Tetanus components can be associated with other bacterial components such as Hib, whole cell and acellular Pertussis as well as viral components such as IPV and Hepatitis B. To date, the most complex vaccines combine six valencies. Among these combined vaccines, the French authority is involved in the control of the following products : T; T IPV; DT; dT; DT IPV; dT IPV; DTwP; DTwP IPV; DTwP Hib; DTwP IPV Hib; DTaP IPV; DTaP IPV Hib and DTaP HBV IPV Hib. The batch release activity for these products represents 341 certificates issued for year 2003.

For most of the products, the compliance of Diphtheria and Tetanus potency assay is evaluated by the *in vivo* challenge test according to the relevant European Pharmacopoeia monographs. The protective activity is estimated by comparison with a reference preparation. However, the French authority also performs another potency test specific to non aluminium adsorbed vaccines.

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Indeed for these vaccines issued from an old formulation, compliance of Diphtheria and Tetanus components is evaluated by the capability of the vaccine to protect at least 80% of guinea pigs after challenge with 10 diphtheria or tetanus minimal lethal toxin doses. For year 2003, 45 *in vivo* challenge and 10 NIH assays were performed for Tetanus and 41 *in vivo* challenge and 4 NIH assays for Diphtheria.

Considering the increased laboratories activity in relation with batch release and particularly the workload due to *in vivo* potency testing in terms of time and animals consumption, a reflection has been initiated at the European level to limit and optimize these *in vivo* controls. In the context of the three Rs, a strategy was implemented at Afssaps.

This strategy is based on three combined approaches:

- Replacement of multi-dose assays by single dilution tests in accordance with the Manual of Laboratory Methods for testing of vaccines used in the WHO expanded Programme on Immunization [1]
- Reduction of *in vivo* testing in accordance with the European OCABR guideline [2]
- Implementation of *in vitro* alternative methods.

This strategy has been implemented thanks to the availability of numerous Afssaps data relative to each product over many years.

The implementation of such a potency testing scheme assay needed to be previously validated by analysing the criteria assuring the consistency of historical data.

Concerning the single dilution assays, the essential criteria is that no discrepancy is observed between results obtained with single and multi-dilution assays.

In this case, only one dilution is injected to the animals and the vaccine is considered as compliant if results are significantly higher than those obtained for the reference vaccine.

The replacement of multi dilution by single dilution assays for the Diphtheria or/and Tetanus components in various vaccines has been studied by Afssaps since 2001.

The single dilution has been implemented for DTwP combined or not with IPV and for dT vaccines. Implementation for dT IPV vaccine is in progress. Other vaccines have not been approved as candidate for the single dilution assays because they did not fulfil the criteria but new analysis on complementary data could be proposed and re-examined when available.

In the same time, after discussion within the OMCLs network the possibility that the OMCL may limit *in vivo* potency retesting has been approved, provided that sufficient data showing consistency of the component potency are available.

After analysis of historical data, Afssaps submitted proposals to reduce *in vivo* retesting and obtained approvals for Tetanus and Diphtheria components in some combined vaccines.

To assess the impact of this modified testing strategy, the number of animals saved by this double approach has been calculated. For each test the use of single dilution assays for D and T allows a reduction of respectively 66% of guinea-pigs and 75% of mice.

In addition, taking into account the principle of reducing *in vivo* testing, the reduction of 1500 guinea-pigs and 3900 mice was achieved for year 2003.

In the same time to reduce the number of animals used, alternative methods have been developed.

Thus, a Tetanus C-fragment ELISA has been developed and validated at Afssaps to quantify the tetanus antigen content in the vaccines. To date, internal control limits have been established based on historical data and a control chart of the NIBSC tetanus reference has

## Serological Potency Tests for Diphtheria and other Vaccines

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been implemented. This test is routinely performed on all released DTwP and DTwP IPV vaccines to monitor the tetanus component consistency.

Developments for implementation of this test for other combined vaccines are in progress.

Keeping in mind the need to evaluate other ways to reduce the *in vivo* testing, Afssaps participated in collaborative studies aiming at validating serological methods for the Tetanus component such as T ELISA and Tobi test.

In the same way, the current collaborative study BSP034 has explored the possibility to use the serum from the same animals to test diphtheria and tetanus toxoids.

The ELISAs and the Vero cell assays were performed according to the operating procedures previously established by the NIBSC laboratory. Reference guinea pig antitoxins were provided by EDQM and diphtheria toxin was originated from Aventis Pasteur manufacturer.

Some minor changes were introduced in the Vero cell assay concerning the culture medium with the use of M199 instead of MEM medium for the culture of Vero cells and concerning the viable cells which were revealed by UPTI BLUE® redox indicator.

The results of the three assays performed by the participants and also by Afssaps do not demonstrate major difficulties for future implementation of the method. It will have to be carried out in laboratories with experience in ELISA assays and cell culture. However, it can be noted that the delay to obtain results with the Vero cell assay is increased compared to D ELISA because of the incubation step between the cells and the sera.

The advantages and disadvantages of the implementation for routine batch release of Diphtheria and tetanus potency assays by serology on the same animal serum were evaluated.

The following advantages could be identified :

- The number of animals will theoretically decrease because of the use of the same sera for diphtheria and tetanus assays;
- Animals are sacrificed after bleeding and are less suffering compared to the lethal end point challenge;
- The variability of results issued from Vero cell assay and mainly from ELISA assays is improved compared to those obtained with *in vivo* challenge assay;
- The serological assays are reliable in laboratories with basic experience in ELISA and cell culture methods
- Functional antibodies are quantified by the Vero cell assay.

However, some disadvantages can also be identified:

- The total duration of the assay combining immunization plus serological assay is increased compared to the *in vivo* challenge assay (respectively about 7 to 8 weeks and 5 weeks).
- Guinea pigs were selected for immunisation to get optimal double tetanus and diphtheria responses. However, compared to the mice which are used for the tetanus assay, the supply and the handling of this laboratory animal is more difficult. Moreover, guinea pig is about 10 fold more expensive than a mouse.

Lastly some validation issues need also to be carefully considered before implementation of the serological assays.

- Preliminary validations for the assays themselves need to be performed in each laboratory and to qualify the staff.

## Serological Potency Tests for Diphtheria and other Vaccines

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- The immunization step needs also to be validated for each type of vaccine.
- The use of a single dose should be quickly implemented to reduce the number of animals used and to reduce the workload.

Indeed, as presented before, for the time being, the testing strategy implemented in OMCLs laboratories leads to optimize the use of animals and to obtain the results in acceptable delays. We have calculated that the implementation of Diphtheria and Tetanus ELISA with multi-doses immunisation and without reducing schemes will increase the animal number by approximately 2.5 fold with the need of more than 4 000 guinea pigs each year.

To conclude, some points still need to be discussed before considering that such a strategy is largely applicable. It concerns the development of serological assays with the same sera on other components. This will be of interest for combined vaccines including acellular pertussis or IPV components. But again this means revalidation of the design of the tests and should be envisaged only on a product specific basis.

It should also be kept in mind that even in case of implementation of serological assays, the proficiency testing for the *in vivo* challenge test should be maintained as this test will be always considered as the “gold standard assay”.

We also have to consider the implementation of these assays by serology for OMCL batch release in conjunction with manufacturer’s position. Indeed any modified OMCLs testing strategy should be feasible within the 60 days delay foreseen by the Directive on batch release.

Finally, it remains difficult to establish a global position for the OMCLs considering their diversity in terms of structure, activities, workload and strategy which is already implemented to reduce the use of animals. It is likely that advice and general guidance can be drafted within the OMCLs network to improve current potency testing practises but in all cases they should be evaluated on a case by case basis that is for a given product and for a given OMCL.

### References:

- [1] Manual of Laboratory Methods for testing of vaccines used in the WHO expanded Programme on Immunization (WHO, ed). Use and validation of a single vaccine dilution assay for testing of Diphtheria, Tetanus and combined vaccines. *WHO/VSQ/97.04, Part III*, pp 162-174 (1997).
- [2] Mechanism for reducing *in vitro* testing by OMCLs during batch release *PA/PH/OMCL (98) 11*

**SESSION II:**

**PERSPECTIVES FOR COMBINED VACCINES CONTROL**

*Implementation of diphtheria and tetanus potency assays by serology on the same animals for routine batch release*

**Manufacturer's point of view**

Dr F. Brunel, Aventis-Pasteur (F)

Dr F. Brunel's slides are available on page 7 of this symposium-SessionII.pdf  
[http://www.pheur.org/site/page\\_dynamique.php3?lien=M&lien\\_page=4&id=2](http://www.pheur.org/site/page_dynamique.php3?lien=M&lien_page=4&id=2)

*Abstracts: In order to reduce the use of laboratory animals and the variability of potency tests currently performed on vaccines, it is advisable to replace challenge potency tests with immunogenicity tests. In this frame, EDQM initiated standardization programs for developing alternatives to tetanus and diphtheria challenge tests used for the quality control release of single and multivalent vaccines for human use. Aventis-Pasteur experience in those tests will be presented in view of the test performance and industrial constraints we can expect. The approach of inter-method correlation, the validation steps and the analysis of results with regard to current specifications are key elements to be considered before routine implementation of this new generation of tests. Moreover, we will share our point of view on new approaches such as in vitro characterization of antigens which we considered opportunities to go further in the reduction or even elimination of in vitro tests. We do believe that these new analytical tools would allow to ensure the consistency of vaccine manufacturing processes in many cases. However, limitations to those approaches will be shared in the frame of the testing purpose, e.g. release, stability, process change documentation.*



**SESSION II:**  
**PERSPECTIVES FOR COMBINED VACCINES CONTROL**  
*Implementation of diphtheria and tetanus potency assays by serology on the  
same animals for routine batch release*  
**Manufacturer's point of view**

Dr M. Duchêne, GlaxoSmithKline Biologicals (B)

Dr M. Duchêne's slides are available on page 14 of this symposium-SessionII.pdf  
[http://www.pheur.org/site/page\\_dynamique.php3?lien=M&lien\\_page=4&id=2](http://www.pheur.org/site/page_dynamique.php3?lien=M&lien_page=4&id=2)

The application of the serological potency tests for Diphtheria and Tetanus-containing vaccines has been evaluated in comparison with the challenge assays throughout various perspectives:

- Quality assessment of the vaccines tested
- Implementation process for routine use
- Industrial considerations
- Potential future of this application

**Quality Assessment of the Vaccines tested**

The Diphtheria and Tetanus challenge assay has been used by GSK Biologicals for more than 30 years: an impressive amount of experience has been accumulated over time for the following purposes:

- Routine release
- Development of new combined vaccines
- Validation of manufacturing changes
- Establishment of consistency
- Stability profile of D and T-containing vaccines

All vaccines passing this test have proven efficient in the field.

But the challenge assay also results in the use of a huge amount of animals; it requires a rigorous management and monitoring of the various parameters to enable a high success rate and to reduce the variability of the test. This test is time- and cost consuming (animals and facilities); it can be a potential bottleneck to the production capacity and the information on the vaccine characteristics is often limited to a pass or fail situation.

GSK Biologicals' present experience with the serological assays proposed as an alternative to the challenge tests is very limited; the serology by a classic ELISA test is quite simple and easy to handle but does not refer to a functional test. The alternatives by seroneutralisation on Vero cells for the Diphtheria or by the TOBI test for the Tetanus component are functional tests all right but we still have to demonstrate their correlation with the clinical performance.

In our hands, we have observed a good correlation between the challenge data and the Vero assay when the test is performed at 6 weeks but we have not observed this correlation when

the test is carried out at 4 weeks as recommended for the classic challenge assay. There is no explanation to this discrepancy and, so far, the serological tool cannot be used for the assessment of the quality of the vaccines tested. Nevertheless, the good result at 6 weeks demonstrates that there is a close correlation between the abs quantification and the neutralisation data: this would allow the use of this serological assay for the routine evaluation of the commercial vaccines once the formulation of the vaccine and its critical parameters have been established. If limited to routine release, an Elisa serology is to be recommended since it is easier to perform, to standardise and to harmonise.

### Implementation Process

Since the Abs response can vary from one vaccine to another and since we have to discriminate good and less potent vaccines, it is important to demonstrate that the dilutions inoculated are within the linear dose response curve by establishing the complete dose-response curve for each vaccine. These curves have to be done at least three times so as to select the appropriate dilutions to be performed on each new vaccine batch.

Afterwards, the variability of the full test has to be established by assaying the reference preparation on various occasions so as to define the validation criteria for each future assay: the reference preparation can be the same for each vaccine formulation. Practically, this means that numerous experiments have to be carried out to determine these parameters: the relevant dilutions to be inoculated and the validation criteria.

Fortunately, the parameters related to the serology have all been included in the inter-laboratory studies and do not have to be defined still. For the sake of consistency, it is also relevant to test different vaccine lots in order to establish the variability of the manufacturing operations from one batch to another as well as the consistency limits.

In sum, this implementation process represents a huge amount of work and time before entering routine application.

### Industrial Considerations

Presently, the challenge assay for the Diphtheria and Tetanus potency on each vaccine lot for the routine release and for the establishment of the vaccine characteristics (manufacturing changes, stability, development,...) represents 40 to 60 % of the total *in-vivo* testing capacity; out of this capacity, about 75% and 25% are respectively used for guinea-pigs and mice. Therefore, from a purely industrial viewpoint, these tests are critical parameters for the production capacity, the cost and availability of the vaccines.

The recent change to the Ph. Eur. allowing a single dilution had a significant impact since the amount of animals have been reduced by 60% and the Animal House capacity has increased by 50%.

The conduction of serological assays using the same animals for both ags once established will result in a large reduction of animals but only by a 2-fold factor; the cost per test will increase due to additional laboratory workload. Regarding the test capacity, there will be no improvement since the test is performed on guinea pigs and over a period of 6 weeks instead

of 4 weeks. At this stage, there is no benefit – or only a marginal benefit – to the shift of testing.

Nonetheless, the real advantage could be more obvious once the single-dilution test is also validated for the serological test but again this will be marginal compared to the present situation for routine release activity. Additionally, the application of the new serological assay for routine release in parallel with the maintenance of the challenge assay for the other testing purposes (development, stability) will result in an increased complexity due to the management of different potency assays.

### Potential Future

Extrapolating from equivalent tests applied to other vaccines, we can assume that this serological test will allow less variability from test to test and will give a better success rate: theoretically, we can also assume that the information obtained with this test will be more precise than the simple pass/fail status of the challenge assay. This means that this analytical tool will enable a better refinement of each formulation.

In addition, the same animals could be used to test other antigens usually present with Diphtheria and Tetanus components like acellular pertussis components. However, this possibility is restricted to antigens still tested with an *in-vivo* assay and if the same dilution can be used for all these antigens.

The application of this new test can be seen as a further layer of complexity for the manufacturers: indeed, there will now be three methods for the same antigens depending on the country of destination (US, EU, WHO). Conversely, this can be seen as an opportunity to harmonise the Diphtheria and Tetanus potency assays, at least for routine release purpose.

The implementation of this test gives another opportunity: since serum materials are now available, they could be exchanged with the national release authorities. This would allow avoiding the classic repeat testing on animals and sharing the same *in-vivo* test with the official control laboratory. This new way of working is impossible with the current challenge assay and would mean a significant breakthrough in the reduction of animals for the entire release purpose of vaccines.

Finally, the use of a serological marker will demonstrate that there is no need to have a complete functional test for routine release activity; this could be the first step to the development of an *in-vitro* testing to routinely assess the quality of a well-established vaccine.

### Conclusion

As a conclusion, GSK Biologicals' viewpoint is that there is no real incentive or benefit from the application of this new assay. The impact on the animal welfare is rather marginal and does not balance the significant investment, cost and complexity this new assay will require. Nevertheless, it opens the door to several new opportunities for the routine release of the Diphtheria and Tetanus-containing vaccines. Among these, sharing the *in-vivo* test with the NRA would surely allow a major progress for all parties concerned, for the animal welfare, for the release authorities and for the manufacturer while maintaining the quality of the vaccines released.

## DISCUSSION

**Dr D. Sesardic:** I have a question for regulatory authorities about the implementation of the single point assays. As a result of large variability generally observed in potency testing by challenge for diphtheria we found it quite difficult to implement the single point assay; therefore I was questioning how you were able to do that. For Tetanus we were able to achieve a single point assay because as was pointed out, in order to go from a multiple point assay to a single point you need confirmation of consistency of a test and you are never really able to see that in challenge assays for diphtheria. We are also thinking about moving towards serology, as that would also increase the ability to move faster to single point testing because the variability of the assay is reduced.

**Dr D. Garcia:** In fact what I would like to present is that for the time-being we have implemented a strategy which allowed the use of a reduced number of animals and for some vaccines, where the single dilution could be implemented, to move to serological assays means that you need to go back to multi-dilution and to revalidate it and it's, as Mr M. Duchêne explained, it's time-consuming and costly because we have to use new animals. Maybe it's because French authorities have a lot of batch releases to perform and we have an important work load, so it appears difficult for us to introduce new validation in parallel to our batch release activities. This is why, for the time-being, it must be difficult to introduce and maybe we can wait for serological assays for another component, for example if we can associate acellular pertussis batch releases it will be of interest to re-validate and to perform the serological assays for the three components. We are not against the implementation of these serological assays, but the present strategy allows the use of a very reduced number of animals.

**Dr R. Winsnes:** Thank you. First it's Dr D. Wood.

**Dr D. Wood:** Two questions - one to Dr M. Duchêne and one to Dr F. Brunel. First to Dr M. Duchêne, could I just clarify what you intended by saying you flagged up this concept of stopping the testing by the National Registration Authority (NRA). I think what you are proposing is that the manufacturer, for example, would immunise the animals and then share the sera with national control laboratories, so that the national control laboratory could then for example carry out the serology testing. Is that what you are actually proposing?

**Dr M. Duchêne:** Yes this is precisely what I propose, but the reverse can work too.

**Dr D. Wood:** Thank you. I think that's a useful clarification that one set of animals could be used for obtaining two independent sets of results on the acceptability. I think that's a good concept actually – thank you.

For Dr F. Brunel, in your validation of the single dilution assays I may have missed, but what number of animals did you decide to use? Is it the same number of animals that you use in your four dilution test or have you increased the number of animals compared to the number of animals you inoculate per dose in the four dilution test?

**Dr F. Brunel:** I think that we will use ten animals per group and so it's why we have reduced the number of animals, but not so drastically that if we use the current number of animals for

the four doses current test. The data I have shown for the concurrence between the two methods are based on the historical data and validation data that we have obtained with this type of test using ten animals per group.

**Dr R. Winsnes:** Peter Castle the floor is yours and Gupta Rajesh afterwards.

**Dr P. Castle:** First of all a comment on the idea of having the sera from the same animals tested by the manufacturer and the National Control Laboratory for a check assay, of course that eliminates one important source of variability in the test and that's what the check assay is meant to be controlled on. But I can see that if the manufacturer and the control laboratory alternately vaccinate the animals and pass the sera to the other, then that would be a kind of check on variability from the animals.

A question to Dr D. Garcia. In your one dilution assays, what do you use as a reference preparation? Do you use the EDQM Biological Reference Preparations or do you use a homologous reference preparation and does it have any effect on the ability to move to a one dilution assay?

**Dr D. Garcia:** I think we use an international reference preparation for this assay, because we use the same reference as we used before for the four dilution assay and in fact it has no effect.

**Dr P. Castle:** So you use in fact a monovalent reference, you never use a homologous reference? Maybe Dr F. Brunel could also tell us what Aventis Pasteur does. Do you use a monovalent reference or a homologous one?

**Dr F. Brunel:** It is an official reference, yes, the same that we use in the four doses testing.

**Dr J-M. Spieser:** To my information, for two years you have used our European Working Standard as your working standard - according to the number which we deliver.

**Dr R. Winsnes:** Which reference do you use, Dr M. Duchêne?

**Dr M. Duchêne:** No, we are using an in-house reference material which has been calibrated and validated versus existing international reference and this is an heterologous vaccine preparation.

**Dr R. Winsnes:** Thank you. Then it was Dr R. Gupta who wanted the floor.

**Dr R. Gupta:** I'm thinking of Dr M. Duchêne's idea about exchanging the sera with the regulatory authority is very good. It will not only reduce variability, but it will also save time. I think time is one of the immediate factors. As Dr P. Castle put forward that one time manufacturers immunise the animals and the other time the regulatory authorities. That will also remove concerns from the regulatory authorities about any conflict of interest or anything along those lines.

The second thing, these three presentations were very good to hear – about manufacturer's experience and the realistic approach, particularly the practical implications, that for the short term, it will not be like saving time or resources. Maybe you have to invest more resources

and time but in the long term, particularly on the harmonisation issue I want to just comment on that. I don't know what is the obstacle in harmonising between WHO and Ph. Eur methods. The only difference is the requirements, so if you meet the Ph. Eur requirements, you don't need to do the WHO tests. That's what my question is to the manufacturers.

**Dr R. Winsnes:** Would you like to comment on that Dr D. Wood?

**Dr D. Wood:** I would be first interested to see what Dr M. Duchêne thinks!

**Dr M. Duchêne:** As I mentioned in my presentation I see no reason why this type of testing could not be implemented in the WHO requirement, at least as an alternative in a first step.

**Dr R. Winsnes:** A question from Dr A. B. Arunachalam.

**Dr A. B. Arunachalam:** This is a question directed to Dr M. Duchêne. You mentioned that you have seen good correlation with the six week bleed and not with the four week. I assume this is the Vero cell assay versus the challenge method. You do not look at the correlation between ELISA versus challenge method at four weeks and six weeks, because ELISA obviously picks up both low and high affinity antibodies. So, I was wondering, do you see better correlation at four weeks with ELISA?

**Dr M. Duchêne:** As I mentioned during my presentation there is a very good correlation between ELISA data, challenge data and Vero cell assay data at six weeks. But this correlation does not exist at four weeks. As I mention, we only performed a few experiments. I cannot say that this is the definitive picture, but at least with all the experiments we performed so far, this is the conclusion we have.

**Question from the floor:** Can you give a precise number of what kind of level of correlations you see at four weeks versus six weeks?

**Dr M. Duchêne:** I do not remember precisely the figures, but I think that the Vero cell data is lower at four weeks than at six when compared to the challenge data. I think that ELISA is the opposite, but I am not completely sure.

**Dr R. Winsnes:** Maybe Dr J. Arciniega you want to come in here before Dr D. Sesardic.

**Dr J. Arciniega:** I have several things here. One is just a clarification from Dr F. Brunel. You mentioned the passing criteria for a single dose as the comparison with the upper limit. I think that is the lowest possible limit, right? Not the upper.

**Dr F. Brunel:** Yes.

**Dr J. Arciniega:** The other thing is, you were mentioning some kind of chemical techniques to characterise antigens. But how do you reconcile that with the fact that these antigens are eventually going to be detoxified and then absorbed? How do you manage that?

**Dr F. Brunel:** We have the idea to implement this type of *in vitro* characterisation at the bulk stage, when the antigen is not absorbed and before the vaccine formulation. As a screening test or consistency test we thought before formulation we need to ensure that we have no

problem on the purified bulk. We don't want to implement this kind of test at the final stage when all the antigens are blended together and are adsorbed.

**Dr J. Arciniega:** So if you continue doing a potency test, how do you envision this? Is the potency test going to be simplified because you better characterise the vaccine along the way?

**Dr F. Brunel:** It's a consistency tool for manufacturers to be sure. It's more for us to be sure at an early stage that when we have a problem on a final batch, to be able to say that the problem is due to a particular antigen. We can imagine that if we have a low result in terms of potency at the final combination level that the adjuvant or some other antigen are involved in this decrease of activity. So, for us it's easier to perform this type of test before combination to be sure that we have very well identified the issue, if it exists. It is a consistency test, it's complementary test to the *in vitro* assays. It's not the final strategy to have only this *in vitro* characterisation test. I think it's too early.

**Dr J. Arciniega:** But, can you imagine the possibility of rejecting a lot of antigen with this type of tests, even before it gets detoxified?

**Dr F. Brunel:** Today I can't answer, because I have no sufficient data to be sure that we have a good correlation between confirmational data and quality, but if we imagine that we have successful analytical tools to do that, then why not?

**Dr J. Arciniega:** Right. Regarding the presentation by Dr M. Duchêne, this idea about exchanging sera is intriguing. However, I'm not sure of how practical it will be, because I do not know your commercial practices: if a lot goes to multiple countries, you may need to pay attention to the species you use for the potency test, because you are going to need a lot of serum to exchange with different control authorities. Is that a problem?

**Dr M. Duchêne:** I agree, but we are in Europe. This test has been presented in the European Pharmacopoeia and you have to know that within the European batch release systems only one test is carried out for the different European countries of the EU, so we don't need to exchange a lot of different sera. It is true that this could be difficult for implementation at a larger level. For WHO I think that the release is currently performed by the local national release authorities only. So, sharing the same serum with multiple authorities should be an exception rather than the norm.

**From the floor:** We are open to mutual recognition also outside Europe!

My colleague and I have performed in case of emergencies, this kind of testing for using sera coming from company. But we have a kind of reluctance towards implementing all the time, like getting sera like it's a routine practice, we are reluctant to do that because we believe the testing has to be confirmed right from the beginning up to the end stage using a different set of animals.

**Dr J-M Spieser:** That has already been the case, at the beginning when implementing the batch release. It has always been said that it consists of two independent assays. But of course if all the actors in the system agree to modify that system and get it approved by all those that have to say something in this procedure, so far so good, but it's true that it does not represent a true independent assay.

**Dr R. Winsnes:** Dr D. Sesardic, I think you asked for the floor some time ago?

**Dr D. Sesardic:** You wanted me to answer the question before about the point of potency testing between four and six weeks. It's actually not quite true to say that you would not be able to get potency data at four weeks. It's purely the ability to measure antibodies in Vero cell assays. I wanted to confirm that it's quite a good correlation of course with the observed potency by the manufacturers performed at four weeks by challenge and performed by either five or six weeks in ELISA. It's an issue of the sensitivity of the assay to measure the antibodies at later time point, that's the main reason.

**From the floor:** Also I just want to add because we are comparing with a so-called gold standard which is highly variable. So the data we generate from the animal challenge assay, how reliable is it? Because when it is highly variable and we are considering that as a gold standard, we are always comparing against variable gold standards.

**Dr D. Sesardic:** Potency testing means that you should relate responses to the reference vaccine so they are supposed to be equally effective in the assay. It would make no difference in a potency calculation.

**From the floor:** That's right, I'm addressing more to the US challenge method, where there's no reference vaccine there.

**Dr R. Winsnes:** Over to Dr Gupta.

**Dr R. Gupta:** Two points: one on exchanging the sera. I think I hear from the regulatory agencies that there are some concerns, but I think we should move forward with the idea that the lot release batch was implemented when there was no GMP procedures for vaccines. Nobody is considering GMPs and consistency in manufacture, while giving so much importance to lot release. I think it's a good idea to immunise both alternatively and exchange sera. But again I think this needs to be considered by the manufacturers and the regulatory agencies.

My second comment is on the *in vitro* characterisation which Dr J. Arciniega asked about. I would just like to add that diphtheria and tetanus toxoids are very robust proteins even after adsorption onto the adjuvant, you still get the same antigenicity by flocculation test or even by ELISA using capture method.

On the comment about doing the *in vitro* characterisation, I think it's a great tool for consistency in manufacture and it has been done for many years, flocculation test has been gold standard for many, many years to look at the antigenicity by the manufacturers, I know that they will agree. Manufacturers don't only look at the Lf, they look at the kinetics of flocculation value also. When the Kf go out of range, they know that the quality of the toxoid is not good, even if they get the same amount of Lf. I think what Dr J. Arciniega mentioned that rejecting a lot earlier based on the Lf or other *in vitro* tests will be advisable rather than failing at the end due to the potency test or not performing well in humans.

**Dr R. Winsnes:** Thank you. Dr R. Dobbelaer you wanted the last word?

**Dr R. Dobbelaer:** Just a remark on this exchange of serum. I think the decision whether this is a valid approach from a regulatory point of view that's for the policy makers. I think that the main message here is that the serological allows it to do. It offers the possibility to do it and then the policy makers have to weigh the loss in so called independency versus the gain in animal reduction and again in precision. Not here but by the policy makers.

**Dr J-M Spieser:** Except that there is another very important issue which you have to take into account – liability. Who becomes the releaser? Because up to now all of the legislation have always been built in such a way that the liable person for the release is the manufacturer.

**From the floor:** That will not change.

**Dr J-M Spieser:** Yes, except that you will be playing a great part if it's you, the authority for that batch, who will be the unique person who will immunise the animals and then you have to think about what it means in terms of liability.

**From the floor:** But that's the other step.

**Dr R. Winsnes:** Thank you - a very good discussion. We have heard that manufacturers would prefer as OMCLs, to see more antigens potency assayed by serological methods. So please, Dr D. Sesardic, could you tell us if we could come to that in the near future?



## SESSION II:

### PERSPECTIVES FOR COMBINED VACCINES CONTROL

#### *Possible extension of serological potency assay to other components of combined vaccines*

#### Data from trial phase: BSP034 extension to IPV titration

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Dr D. Sesardic's slides are available on page 19 of this symposium-SessionII.pdf  
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### SEROLOGICAL POTENCY TESTING OF COMBINED VACCINES: REALISTIC OPPORTUNITY?

#### Introduction

All, except one, currently licensed vaccines containing diphtheria toxoid are presented in combination with at least one other antigen, tetanus toxoid. All licensed vaccine combinations, produced by several manufactures therefore include, in addition to diphtheria and tetanus toxoids, types 1, 2 and 3 polioviruses, either 2, 3 or 5 pertussis antigens, *Haemophilus* Type b (Hib) glycoconjugate component and in some cases Hepatitis B (HepB). Different animal models or protocols are used to confirm potency, as a batch release requirement, or to establish product immunogenicity. In order to test a single lot of hexavalent vaccine approximately 400 animals are required.

Replacement of the direct challenge assays by serology for diphtheria component in combined vaccines provided an opportunity to investigate the possibility if further reduction of animals could be achieved by using the serum from the same animals for testing of more than one antigen. The collaborative study therefore included testing of tetanus component as presented in previous communications. It is clear that a common animal model is required, to study all components in current vaccines, not just to reduce number of animals required for potency testing but also to investigate effect of components in combinations, where possible. Antibody responses to diphtheria, tetanus, Hib and HepB have all been reported to be reduced or compromised in some clinical trials with some vaccine combinations.

This presentation summarizes NIBSC experiences in potency by serology of diphtheria and tetanus components in several multivalent vaccines. Using serum samples prepared by several participants from collaborative study [1,2] it was also possible to investigate dose response to all other antigens in combinations i.e IPV, pertussis, Hib and to limited extent to HepB.

#### Materials and Methods

***Potency of diphtheria and tetanus by serology*** Fourteen different combined vaccines were tested in three separate trials using same lot of DTaP as reference vaccine. Effects of IPV and Hib on potency were investigated by including same production lots with additional components. G. pigs

## Serological Potency Tests for Diphtheria and other Vaccines

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(8-10 per group) were immunized once s.c. with 1.0 ml of vaccines with 3 dilution steps in the range of 1:5, 1:15 and 1:45. Animals were bled at day 35 (5 weeks) and serum tested by Diphtheria and Tetanus-ELISA [1]. Potency was calculated by parallel line analyses in relation to a reference vaccine.

**Serology of other antigens** Serum samples prepared for use in the collaborative study for refinement of potency testing for diphtheria toxoid vaccines were used. These were from Phase I and III trials as prepared at NIBSC and were also donated by five participating laboratories from their own trials from Phase II. In all cases serum samples were from individual g. pigs (8-12 per group) injected s.c. once with 1.0 ml of vaccines diluted from ca. 1:5 or 1:10. Animals were bled 5-6 weeks later.

Serum samples from combinations containing IPV were analyzed for neutralizing antibodies on Hep 2C cells for Types 1, 2 and 3 polioviruses. Results were expressed as number of sera responding positively, above selected cut of point, out of total of 12 individual samples tested. Antibody responses to three pertussis components, pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN) were performed by ELISA using antigens and protocols provided by GSK (Belgium), and expressed as dilution titres. Antibody responses to Hib polyribosyl ribitol phosphate (PRP) were performed by ELISA using Wyeth antigens and also expressed in dilution titres. One set of samples was also tested in Abbott Ausam Kit for antibodies to HepB.

### Results and Discussion

**Potency of diphtheria and tetanus by serology** The studies confirm that it is possible to calculate statistically valid estimates of potency for both components in 14 different vaccines tested in three separate trials during 2003-4. Only in one case it was not possible to calculate potency due to non-parallel response. 95% Confidence limits were generally within 60-160% and in most cases between 70-140% for both diphtheria and tetanus estimates when 3 vaccine dilutions groups were used with 8 g. pigs per group. Potency in a single animal model of two lots of DTaP<sub>3</sub> IPV with and without Hib required 120 g. pigs in one assay, instead of 148 g. pigs and 424 mice in two separate assays as per current Ph. Eur. direct toxin challenge methods [3]. Potency of two components (diphtheria and tetanus) using serum from the same animals provides opportunity for reduction in number of animals; increased precision of potency estimates; provides opportunity for further reduction in number of animals per group from 8 to 5 and/or in number of groups and eliminates animal handling post challenge procedure, for up to one week. The same assay also confirmed inhibitory effect of IPV component and modulating effect of Hib on tetanus potency. The lowest potency for diphtheria component was in DTaP<sub>5</sub> IPV Hib combination.

**Serology of other antigens** Reduction in total number of animals would be even greater if potency could be monitored for additional antigens in combined vaccines.

Potency for IPV is by serology using rats, g. pigs or chickens for immunizations [3]. Rats are the generally preferred species where animals are given high immunizing dose and bleeding is at 3 weeks post immunization. Limited information is available on serology for combined vaccines for IPV in g. pigs at lower immunizing doses with bleeding at 5-6 week post immunization, an optimum time of bleeding for potency of toxoid antigens. Our studies on serum analyzed from two combinations (DTaP<sub>2</sub> IPV Hib and DTaP<sub>3</sub> IPV) made by two manufacturers and produced in five laboratories confirmed excellent and reproducible dose response for neutralizing antibodies against

## Serological Potency Tests for Diphtheria and other Vaccines

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all three IPV serotypes. Data suitable for potency calculation was obtained but long time required for neutralization assay makes this approach less practical for batch release purposes.

Potency for aP is by serology in a mouse model [3], which is generally considered as unsatisfactory and there are no defined specifications for potency by serology. Availability of serum from g. pigs makes it an attractive opportunity to investigate serology of aP components. Serum was analyzed from four vaccine combinations made by two manufacturers at three separate sites and included aP with 2, 3 and 5 components. Useful dose response and one generally suitable for potency calculation was observed for PT, FHA and PRT in all combinations where these antigens were present in vaccines. Antibody responses for PT and FHA were higher for DTaP<sub>3</sub> than for DTaP<sub>2</sub> combinations and anti-PT response was lowest for DTaP<sub>5</sub> IPV Hib product. The antibody response to PT appeared to be less effected by the presence of other components in vaccine or donating laboratory, but was product specific.

There are no requirements for animal test for potency of Hib component in vaccines. It is required that during development stages, vaccine must be able to induce T-cell dependent antibody response (3). Serum was analyzed from four combinations made by two manufacturer and included DTaP<sub>3</sub> IPV Hib, DTaP<sub>3</sub> IPV HepB Hib, DTaP<sub>2</sub> IPV HepB Hib and DTaP<sub>5</sub> IPV Hib products. Anti-PRP responses were high with reasonable dose response for the two combinations made by one manufacturer containing either aP<sub>2</sub> or aP<sub>5</sub>, but low with no suitable dose response with combinations containing aP<sub>3</sub>. Interestingly, exactly the same information was observed in a rat model after a booster dose and 42 day bleeding and reported to be in line with clinical studies. However, neither of the models appears to be suitable as a batch release potency test because of generally non linear dose response.

Serum from DTaP<sub>3</sub> IPV HepB vaccine was also analyzed for antibodies to Hep B surface antigen. Immunising doses of 1:5, 1:15, 1:45 and 1:135 produced excellent dose response with ED50 (50% of serum showing positive response) at the second dose.

In conclusions this study confirms that guinea pigs provide suitable model for serology of all key antigens in current combinations with a potential to reduce number of animals used in potency testing.

**Acknowledgement** Author would like to thank participants of Phase II collaborative study for donation of samples and Dr Morag Ferguson and Paul Chamberlin for performance of HepB ELISA.

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- [2] Winsnes R., Sesardic D., Dasss A., Behr-Gross M.-E.: Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccines. Summary of the study report. EDQM document *PA/PH/BIO (04) 40* (October 2004).
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**SESSION II:**  
**PERSPECTIVES FOR COMBINED VACCINES CONTROL**  
*Possible extension of serological potency assay to other components of  
combined vaccines*

Experience from an OMCL in titration of DT Pertussis vaccine

**Feasibility study to determine acellular pertussis and  
IPV antibodies in guinea-pig serum**

Dr A. Maes, Institut Supérieur de Santé Publique (ISSP), (B)

Dr A. Maes' slides are available on page 27 of this symposium-SessionII.pdf  
[http://www.pheur.org/site/page\\_dynamique.php3?lien=M&lien\\_page=4&id=2](http://www.pheur.org/site/page_dynamique.php3?lien=M&lien_page=4&id=2)

The goal of the experimental part is to investigate whether it is feasible to generate antibodies in guinea pigs against IPV and acellular pertussis components in combined vaccines and to measure both these antibodies in the same serum samples.

Since 1996, the European Pharmacopoeia has started a collaborative study to validate a serological method for the potency testing of tetanus toxoid vaccines and in 2001 for diphtheria toxoid vaccines. It was decided to use an analogous protocol for the experimental part, thus in principle allowing the possibility to use a single guinea pig serum sample for the simultaneous determination of diphtheria, tetanus, acellular pertussis and IPV antibody titres generated by combined vaccines.

Female Dunkin Hartley Specific Pathogen Free (SPF) guinea pigs were purchased from Charles River weighing between 250-300 g. The 10 guinea pigs were housed in the same cage. One guinea pig was used for obtaining negative control serum. The choice of the guinea pig was based on the Diphtheria and Tetanus collaborative studies organized by EDQM allowing the possibility to use a single guinea pig serum sample for the simultaneous determination of diphtheria, tetanus, acellular pertussis and IPV antibody titres generated by combined vaccines.

An Infanrix-IPV, a combined diphtheria, tetanus, acellular pertussis and inactivated poliomyelitis vaccine (DTPa-IPV) manufactured by Glaxo SmithKline Biologicals (GSK Bio) (Rixensart, Belgium) was used to vaccinate the guinea-pigs. The vaccine has the following composition per dose (per 0.5 ml)

Diphtheria toxoid	≥ 30 IU
Tetanus toxoid	≥ 40 IU
Acellular Pertussis	
Pertussis Toxoid (PT)	25µg
Filamentous haemagglutinin (FHA)	25µg
Pertactin (69K-PRN)	5µg
Inactivated Poliomyelitis Virus	
Type I	40 DU
Type II	8 DU
Type III	32 DU
Aluminium Hydroxyde	0.5 mg

As an anesthetic agent a combination of Xylazine hydrochloride 2% (Rompun®, Bayer) and Ketamine 100 mg/ml (Anesketin, Eurovet Animal Health) was used. Approximately 2.5 ml blood was collected by cardiac puncture forty-two days after immunization of the guinea pigs. The choice of the time interval was again based on the Diphtheria and Tetanus collaborative studies organized by EDQM

For the determination of IPV antibodies in guinea pig serum a neutralising antibody test has been used as described in the Ph. Eur. 2.7.20: Neutralising titres against all 3 poliovirus types are measured separately using 100 CCID<sub>50</sub> of the Sabin strains as challenge viruses, Hep2 cells as indicator cells, and neutralisation conditions of 3 h at 35-37 °C followed by 18 h at 2-8 °C where necessary for consistency of results. Results are read following fixation and staining after 7 days of incubation at 35 °C.

For the determination of acellular pertussis antibodies an ELISA has been performed as described in Ph. Eur.: Microtitre plates are coated with the purified antigen and twofold dilutions of sera from guinea immunised with vaccine are made on the plates. After incubation, the plates are washed and a suitable solution of rabbit anti-guinea-pig IgG enzyme conjugate is added to each well and incubated at 22 °C to 25 °C for 1 h. After washing, a chromogenic substrate is added from which the bound enzyme conjugate liberates a chromophore which can be quantified by measurement of absorbance. (Ph. Eur., 2.7.16. Assay of pertussis vaccine (acellular))

From the results presented above it can be concluded that, after vaccination with one single human dose, guinea pigs develop measurable and possibly also quantifiable antibodies against the three IPV antigens and quantifiable antibodies against the three acellular pertussis antigens present in a DTPa-IPV combined vaccine but acknowledged that this is only a preliminary step in the replacement of the currently approved serological assays using separate immunogenicity assays for each group of three antigens. To achieve such a replacement and the associated animal reduction, further studies should be performed. These would include:

- The determination of the optimal dilution of the vaccine dose: In our study, the guinea pigs were vaccinated with one human dose to maximize the probability of a measurable antibody response. In the collaborative study for diphtheria vaccine, the four dilutions used ranged from 1/80 to 1/5 of a human dose. This range was also suitable for Tetanus vaccine. Therefore the anti-IPV and anti-aPer antibody response of guinea pigs should be evaluated at these lower doses. An alternative approach could be to evaluate the anti-diphtheria and anti-tetanus response to higher doses of vaccine antigen. A “compromise” dose range may be established.
- The optimal time interval between immunization and bleeding: again, ideally, the same interval should be chosen as during the validation study of serological methods for potency testing of tetanus and diphtheria toxoids. However, based on our preliminary results for IPV, it is observed that type 3 gives a weak response (low antibody titres with one human dose). The 42-days time-interval of the collaborative studies may be too long for IPV. In addition, the 42 days were also chosen to enable a parallel assessment of guinea-pig immunity to tetanus and diphtheria by both challenge and serology and a shorter time interval may be equally suitable. Again it may be possible to establish a “compromise”.

- The correlation between the current serological assays performed in mice (Ph. Eur. 2.7.16 and 2.7.20) and the serological assay performed in guinea pigs should be evaluated.
- Vaccines of different manufacturers and different composition should be included in the study. If possible a vaccine of borderline quality should be included.

### DISCUSSION

**Dr S. Gairola:** I have questions for both speakers. I'm going to start with the second speaker first. One is a clarification – you use a single dose to immunise the animals (the guinea pigs), is that correct?

**Dr A. Maes:** One dose.

**Dr S. Gairola:** The other question, I will preface with some experience. When we were licensing the acellulars in the US, somebody approached us with a guinea pig model and we didn't take it because that particular manufacturer was unable to show us that the guinea pigs they were using were *bronchiseptica* free. *Bronchiseptica*, as you know, shares Filamentous Haemagglutinin (FHA) with pertussis. So, the control serum show titles to FHA. Do you have data on your guinea pigs – if there was a base line response to FHA?

**Dr A. Maes:** We didn't have a lot of control serum but we did have a base line of the undiluted serum. We just tested undiluted negative control serum and we measured 0.5 OD, which was quite high, but that was undiluted. If you test undiluted serum from guinea pigs, which are vaccinated we obtained ODs around 3.0. You do have a base line but there is enough space with the true response of the antibody concerned.

**Dr S. Gairola:** The question for Dr D. Sesardic – I have to take issue with something she said. She said something along the lines that current mouse test, single dose test, for pertussis is unable to differentiate between vaccines. I would like to ask if she could clarify or mention what data she's using in order to make that contention? What, in her opinion, is the method not showing?

**Dr D. Sesardic:** I have no direct experience in mouse testing of pertussis but it is performed at NIBSC and for the Ph. Eur. test it requires a single dose against PBS controls. So we are aware that you cannot actually discriminate between the lots, but I've also been told that essentially you have very large differences to antigens in different products which do not necessarily correlate with what you see in protection assay in mice. So there are two different questions here. How do you correlate serology to protection in mice because serology will provide product specific information and how do you extrapolate that into the information to be useful for batch release.

**Dr S. Gairola:** Why do you want to make that correlation to protection in mice? What's the purpose of that?

**Dr D. Sesardic:** The purpose of the challenge potency in mice is to monitor the functional response to the bacteria strain.

**Dr S. Gairola:** But that model doesn't correlate with protection in humans.

**Dr D. Sesardic:** I have no direct experience to answer that.

**Dr S. Gairola:** We have examined the data and it doesn't. Why do you want to correlate with something that doesn't correlate with protection in humans?

**Dr D. Sesardic:** I believe that's something that you should have a lengthy discussion with my colleagues at WHO level. I don't think it's appropriate for me to discuss it here. I believe that several challenge assays have been developed for acellular pertussis vaccines, but whether they have relevance or not is beyond the discussion that I have. I believe protection models may not be as discriminate as serology but they should give you an overall picture of combination of different antigens that play a role in protecting against challenge with bacteria.

**Dr R. Winsnes:** Dr Dobbelaer, would you like to comment?

**Dr R. Dobbelaer:** The only comment I have is that this opinion comes, I guess, from a ranking experience which, if I remember correctly, more or less started on the upper level where the whole cell pertussis followed very closely by the five component. Then you had at the lower end, and this is about inter-nasal and respiratory challenge models and more or less the same ranking in humans. That's the data that I guess Dr Sesardic is referring to. Of course the serology model can distinguish between vaccine quantities. Whether it does differentiate quality, I don't know. That's where the opinion comes from.

**Dr S. Gairola:** What I think you are referring to is a published paper that the endless subject to a collaborative study precisely organised by NIBSC and the conclusion of our collaborative study was that that ranking didn't stand when we examined on a blinded basis.

**Dr R. Winsnes:** Dr R. Gupta?

**Dr R. Gupta:** I would just like to comment on this 3Rs paper and also the other paper. This is good progress. Manufacturers want to know if this approach has to be successful or practical, so it could be applied to further antigens. It seems that with 3R's results particularly these are encouraging results with the acellular pertussis antigens, hepatitis B and IPV. About the Hib conjugate vaccine, I would just like to make a comment that we should not go back. We had a meeting in Brussels in 1996/97 and based on the data and our own model with the guinea pig we developed a model for the Hib potency test in guinea pigs. This was a very good model for development purposes I would say, but not for the lot release. That was when we decided that animal model is not a good indicator for a lot release. That's what Dr Sesardic confirms from her data in this study. For IPV and Hib and the acellular pertussis potency components, I think the results are very encouraging.

**Dr R. Winsnes:** Anybody else who wants to comment on this topic?

**From the floor:** My question is to Dr A. Maes. Did you heat inactivate the sera? Or have you not investigated on heat-inactivated sera?

**Dr A. Maes:** No, I didn't.

**From the floor:** My second question. Why do you house non-immunised and immunised animals together?

**Dr A. Maes:** In fact, it is just variability. Putting guinea pigs in different cages increases the variability between the animals.

**From the floor:** But normally we do it.

**Dr A. Maes:** The guinea pig, which wasn't immunised was marked, so it was identified. I don't think it's a problem to house them in one cage.

**Dr R. Gupta:** Can I just mention something about the heat inactivation of sera? We have a bad experience with human sera and pertussis antigens. So don't do it if there is no need. It modifies the response.

**From the floor:** A colleague of mine made investigations on pertussis antigens and for one antigen she had to inactivate the sera and that is why I asked this question.

**Dr R. Winsnes:** Thank you. I think we shall close here.



**SESSION III:  
TRANSPOSITION INTO REGULATORY REQUIREMENTS**

**Summary of the meeting and definition of action plan**

Dr R. Dobbelaer, BSP Steering Committee

Open discussion with the audience. Participation of World Health Organization (WHO), US Food and Drug administration – Center for Biologics Evaluation (FDA-CBER), and European Vaccines Manufacturers (EVM) representatives

Slides are available on page 2 of this symposium-SessionIII.pdf  
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**Dr P. Castle:** Could I now ask you, Dr David Wood, what the WHO perspective is on this?

**Dr D. Wood:** Thank you. Basically you've asked the question, if the European Pharmacopoeia were to introduce the serological test and the one-dilution test, would this be compatible with the WHO position and the short answer to that is, yes it would. The WHO position is actually set out in a document that is included in your reference documents folder, supplied as background information to this meeting. The WHO position since 1990 has been that serological tests could be used and also a single dilution test could be used for diphtheria potency testing. The document you have in your binder gives clarification on the approach that WHO would recommend. In essence what we say is that once consistency of manufacture has been established then the serological testing of the diphtheria component and also a one-dilution test may be used for routine batch release. We give criteria for how you would show that you have established consistency of manufacture. We suggest that at least ten batches would need to be tested and shown to give consistent results in the full potency test in order to make the switch to the single dilution test and also to use serological assays.

As regards to serological assays, we suggest that the ELISA test may be used, provided that it has been validated against a functional assay. So in summary I think the WHO position is very much in line with the proposed position that you are suggesting for the European Pharmacopoeia. It may be useful for the group to know that we are in the process of undertaking a full revision of the WHO recommendations for DTP. So if there are any minor differences between the approaches that have been suggested in the Ph. Eur. and the WHO, then there is an opportunity to do some fine-tuning so that we can be as closely aligned as possible. The other interesting point that has been made is the inclusion of some guidelines on how the test would be performed, for inclusion in the monograph. Again that is something we would very much welcome. We too have what we call a manual of methods for performance of these tests and we would very much like to be able to include details in the form of an SOP or something like that, as to how the tests should be performed in the manual. Again there would be benefits of course to harmonise with what is going on at a European level.

As a final point from a WHO perspective, we would very much like to encourage the future investigations that we've heard of, in terms of test multiple antigens in the same sera and also

the development of the *in vitro* antigen assays. So I think from a WHO perspective we're very pleased to see these developments and would encourage these to be implemented.

**Dr P. Castle:** I think that you couldn't have said anything that would have pleased me more! You know in Group 15 when we are working on vaccine monographs we always struggle to maintain compatibility with WHO so I think in this case it shouldn't be too much of a struggle to do that. I think we are on the same wavelength as far as trying to draft some guidelines are concerned. I said to Dr A. Artiges (before the coffee break) that when I heard about the work from Aventis Pasteur about characterising of the antigen up stream, that this really is part of, I don't know whether it's a minor or a major, revolution. I suppose all revolutions start small and then get out of hand, but the Process Analytical Technology revolution which is going on in the pharmaceutical industry, is about understanding the process and getting information early, multi-variate data which tell you a lot about the process, it's not a question of acceptance or rejection, but it's process understanding technology. I know that when we had a WHO meeting in Bilthoven a few years ago there was a lot of discussion and encouragement to do this. It obviously takes time to build up the database for that. But I think also maybe we need to mention that as something that manufacturers should be looking into.

Can we now move over to Dr J. Arciniega and ask him to give some perspective on the FDA on these issues?

**Dr J. Arciniega:** Yes I would like to make some comments and pose some rhetorical questions, and then I will go to some specific questions that Dr P. Castle gave me by e-mail regarding my participation.

One of the first things that I believe is on the way of harmonisation is the use of words. I mean, I still don't know, by the context I have an idea, but what does OMCL stand for? I think it's very clear for you, but not for me. You don't need to answer the question right now. Eventually I will know.

**Dr J-M. Spieser:** We can – Official Medicines Control Laboratories.

**Dr J. Arciniega:** Thank you. This is just an example of the situation. Words sometimes divide us more than unite us. For instance, what do we mean when we ask for a potency test? What are we expecting a potency test to do? What is validation? I gave a talk some time ago and read something very nice somewhere, unfortunately I don't recall the author, but it said that validation, like beauty, is in the eye of the beholder. What validation means for you may not mean validation for me. I made a comment regarding the use of the word correlation between methods. I said that you can have very good correlation without having very good agreement between the two outcomes, because there is a factor, a multiplier there. So those are three terms I was concerned about during the conference.

I would also like to pose a couple of questions. How fast do we want to go and how far? What is the combination of these two things? I get a little anxious about the fact that we are probably caving into certain priorities versus other priorities. I mean what are we more concerned about? Are we interested in having assays that tell us what we want to know, provided that we have a good definition of what we want to know, or do we care more about the fewest animals we can use? I think we have to always balance these two, and that's a little complicated.

Also I want to mention that what you know as the NIH method or the US method is used in different versions in other countries in the world. Actually at the beginning of the year we had a meeting in Brazil with the participation of several Latin-American countries that have OMCLs able to do testing. The consensus was that not even in their dreams they can start doing the 3-dose challenge model for lot release of Diphtheria and Tetanus vaccines. Not even for licensing. It's beyond their possibilities to do that. We therefore have this situation in which WHO recommends a method that cannot, or is not, being used by a number of countries in America. Even Canada uses our procedure on a routine basis.

We have to do something about it. I mean in a meeting trying to clarify the WHO regulations, I insisted, and it was rather a heated discussion, that if you don't show or recognise that you have a problem, you will never be able to start solving it. So I asked that the method used in the US would be included as an alternative method, but eventually that was not approved. So the US method is mentioned in the amendment but not as an alternative method, nor there is recognition that the method is being used in several countries.

Now let me talk about the specifics of what is expected from the US perspective regarding the acceptance of a new test method. Many times we are asked to speak for the FDA. There are very few individuals who can do that and I'm not one of them. I have my opinion and my opinion probably has some weight on the FDA decision, but I'm not speaking for the FDA, I'm speaking for myself. The problem with many of the methods described yesterday and today is that there is no real incentive for the national authority to be proactive and change the current methods on basis different than harmonisation. We are recommending release of D and T-containing vaccines using a very basic design: we only need 12 guinea pigs to release for Diphtheria and Tetanus. So any modification that we do to the test is going to increase and not decrease the number of animals that we have to use. It's not an incentive in that regard. However, we're always open to manufacturers coming to us and asking for a change. What change are we willing to accept and how are we going to qualify if that change is acceptable? Dr Winsnes asked me to mention the sections in the regulations where this is described. I'm not going to read the whole thing, you can read it yourself, but I'm using the wording of a section of the USCFR, which is called '*Equivalent Methods and Processes Indices*', Title 21 in section 6- 10.9 and says

'modification of any particular test method... shall be permitted only under the following conditions: the applicant presents evidence in the form of a licence application or a supplement to the application submitted in accordance with... (different sections), demonstrating that the modification will provide assurances of the safety, purity, potency and effectiveness of the biological product equal to or greater than the assurances provided by the method (to be replaced).'

Then it sends you to '*Changes to an Approved Application*.' So this is specific for a product that is already licensed.

*Section 601.12 'Changes to the Approved Application' indicates that*

'a supplement shall be submitted for any change in the... controls that has a substantial potential to have an adverse effect on the identity, strength, quality, purity or potency of the product as they may relate to the safety or effectiveness of the product.'

Then it goes to mention specific examples of changes that have to be submitted in a license supplement – ‘changes in the qualitative or quantitative formulation or other specifications, as provided in the approved application or the regulations.’ We have licensed all the D and T-containing products that are currently used in the United States using the so-called NIH method. Changing that method is going to require a supplement considering the above wording. Basically you are going to change the way you express potency. I don’t know what level of validation you will require to do this. I cannot predict it. You may compare methods, but the most likely possibility is that you will have to show that clinical efficacy of several lots tested by the new potency tests to ensure that the product tested with the new method is of equivalent effectiveness than the lots that were released using the previous method. I think that’s all I have to say.

I thought about commenting on the guinea pig reference serum, but I think that I will stop here as there are other people who need to talk. Thank you.

**Dr P. Castle:** Thank you very much. I think that’s a very clear account, very useful. Can we move over to Australia, to Dr D. Meek, to see whether he can enlighten us about what would happen in Australia?

**Dr D. Meek:** Literally the basis of our system is the British Pharmacopoeia which for vaccines is essentially the same as the European Pharmacopoeia. Hence if the new test is incorporated into the Ph. Eur. then that would become an acceptable requirement for us. As Dr J. Arciniega said, if companies are looking at introducing a new test, then we would need to see data by way of what we call category 3 application for modification of their production process. We would need to see data that they are getting equivalent distinguishing between clinically valid and substandard batches by the old method and the new method.

**Dr P. Castle:** Yes thank you. Of course I haven’t asked European regulators because I guess they would give the same answer, they would want to see data and lots of paper. But I don’t know which European regulator I should ask, so if anybody wants to volunteer, then that would be interesting. Otherwise, Health Canada, can you give us some information?

**Dr N. Shakarchi:** We already, as you know, use the North American assay for our potency evaluation of tetanus and diphtheria. However, since 1999 we have started implementing the euthanasia programme to eliminate suffering in our animals and so we are using less animals than required by pairing testing and using the same animals for the immunology for tetanus and diphtheria. So we basically reduced the number of animals to about 12 again, however, we do the 3 dilutions for the diphtheria rather than a single dilution. We are using the equine anti-serum standard obtained by the FDA. So we did the 3Rs as much as possible and we reduced the number of animals tested per lot. We do a lot by lot testing for combination vaccines. Random testing for Diphtheria and Tetanus and we did reduce the time of test by euthanasia to 5 days. Reducing the suffering of animals normally at end points is the factor and we did extensive work on that presented by Dr J. Calver and he did a lot of work and myself – we were involved in that too in 1995/96 and 97. Currently we do that also for other assays. All of our animal assays use euthanasia to eliminate suffering.

**Dr P. Castle:** You in fact use euthanasia for moribond animals, is that right? You validate that against letting the test go to the end?

**Dr N. Shakarchi:** Yes the end point was observed in parallel with the same animals and we monitored the end point.

**Dr P. Castle:** Did Dr J. Calver speak about that at the Tetanus Meeting, Dr J. M. Spieser?

**Dr J-M Spieser:** Yes.

**Dr P. Castle:** So it will be in the papers here.

**Dr N. Shakarchi:** We did that for Botulinum evaluation as well. We mentioned the Diphtheria and Tetanus and Botulinum assay. However, we are in consultation with our counterpart in the US and the manufacturers in North America and we are collaborating with the Europeans. We did Phase 3 with you. However, we do require some collaboration between the manufacturers to agree on the issues in the current situation. I'll give the microphone to my colleague, Dr Sontakke.

**Dr S. Sontakke:** I believe the question is what is our current position regarding the test?

**Dr P. Castle:** Yes, if the manufacturers want to change, what would you require from them in way of data, in the way of doing any work to introduce the test for the vaccines that they provide to Canada?

**Dr S. Sontakke:** The current example I could give is of vaccines licensed in Canada now. The manufacturers have done their own testing and we did our own testing. So they can provide their data but we will do our own testing based on North American method. Just to make some more clarity regarding the clinical efficacy, I would like to ask a question to Dr C. von Hunolstein, what was the method used for measuring international units, so that it would be more clear whether the clinical efficacy was measured by North American method and which reference standard was used?

**Dr C. von Hunolstein:** You mean which assays were used for testing these sera? We accepted the Vero Cell assay, double antigen ELISA, DELPHIA and TOBI. The result obtained in each of these assays were standardised against the results obtained by testing the same sera by the Vero cell assay (see article in Vaccine 2000, 18: 3287-3296.)

**Dr D. Sesardic:** Conclusions made in human serological studies do not have relevance to this particular situation. I do, however, question one of the conclusions that have come from the serological responses regarding the use of TOBI and ELISA assays for tetanus potency testing. I question the need to continue calling the TOBI assay a functional surrogate method thus giving the wrong sense of illusion that TOBI is a functional method of any sort. I think that the Italian group have also confirmed that TOBI is just another immuno-assay measuring different population of antibodies.

**Dr J-M Spieser:** Dr Dobbelaer says that he made a mistake in his presentation – it was not meant to be TOBI, it was meant to be Vero.

**Dr R. Dobbelaer:** But it was meant functional and it has been deleted now. Just before you go on and just to maybe either add some confusion or to clarify as to your question about

international units, it was my impression that you also referred to the international units for vaccines. That's against the European and WHO calibrated standards. So these are vaccine international units which are different from the antitoxin units used in the clinical evaluation. Just to make that clear.

**Dr S. Sontakke:** I believe in your studies international units were obtained on the sera? Against equine antitoxin?

**Dr R. Dobbelaer:** Yes.

**Dr R. Gupta:** Can we talk briefly about that? One of the confusions is the use of the guinea pig versus the horse sera and the different correlation between methods. I mean it was obvious from the collaborative study in the US for potency of diphtheria toxoid using Vero cells. It was found once much more a fact that has been known for a long time that low avidity antibodies give different titers when tested at different doses of toxin. It was recently shown, if I remember correctly, by Usha Dular in Canada, that there was a correlation between Vero and and sero neutralisation titers in animals, but the titers were significantly different. Again I'm using correlation and I shouldn't, because probably these correlate, but the outcome was very different between 10 and 50 times differences between titers obtained by neutralisation in the animals and Vero cells. In the US potency test of diphtheria toxoid low avidity antibodies are generated at 4 weeks of immunization as compared to the high avidity equine antitoxin. Therefore this idea of using a low avidity antibodies guinea pig reference (calibrated by neutralization test in animals at high toxin dose against equine standard) was there in order to avoid that problem with differences in titers at high and low toxin doses, but I think the use of the guinea pig serum is problematic for other purposes. I'm not going to mention all the details, but from a logistic point of view this serum was obtained after a single immunisation, so it is a low avidity serum. One thing is, this reference if it becomes an international reference first of all the availability is going to be difficult because you cannot produce large quantities of this serum. Secondly the serum should be used for other purposes, at least the horse serum is used for other purposes. But this guinea pig serum does not have any other application, except for the US potency test. My contention is that the species of the animal and Dr Castle apparently confirmed that, is not relevant here for toxin neutralization test in Vero cells. What is relevant is the quality of the antibodies. On the whole you have high avidity serum and in humans you have high avidity serum and the assays correlate very well. We have done it, probably many Europeans have done it. Vero and animal neutralisation tests using human serum, they agree, many results are fairly good - one dilution factor above or below, if you use it for immune-globulins or for serum for clinical purposes. There is not such good agreement in the conditions I mentioned for the guinea pig sera. So I hope that clarifies a little the situation.

**Dr P. Castle:** But you know there is not a guinea pig reference serum in the potency model that we have been looking at. The reference preparation is a vaccine. There is a run control which is a guinea pig serum, but this control is going to be the same kind of preparation that you are assaying and those of guinea pig sera. So, I think that all those issues don't really concern the models we were talking about yesterday. Of course you would have difficulty preparing very large volumes of the guinea pig reference serum. Guinea pigs are much smaller than horses. To me that is a side issue.

**Dr R. Gupta:** It is a side issue, because we are not talking about harmonisation. If we would like to harmonise we would have to reconcile the fact that in the American system of potency test you express the response in terms of international units of antibodies produced and in this system (Ph. Eur.) you don't. So, if we are looking for something that is common we may need to start talking along those lines.

**Dr S. Sontakke:** Just to make more clear on the lot release programme, in Canada we will try to use European methods, EDQM methods and we will try to see if there is any correlation with our North American *in vitro* potency testing. In fact that has to be decided after two or three years of study and also it will be widely discussed internally before it may or may not be implemented into the routine practice.

**Dr P. Castle:** Thank you. That is a very interesting and clear statement. Do we have other regulatory authorities in the room who would like to make any statement on the same lines? No, I didn't identify any others in the list. No, so it's your turn, Gupta.

**Dr R. Gupta:** I would just like to make some comments on these horse equine tetanus/diphtheria anti-toxin. I think these were established in the early twenties and there was no correlation with human protection at that time. These were arbitrarily established and arbitrary units were given. Later on it was determined that the protective levels were .01 international units. I think doing the equine standards and titrating the guinea pig sera actually is against the principles of biological standardisation. What I want to say here is that for all the clinically serology that is done, ELISA is the accepted method for tetanus for clinical serological. There everybody calibrates their own human working standard against equine standards. You cannot use the equine standard in an ELISA for human serology. Using human serum calibrated against equine standard in clinical serology is more important than doing a guinea pig test using guinea pig sera calibrated against equine standard for licensing a vaccine. I think you have to make a bridge to correlate with the international units which are regularly established because the international units define the protective levels. I just want to caution that when you give international units it should be only given international units if you do a neutralisation test or a functional test. When you do an ELISA, TOBI or any other test you should not call it international units. When we publish we always call it ELISA international units because giving international units by ELISA I think is again wrong. You are giving the wrong signal. International unit means functional antibodies. I think correlating with all of these potency issues are separate issues.

**Dr P. Castle:** Yes. I still feel that as far as Europe is concerned, they are side issues. We needn't take issue with a statement that you can call it international units if it's guinea pig serum but you do toxin neutralisation. In practice that may be the case but then again it's not the same serum that you're comparing with.

**Dr R. Gupta:** Can I just add something? The problem with these is that some of these reagents end in the hands of people that don't understand many of the complexities here. My concern is that NIBSC send guinea pig material to which some units were assigned. Assuming that this is equivalent to the horse WHO reference to countries for release testing only by a specific method. We don't know that they are not equivalent in a sense – that is my concern - if this material label is only to be used for this particular purpose that is fine with me.

**Dr D. Sesardic:** I think it was quite clear that there was absolutely no actual requirement of using that particular guinea pig serum that we have made for the collaborative test. The reason why the same was used by everybody in collaborative study was to minimise variability and to give us more meaningful information in a collaborative study but there's no requirement to use the guinea pig serum thereof. I do in principle agree with what Dr R. Gupta says, that us calling it IU may not be really appropriate. We call it this purely for the sake of argument, if you know what I mean. I believe that Arnold Daas will be able to say statistically that there would not have been that much difference if we didn't use the relative values in serology in terms of information required for the potency calculation. In fact if we did not use the run control at all we would have got exactly the same conclusion. Guinea pig reference serum is not an absolute requirement.

**Dr P. Castle:** Dr R. Dobbelaer has passed me a question. It says, 'should it be possible to express EU serology in international units for US compliance purpose?' I understand that, that wouldn't be the case, Dr J. Arciniega, because your units are for 2 doses of vaccines, is that right?

**Dr J. Arciniega:** No. We have been discussing, in the context of WHO, on possible ways to achieve harmonisation. I didn't bring the slides with me, because that wasn't the purpose of this meeting, but I gave that presentation to a meeting in WHO headquarters and we asked, for instance, if there is any way to make this correlation. Specifically, how many International Units of toxoid induce 2 International Units of antitoxin in a guinea pig? I don't know. I mean if at least in theory 30 IU or less of D toxoid are always able to induce more than 2 IU of antitoxin per mL of serum, we are done. There was no more discussion about this. I don't know if that's the case. Plus it's not only theory, it's more than theory because the animals in the US are injected with either 0.5 or with 0.75ml of vaccines. Let's say for the sake of argument that they are immunized with 0.5 mL: if 30 IU of D toxoid are contained in 0.5 mL of vaccine and they induce always, or with reasonable frequency more than 2 units of antitoxin, I think the methods are pretty harmonised already. I haven't seen these data and it's difficult to obtain this information. It would be interesting to know, it would facilitate everybody's life.

**Dr P. Castle:** I said that I hadn't asked any European regulators, I wouldn't know who to ask. The European regulator sitting next to me is asking me questions apparently. I'm going to ask him to answer his own questions. In the EU are variations simplified when the method is published in the European Pharmacopoeia?

**Dr R. Dobbelaer:** I was reacting to the fact that one of the hurdles would be to have to introduce variations in the US or in whatever region in Europe. To my knowledge in Europe at least the variations are simplified by the fact that a method is published in the European Pharmacopoeia.

**Mrs A-M. Georges:** I would just like to make some comments on behalf of the European Vaccines Manufacturers Association. One of these remarks refers to what Dr R. Dobbelaer just said, to the way variations will have to be introduced. As Dr R. Dobbelaer said, when a manufacturer is modifying something in his quality control methods in order to comply with the European Pharmacopoeia and provided that it's done within 6 months after it is published, it's even not a variation anymore in the new law it's just a notification to the authority saying, "I'm a manufacturer, I now comply with the new rules in the Pharmacopoeia" and that's all.

But in this case I worry, and I suppose manufacturers in general, because the methods will not be compulsory and manufacturers will have to keep the two methods alive. I mean the challenge method and the new method. There is no way to force the manufacturers to submit the variations first of all because the alternative is still there, also because for stability testing and in case of manufacturing process the old method should be used, then there is no way to force them. My second remark would be: suppose that the manufacturer says, "I will comply with the pharmacopoeia, I will use the new method." I don't believe that the regulatory authorities would agree not to have all the data. I imagine that all the validation data and everything should be submitted.

**Dr P. Castle:** But then that contradicts what you said at the beginning. At the beginning you said if it's complying with the Pharmacopoeia and this strange 6-month rule, I don't where this came from – the title is very strange, maybe it came from our 6 months between publication and implementation? Oh my Goodness me. If for a vaccine stating that it complies with the Pharmacopoeia means it has notification then normally the authorities don't want data, but I can't really believe it.

**French speaker:** Me too. This is exactly what I mean. I can't believe that the authorities will not ask for details.

**Dr P. Castle:** Dr R. Winsnes, how would the Norwegian authorities see this?

**Dr R. Winsnes:** I think I'm biased. I'm too involved in this and would prefer somebody else to answer! However, I would accept the data required according to Ph. Eur. for the alternative method for potency testing of tetanus vaccines. Similar requirements were proposed for diphtheria component potency in my second presentation. I wonder if an incentive for the industry would be that if we did away with the lethal assays. Diphtheria intradermal challenge assay is very cumbersome and I know that several manufacturers and licensing authorities do not want to do it. They have difficulties with it and also with the paralytic challenge assay for tetanus component in vaccines. If you stop with paralysis you have to take out your animals of the cages, see how they walk, see if they have stiff limbs. You should also look at them three times a day. That will be more costly for you, but I think it's a shame that we have lethal assays, that the animals must die of tetanus and diphtheria. I think it is very sad to look at them, so may be deletion of the lethal challenge assays could be an incentive. The animal protection people are very strong these days, and they have made us start this project. When we discussed this problem for tetanus vaccines in Group 15, people from OMCLs said, "No don't take away the lethal assay now. You can take it away when you have found that serological methods can be used instead." So they did not want to go from lethal to paralytic challenge assay to validate that and then from paralytic challenge assay to serological assay. The same can be said for diphtheria toxoid-containing vaccines. So I don't think we should forget that now.

**Dr J. Arciniega:** Can I mention something briefly regarding these particular comments? Two issues: one is Latin America. There is only one country of the five that were represented in the meeting in February that was using the lethal US titration of antitoxin for diphtheria and, this country is moving towards an intradermal test, so there are three-R activities going on in Latin America. Also in the United States we are in the process of talking with manufacturers regarding the use of the VERO assay to titrate the serum of the animal immunised with the

vaccine. So that would further reduce the number of animals we use, from 12 to 10 for two antigens.

**Dr P. Castle:** I think one should recall that in the European Pharmacopoeia we have a general requirement for the use of humane endpoints. This is in the General Monograph on vaccines for human use. You have to use humane endpoints. That is another case of taking a horse to water. I have to say that we have found it politic ourselves not to drink because of the imminence of serological assays, we didn't want to eliminate the lethal endpoint because the database that people would have to be able to introduce serological assays.

Dr D. Wood you wanted to add something?

**Dr D. Wood:** Not on this particular issue. This is to go back to something that Dr J. Arciniega was raising earlier. It was just to pick up on the point that he was making about looking at the "correlation" between results in WHO European methods of potency testing and the US method. As you were saying, that would be one way of moving forward in this area. So I would actually throw a question out to the manufacturers present to say if there is such data available from the tests that they may have carried out using both systems on similar batches. Are those data available?

**Dr P. Castle:** Yes I think that's interesting – an appeal for data from the manufacturers if it's available. You're going to offer some data Dr R. Gupta?

**Dr R. Gupta:** I think this data was presented almost 10 years back or even earlier. It is in the Proceedings so I can at least find out. I was looking at Carolyn Hardegree's papers on this and she had presented a few cases. We are talking about hundreds of units here. It was the European test, it was presented by the Merieux Institute.

**Dr J. Arciniega:** Then the equivalent to more than 2 units in the US will probably be 100 units or more of toxoid, which is probably not what the manufacturers want. I would like to see something done with the bottom line which is 30 units. Even a mock vaccine, not a clinical lot because, as we were discussing before, consistency of manufacturing dictates that the vaccines being manufactured are way above that limit of 30. But as the limit is 30, let's work with the limit and see. Otherwise we are going to force the people to formulate vaccines higher. The best we can say today is that two units are induced by 100 units or more, and that's not consistent with the European regulation.

**Dr R. Gupta:** But you don't want to formulate vaccines at 30 units, to pass 30 units. It is a variable test. I think the data which was presented in earlier meetings, mainly by Merieux Institute.

**Dr P. Castle:** In the European Pharmacopoeia we require 30 international units as the lower limit. So you have to formulate at least at 60.

**Dr R. Gupta:** Yes that was my point. I presented that because they were marketing vaccines in the North American market and Europe also, the same lots. I agree that they were 100 units or more but they all passed the US test. I think there were a couple of instances where some vaccines from US passed the US test but could not pass the European test. These data were presented in our earlier meetings.

**Dr J-M. Spieser:** This discussion brings me to the fact to say we should clearly define what is the rule, because it is not like Dr J. Arciniega is saying. Everyone will understand different things and it will pile up and pile up. So the idea in my mind was that at a given time, there would be a guideline which would really try to define as much as possible what is meant, what data should be there and as it's here in the 2<sup>nd</sup> bullet, to implicate the licensing authority to make sure that there is not a second demand once you have fulfilled that one.

**Dr P. Castle:** Yes I would be all for that, I must say. Mrs A. M. Georges?

**Mrs A-M. Georges:** I still have another regulatory remark. I believe that the data to be submitted will be equivalent to what we submit for a type 2 variation for a major variation. My second question relates to what was said yesterday, that if a manufacturer validates the test in a large combined vaccine, this may be valid for the smaller one. Then if the test is validated for diphtheria in the hexavalent vaccine it's ok for the TP, for T, P, IPV and so on. But our problem in Europe with European Regulatory system, it's quite complicated. Some vaccines have been approved by the centralised procedure, other ones have been approved in mutual recognition and the oldest ones have been approved nationally. Then how could we be sure? Suppose that the manufacturer submits to the EMEA the validation on the hexavalent vaccines, that the authorities at the other level accept those data and will not require the test to be done again on the T, Pa, D, T, P, IPV and so on, because those are different vaccines. The large manufacturers, just for your information, have 15-18 vaccines containing diphtheria.

**Dr P. Castle:** Yes I can see your point and I think that the second bullet point there implicates the licensing authorities. That must be the next stage for us, indeed - an EMEA guideline, a CHMP guideline, an European Pharmacopoeia guideline? Not a European Pharmacopoeia guideline, no.

**Dr J-M Spieser:** You have the chance to have sitting next to you the chair of the vaccine expert group. So we can perhaps ask the vaccines expert group how pro active the vaccines expert can be in having such a guideline taken through the system?

**Dr P. Castle:** Can you write the question down to ask officially please? I have had good experience with animal welfare issues with the immunologicals working party of having this kind of guidelines. We take the horse to the water and the immunologicals working party pushes its head down and in the end it has to drink. So I think that there must be some scope for co-operation there with the licensing authorities and my neighbour from the vaccines expert group who will no doubt receive an official request to deal with this issue.

**Dr J-M. Spieser:** In addition there are so many common experts who hopefully will be able to contribute to defining the same rules depending on which hat they are wearing.

**Dr P. Castle:** I feel that the symposium is drawing to a close. I think the participants are probably exhausted. I hope that the subject matter is also. Are you adding bullet points, Dr R. Dobbelaer, to your summary?

**Dr R. Dobbelaer:** I just added our elements of discussion, because I think that's useful, on the different regions and positions of the different regions.

**Dr P. Castle:** I guess that everybody will receive Dr R. Dobbelaer's slides and within a few months they will receive a transcription of the discussion at this session and the other sessions.

**Dr K-H. Buchheit:** We have discussed a lot about the guinea pig run control, so we would like to know for the further work of the BSP, whether or not there is a general need to establish such an official material. This is the first question. For the second question I think it's a bit premature with regards to the standards and vaccines that you use for the assays. I think that we have to await the validation studies, whether or not we can have single standards or whether or not we need to use the homologous standards, which I would personally regret because this is the end of standardisation as far as the standards are concerned. With regards to the first question I would like to have an answer from the participants here, whether or not you would need a run control or whether you can have that from other sources.

**Dr P. Castle:** Maybe Dr. Sesardic is the one to advise us on that. I mean do you need a single run control for all the participants or can you make your own run control in-house? Is there any advantage to having a common run control? I know that for the Phase 1, Phase 2, Phase 3 there was an advantage, but in the long term?

**Dr D. Sesardic:** I mean it is clear you cannot use a European vaccine standard in the assay because it's only monovalent and if you want to measure potency to two components you need the standard that already has a mixture of two. Whether you actually need a common run control or not, will have to come from the users. I can only say from my experience that we have used a common vaccine standard that means we have used a single product, a DTaP product to calculate potency in other combinations.

**Dr P. Castle:** I must say that's a very interesting point and something I hadn't thought of, about the double reference preparation. The question is really about guinea pig serum, that you use as a run control. Do we need to have a Biological Reference preparations (BRP) of that or can people simply make their own run control in-house? Is there any advantage to having a BRP?

**Dr D. Sesardic:** As I said before it will help to have it, but it's not essential. It should be available for people who want to set the assay up. We have ca. 3000 units of it at the NIBSC and can provide it as well as negative control sera to monitor the assay. It is there to be used. People can buy it and implement their own, but make their own separate element for long term.

**Dr J-M. Spiesser:** I can imagine that the question there is, it's depending on the number of run controls you need in a year. You would if you are a big consumer make your own one. If you are a mid-sized or small user you would probably be happy to buy it. It looks to me that it would be worthwhile to have one. Is that in agreement?

**Dr P. Castle:** Do you have any other comments?

**Dr M-E. Behr-Gross:** A small comment in relation to this run control. We prepared one for the tetanus study and we produced quite a big batch and we used to prepare this reference serum the current tetanus vaccine BRP. I think that's maybe an interesting approach. Another approach could be to use vaccines which are currently marketed and to ask manufacturers to

prepare in their routine controls some pools, then to make a blend and use this material. This is also a question to the audience, what would be the best approach to prepare such reference because we have used this approach for other products in the field of veterinary vaccines, for example using blended materials from manufacturers. This has a certain added value and maybe a multi component reference could also be used in the tests we might be able to validate in the future for other components.

**Dr P. Castle:** I think those are issues which will be for the Steering Committee of the Biological Standardisation Programme.

Does anybody else want to raise any issues before we close the symposium?

**Dr R. Winsnes:** After what we have heard now I thought that you probably would say, "Should we continue with collaborative studies to look at the pertussis antigens to see if we could measure them also in the same animals?" Or is it only I who thinks so?

*Several participants expressed that they had participated in the past in pertussis acellular ELISA assays with NIBSC and the EDQM and are ready for further collaboration in the future.*

**Dr P. Castle:** I will hand over now to Dr Spieser to close the symposium.

**Dr J-M. Spieser:** Thank you very much. I would wish first of all to thank you all for having contributed so actively to this meeting. We thought it was timely because it was just at the closure of these studies which we have run. First of all thank you to all those who agreed to be speakers, to be moderators. Thank you to all the participants who actively contributed to this symposium. As it was said all the presentations will be on the web site shortly. We will do our utmost to have as rapidly as possible the transcriptions. There are some meetings in the near future where it would be helpful. I think of the first one in a months time in WHO, to make sure that we continue to build on the same issue.

Thank you also to all the industry participants who have donated material for this study and to have participated. Thank you to all the OMCLs who have participated and last but not least thank you to the two project leaders and my two colleagues, Mr A. Daas and Dr M. E. Behr-Gross. They have altogether, often being really in the target and have passed sometimes overtime over the weekends to make all the reports and study available to you to have this interesting debate.

So, have a nice visit of Budapest for those who are staying and have a nice, safe journey back. Thank you very much.



## BIOGRAPHICAL NOTES

**Dr Juan L. Arciniega, DSc**, is a Microbiologist at the Center for Biologics Evaluation and Research of the US Food and Drug Administration. He received his doctoral degree in Clinical Biology from the National School of Biological Sciences in Mexico City in 1987. Before joining the Laboratory of Pertussis at CBER in 1989 as a Visiting Fellow, he worked for nine years in different capacities at the Mexican National Public Health Laboratory, most recently as Underdirector for Biological Control, with responsibility for sanitary testing of food and biologics. He was also a founding member of the Permanent Commission of the Mexican Pharmacopoeia (Biologics, and Bioassay and Statistics Committees. His research and regulatory activities have focused on pertussis, diphtheria and anthrax vaccines. Currently Dr J. Arciniega is a member of the Laboratory of Methods Development and Quality Control, Division of Bacterial, Parasitic, and Allergenic Products, where he collaborates with other laboratories in the division on the development of quality control methods for bacterial vaccines. Additionally, he actively participates in regulatory activities, such as vaccine licensing, batch release, and facility inspection (product expertise). He has published several papers in his field and cooperated with the World Health Organization and the Pan American Health Organization as a Temporary Advisor and as mentor of young Latin American vaccine regulators.

**Dr Agnès Artiges** graduated in pharmacy from the University of Bordeaux (France) and has a PhD in the same subject, as well as a degree and a PhD in law, the latter from the University of Paris, France. In her postgraduate law degree she specialised in European Institutions.

She was Assistant and Assistant Instructor in the Toxicology Laboratory of the Faculty of Pharmacy of Bordeaux before joining the French Ministry of Health in 1971. During her career with the Ministry, she has held the posts of Head of the French Pharmacopoeia, Head of the Registration Authority for Medicinal Products for Human Use and Head of the Sub-directorate of Scientific and Technical Affairs.

In addition, she was Chairman of the European Pharmacopoeia Commission from November 1989 to November 1992 and a member of the former Quality Working Party of the Committee for Proprietary Medicinal Products (CPMP) of the EC and was Chairman of this Working Party from December 1991 to March 1993.

Dr A. Artiges left the French Ministry of Health in April 1993 to take up the post of Director of the European Directorate for the Quality of Medicines (European Pharmacopoeia and European Network of Official Medicines Control Laboratories/OMCL) - Council of Europe.

**Dr Marie-Emmanuelle Behr-Gross** earned a pharmacy degree at the Louis Pasteur University of Strasbourg (F). After a spell in pharmaceutical practice, she worked in research on immuno-pharmacology. After completion of her PhD she became a lecturer at the Department of Pharmaceutical Sciences of the Louis Pasteur University and also gained some experience in product development. She is currently a scientific officer at the European Directorate for the Quality of Medicines where she is in charge of co-ordinating projects belonging to the Biological Standardisation Research Programme co-sponsored by the Council of Europe and the European Community.

**Dr Florence Brunel-Veilleux** obtained his degree in biochemical engineer from “The Institut National des Sciences Appliquées” in Lyon (France). She received her PhD in Immunology in 1999 from the Claude Bernard University, Lyon (France). In 1999, she was appointed by Aventis Pasteur as Head of QC Immunology/ *in vivo* Testings Laboratory. She is currently QC manager in charge of technical support in the QC Development department.

**Dr Karl-Heinz Buchheit** obtained his degree in pharmacy from the Johann-Wolfgang-Goethe University in Frankfurt/M. (Germany) and his Ph.D. in pharmacology in 1984 from the same university. He joined Novartis (Sandoz at that time) in Basel, Switzerland in 1984 where he stayed until 1999 as research scientist and group leader in pharmacology in various fields (serotonin receptors, potassium channels, immunosuppression) and in drug development. Since December 1999, he is Deputy Head of Division IV at the EDQM (Council of Europe, Strasbourg, France).

**Mr Peter Castle** graduated in biochemistry from Cambridge University, England in 1968. He worked on drug metabolism and determination of drugs in body fluids at the Pharmaceutical Society of Great Britain for three years before joining the animal health division of Smith Kline & French, UK. Since 1974 he has worked in the Technical Secretariat of the European Pharmacopoeia, now a division of the European Department for the Quality of Medicines (Council of Europe, Strasbourg). He is Secretary to the European Pharmacopoeia Commission and head of the division dealing with development of monographs and general chapters. Co-ordinates work on international harmonisation with the Japanese Pharmacopoeia and the United States Pharmacopoeia (Pharmacopoeial Discussion Group – PDG) and within VICH.

**Mr Arnold Daas** – obtained his master degree in mathematics (statistics and operations research) in 1993 from the University of Nijmegen in the Netherlands. He has worked as apprentice at the Medical Statistical Department of the same university, and as assistant at the Biometrical Department of the Erasmus University in Rotterdam. Since 1995 he works as Statistician/Biometrician at the European Directorate for the Quality of Medicines – Council of Europe, where he is responsible for the design and analysis of data from collaborative studies, mainly in the context of the Biological Standardisation Programme.

**Dr Roland Dobbelaer** is a biochemist by training and currently head of the Section for Biological Standardisation of the Scientific Institute of Public Health in Brussels, where he is responsible for the National Control Authority Batch Release of vaccines and plasma derivatives operating in the EU Network of Official Medicines Control Laboratories. He also advises the Belgian Medicines Board and, as a member of the CPMP Biotechnology Working Party, also the European Authorities in matters of licensing and regulation of biologicals. He is currently chairing the CHMP Vaccine Expert Group, the Biological Standardisation Steering Committee of the European Directorate for the Quality of Medicines (EDQM) and the European Pharmacopoeia Expert Group N° 15 on vaccines. He is also a member of the WHO Expert Committee on Biological Standardisation (ECBS).

**Dr Michel Duchêne** obtained his degree in Agronomy Sciences from the University of Gembloux in Belgium. He worked in the field of food microbiology and bio-technology at the same university and obtained his PhD in 1982. He joined the Company GlaxoSmithKline Biologicals in 1982 as Bacterial Vaccine Production and Development Manager. From 1984 till 1989, he was Vaccine Production Associate Director.

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He was appointed Director, Quality Control in 1989, Technical Affairs Director in 1998 and has been appointed Product Life Cycle Management Director in 2004.

**Dr Dominique Garcia**, graduated in 1985 from Lyon I University and received his PhD in 1993 from Faculty of Pharmacy, Lyon I University, France. She joined, from 1993 to 1996 as post-doctoral fellow, the National Institute of Medical Research (INSERM) immunology laboratories. In 1997 she was integrated in the French Agency for the safety of health Product (Afssaps) in the Laboratories and Control Department where since 2002, she has been responsible for the Bacterial vaccines control Unit. She manages a team in charge of control analysis and technical developments for the lot release of bacterial vaccines as well as animal purified immunoglobulins, and in charge of expertise for the pharmaceutical part of vaccines licensing files.

She acts as WHO expert for National authorities assessment and manufacturer qualification.

**Dr Alexandrine Maes** - 1993-1998: Pharmaceutical Sciences, University of Ghent, Faculty of Pharmaceutical Sciences. 2002-2004: Master in Laboratory Animal Science, University of Ghent, Faculty of Veterinary Medicine.

From Nov 1998- March 1999 Dr A. Maes was Laboratory Manager at Zeneca Responsible of the Quality Control Laboratory. From April 99- Dec 2003 she worked at the Scientific Institute of Public Health, Belgium, Section Biological Standardisation, Scientific Collaborator, Responsible for the TSE risk evaluation of medicinal product for human and veterinary use. From 2000-2003-TSE expert and member of Technical Advisory Board, EDQM, Certification Unit.

As from Jan 2004 Dr A. Maes works at the Scientific Institute of Public Health, Belgium, Section Biological Standardisation as Scientific Collaborator and is Responsible for the batch release of non-living viral vaccines.

**Dr Dorothea (Thea) Sesardic**, BSc (Hons); PhD, obtained a degree in Biological Sciences in 1975 and a PhD in Microbial Biochemistry in 1980, from University of London (UK). She was a Research Assistant at MRC TB Unit at the Royal Postgraduate Medical School and from 1982 to 1990 Senior Postdoctoral Scientist in Department of Clinical Pharmacology, RPMS, Imperial College, where she studied *in vitro* models for drug metabolising enzymes. Dr D. Sesardic joined the Division of Bacteriology at NIBSC in 1991 as a Senior Scientist and was promoted to the Principal Scientist post from 1994. Since 2003 she is a member of senior NIBSC staff and oversees study testing of biological product derived from bacterial toxins (toxoid vaccines, therapeutic toxins and antitoxins).

For the past 15 years Dr D. Sesardic has provided recommendations regarding product safety and efficacy for batch release, at pre-clinical stages, for product stability, extensions of shelf life as well as post marketing surveillance. Dr D. Sesardic is an expert adviser to MHRA, UK DoH, WHO and EDQM, representing UK at EDQM Ph. Eur. Group of Experts No 15 for vaccines and sera from 2001 and advising BPC as a member of panel of experts on Immunological products, from 2003. She is also member of the Botulinum toxin drafting Group. She was responsible for co-ordinating many successful collaborative studies that led to establishment of WHO International and Ph. Eur. standards and alternative assays methods. Her current research interests include development of new assays for testing of existing and future therapeutic products derived from bacterial toxins.

**Mr Jean-Marc Spieser** studied Pharmacy at the University of Strasbourg and obtained his Masters Degree (postgraduate) in Applied Industrial Pharmaceutics at the University of Montpellier in 1973.

After different positions in Research and Pharmaceutical Industry he joined the Technical Secretariat of the European Pharmacopoeia Commission at the Council of Europe in Strasbourg. Jean-Marc Spieser is currently Head of Division IV at the EDQM, an independent division which manages the activities of the Biological Standardisation Programme aimed at:

- developing and validating new methodologies and particularly those *in-vitro* methods which are alternative methodologies to *in-vivo* animal bio-assays and
- establishing the European working standards and reference materials for biologicals (hormones, vaccines and blood derivatives).

and the OMCL Network including for the time being about 85 participating Official Control Laboratories all over Europe involved in both the human and the veterinary field In 1994, he initiated the inter communication between Official Medicines Control Laboratories (OMCL) within Europe by developing a real European Network governed by general common policies and operational guidelines especially in the areas of :

- Quality Assurance
- market surveillance, for pharmaceuticals commercialised in Europe through both systems (centralised and decentralised) and,
- batch release activities by official control Authorities for biologicals .

**Dr Christina von Hunolstein** obtained his degree in Biological Sciences from the University of Rome (La Sapienza) in Italy. She obtained her PhD in Microbiology from the same University in 1985. In 1985 she was appointed as scientist at the Laboratory of Bacteriology and Medical Mycology of the Istituto Superiore di Sanità and in 1990 as senior researcher in the same Laboratory.

She is currently head of the Unit of Bacterial Vaccines of the Departement of Infectious, Parasitic and Immunomediated Diseases of the Istituto Superiore di Sanità. She is involved in control (batch release) of bacterial vaccines.

Her recent main research interest focused on the field of diphtheria (microbiological aspects and seroepidemiology).

**Dr Randi Winsnes**, M.Sc.Pharm, obtained her Drphilos. degree in immunology and microbiology at the Medical Faculty, University of Oslo, Norway, in 1981. She studied for her PhD when working as a scientist at the Bacterial Toxoid Unit at the Vaccine Department of the Norwegian Public Health Institute, where she established and directed for some years the Immunoglobulin Unit and thereafter the Unit for Quality Control of Vaccines and Immunoglobulins. She was declared professor competent in microbiology in 1987. While working at the Public Health Institute she acted as a supervisor for pharmacy students taking a master degree.

In 1988 she was appointed head of the Pharmaceutical Department at the Norwegian Medicines Control Authority, now called Norwegian Medicines Agency. In 1992 she was created a Candidate in Health Administration. From 1995 she has been responsible for laboratory control and assessment of vaccines for human use at the Norwegian Medicines Agency, where she is the head of the Vaccine Section. The Vaccine section is responsible both for assessment of quality, non-clinical and clinical safety and efficacy and analytical controls.

She has been a member of the Nordic Standardisation Group for Medicinal Products, chair of the Norwegian Pharmacopoeia Commission, chair of the Ph. Eur. Expert Group 15 – sera and

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vaccines- for 12 years, where she presently is a member. She is presently chair of the Norwegian delegation to the Ph. Eur. Commission. Furthermore she is a Norwegian representative in the CHMP/Vaccine Expert Group and the CHMP/Biotechnology Working Party at European Medicines Agency.

**Dr David John Wood** - B.Sc (Hons) Microbiology, University of Leeds, UK (1974-1977) - PhD, (Thesis – Virus infections in children with leukaemia) University of Manchester, UK (1979-1982)

Employment history : 1977–1979 Technician, North Manchester Regional Virus Laboratory, Manchester, UK. 1982–1988 Scientist, North Manchester Regional Virus Laboratory, Manchester, UK. 1988–2001: Senior Scientist, National Institute for Biological Standards and Control, Potters Bar, UK. 2001–2003: Scientist, Quality Assurance and Safety of Biologicals, Immunisations, Vaccines and Biologicals, World Health Organisation, Geneva, Switzerland. From 2003–to present: Co-ordinator, Quality Assurance and Safety of Biologicals, Immunisations, Vaccines and Biologicals, World Health Organisation, Geneva, Switzerland.



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