Alternative tests for DT containing vaccines – an overview

Christina von Hunolstein

ISTITUTO SUPERIORE DI SANITA' – National Centre for the Control and Evaluation of Medicines, Rome - Italy

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Outline of the presentation

- Introduction
- Potency assay for D &T
  - Challenge potency assay
  - Serology assay
  - Single dilution assay
- Specific toxicity test for D & T
- Conclusions

Introduction

WHO has implemented the 3Rs principles by their adoption in several guidelines outlining the quality control of vaccines;

The WHO guideline on independent lot release encourage the NCLs:
- to apply the 3Rs principles to minimize the use of animals
- to pursue mutual recognition of animal testing performed by the exporting country's NCL

Potency test of vaccines

- The purpose of the potency test is to assess in a suitable animal model the capacity of the product being tested to induce a protective response analogous to that of the vaccine shown to be efficacious in humans.

- The potency test consists of two stages:
  - during the first stage, a protective response is induced in mice or guinea-pigs (*IMMUNIZATION)*
  - during the second stage, the protective response is measured by direct or indirect methods

Diphtheria potency methods

- Challenge
  - lethal
  - Intradermal
- Serology
  - Guinea pig
  - Mouse

Reference: **WHO IVB11.11 2014 - Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines - (Chapter II.1)**

Diphtheria potency assay in guinea pigs (gp) by challenge methods

Intradermal challenge

- Injection: sc. different dilutions (4) of vaccine and a Reference Preparation; each dilution is allocated to a group of gp (multi dilutions)
- Challenge: 28 days after vaccination, shave both flanks of gp and inject 0.2 ml of each of the 6 toxin dilutions id.(in Lf) into 6 separate sites on each of the vaccinated gp in such a way to minimize interference between adjacent sites
- Reading of results: 48 h after challenge record the erythema. The reaction is positive if the erythema Ø is ≥ 5 mm.
- Calculation of the potency of the test vaccine relative to the potency of the Reference Preparation
- Assay validity: all details in the manual

Lethal challenge

- Injection: sc. different dilutions (4) of vaccine and a Reference Preparation; each dilution is allocated to a group of gp (multi dilutions)
- Challenge: 28 days after vaccination, inject 1 ml of ~100 LD_{50} toxin sc. in the vaccinated gp
- Reading of results: 4 or 5 days (*depends on the country*) count the n. of surviving gp *or use the humane end points*
- Calculation of the potency of the test vaccine relative to the potency of the Reference Preparation Reference Preparation
- Assay validity: all details in the manual

Diphtheria potency assay in guinea pigs (gp) by challenge methods, *cont'*

Humane end points used for determining the toxic effects of diphtheria toxin following **subcutaneous challenge** are as follows:

Stage 1: light pink skin colour at the injection site

Stage 2: dark purple/pink colour at the injection site and rough coat

Stage 3: black colour and tissue hardening at the injection site and rough coat

*Examples of additional parameters that could be monitored in guinea pigs during the **lethal challenge** assay method*

Degree of severity	Coat and skin	Body condition	Behaviour	Belly skin
Normal	Normal	Normal	Bright/Alert	Normal
Mild	Ungroomed	Mild muscle atrophy (MA)	Slow/Response	Light pink
Moderate	Piloerected	Moderate MA	Lethargic	Dark pink
Severe	Hair loss	Marked MA	Moribund	Black and pink

Tetanus potency methods

Lethal

Challenge test in guinea pigs or mice

Paralysis

Challenge test in guinea pigs or mice

Serology

Determination of antibodies in guinea pigs or mice

Reference: **WHO IVB11.11 2014 - Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines - (Chapter III.1)**

Tetanus potency assay by challenge in mice or gp – end point paralysis

- Injection: sc. different dilutions (4) of vaccine and a Reference Preparation; each dilution is allocated to a group of gp/mice (*multi dilutions*)
- Challenge, 28 days after vaccination, inject the vaccinated animals with toxin
  - in mice, sc. 0.5 mL of 50 PD_{50} toxin sc. Over the lumbar region of the spine to produce a distinctive hind limb paralysis of the vaccinated mouse
  - in gp, sc 1.0 mL of 50 PD_{50} toxin mid ventrally directly behind the sternum with the needle pointing towards the forelimb, so that toxin will produce paralysis in the forelimb of the guinea pig
- Reading of results: examine the animals for 96 h, in particular 3 times on the first 3 days and twice on the last day to record the tetanus grade and cull animals when T3 grade is reached (see tables, one specific for gp and on for mice)
- Calculation of the potency of the test vaccine relative to the potency of the Reference Preparation
- Assay validity: all details in the manual

Tetanus grade sign

In guinea pigs

T1: slight stiffness of one forelimb, but difficult to observe;

T2: paresis of one forelimb which can still function;

T3: paralysis of one forelimb. The animal moves reluctantly, the body is often slightly banana-shaped owing to scoliosis;

T4: the forelimb is completely stiff and the toes are immovable. The muscular contraction of the forelimb is very pronounced and usually scoliosis is observed;

T5: tetanus seizures, continuous tonic spasm of muscles;

D: death.

In mice

T1: slight stiffness of toxin-injected hind leg, only observed when the mouse is lifted by the tail;

T2: paresis of the toxin-injected hind leg, which still can function for walking;

T3: paralysis of the toxin-injected hind leg, which does not function for walking;

T4: the toxin-injected hind leg is completely stiff with immovable toes;

T5: tetanus seizures, continuous tonic spasm of muscles;

D: death.

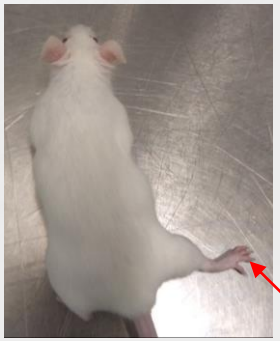
With experience T2 can also be used as an end point

Tetanus grade: 0

Tetanus grade: T1

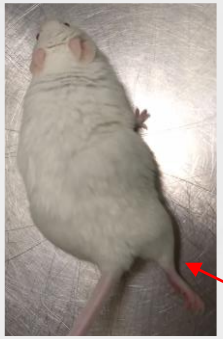
Slight stiffness of toxin-injected hind leg, only observed when the mouse is lifted by the tail.

Tetanus grade: T2



Paresis of the toxin-injected hind leg, which still can function for walking.

Tetanus grade: T3



Paralysis of the toxin-injected hind leg, which does not function for walking.

Potency assay for D or T

by serology

to reduce the amount of distress

imposed by the experiment to the

animals (Refinement)

Potency for D or T by Serology

In vivo

Day o

s.c. immunization with Test vaccine and Reference preparation

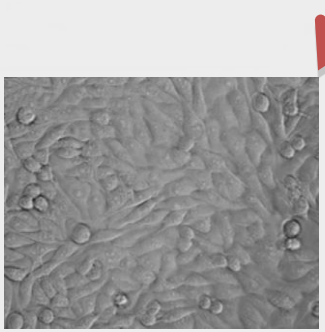
5-6 weeks

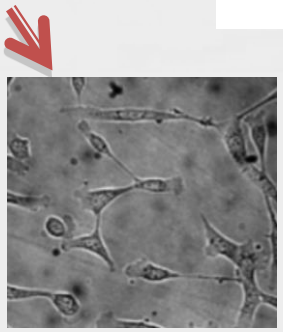
Bleeding of each gp under general anesthesia

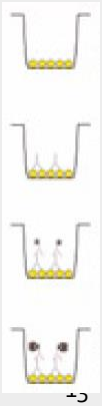
Collection of individual sera

In *vitro* titration of immune sera

By VERO cell assay for diphtheria







ELISA / TOBI

Potency for D and T by Serology using the same animals

In vivo

Day 0

s.c. immunization with
Test vaccine or
Reference preparation

or

5-6 weeks

Bleeding of each gp under general anesthesia

Collection of individual sera

In vitro **titration of immune sera for D & T by ELISA or multiplex Immunoassays**

Same animals can be used for the concomitant potency testing of D & T (aP, wP,) if the dilution range gives an appropriate dose-response for all vaccine components and no deviation from parallelism

Serology for D &T in one set of animals

Kadaml, Patel K, *et al.* from Serum Institute of India

Development and validation of a magnetic bead pentaplex immunoassay for simultaneous quantification of murine serum IgG to acellular pertussis, diphtheria and tetanus antigens used in combination vaccines.
Methods 2019, 158:33-43.

Nice example on how immunogenicity of a combined vaccine can be assessed using one set of animals (potency by serology).

All individual antigens were covalently linked to magnetic beads using Multiple Analyte Profiling Technology - xMAP®; Luminex Corp., Austin, TX which is a flow cytometry-based system based on the use of distinct fluorescent microspheres as carrier of different antigens .

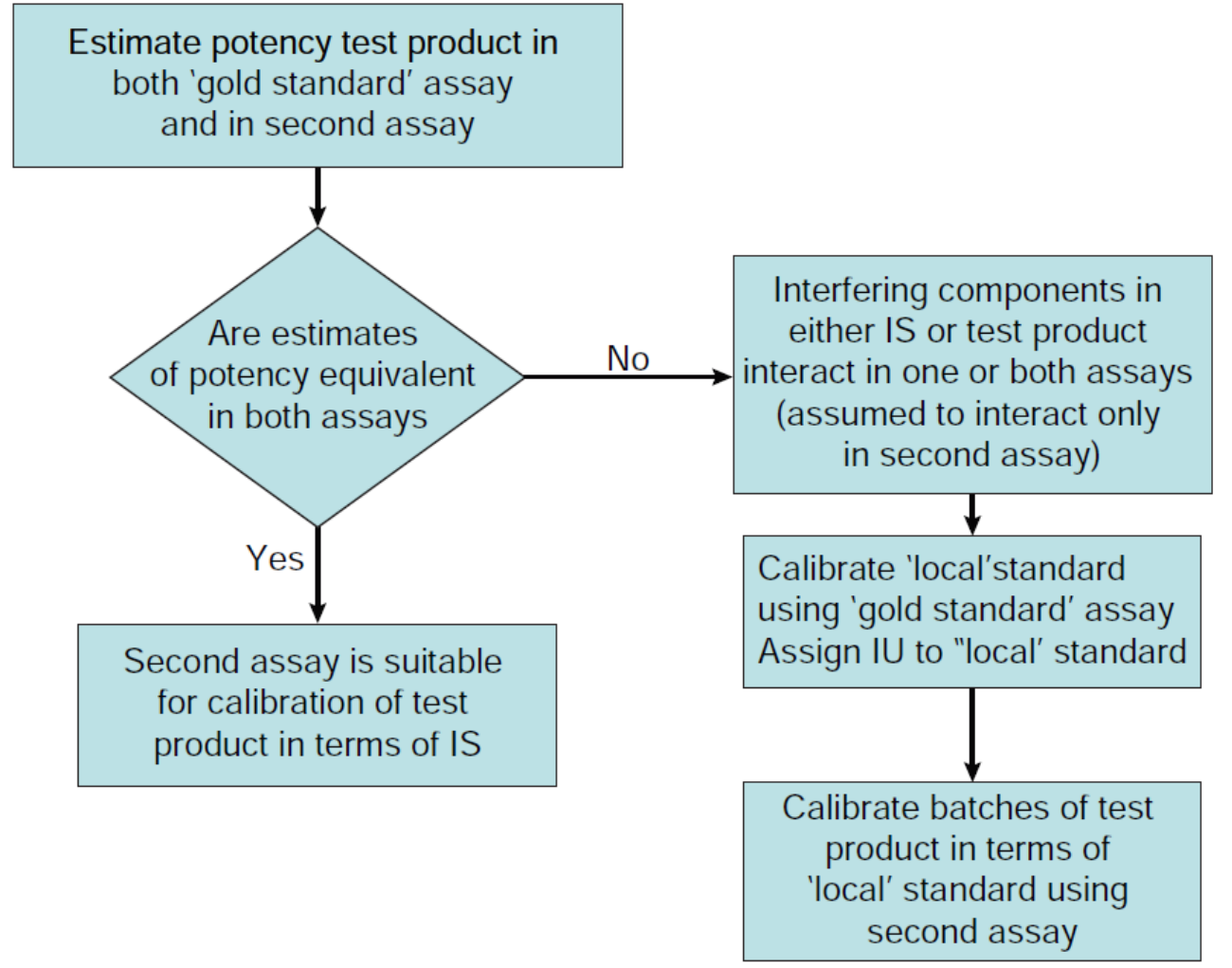
Transferability of IU determined in challenge assay to serology assay

- Activity (expressed in IU) of D & T International Standard (IS) is determined by challenge assay and not by serology assay;

- The D or T IS are toxoids adsorbed to $Al(OH)_3$, while the test vaccines are usually combined with several other antigens:
  wP, aP (wP) , HepB, IPV, Hib

Therefore, the alternative assay may show different specificities for D & T and assay validity criteria might not be met (regression of the dose – response, linearity, parallelism between the response of the test vaccine and the IS)

Assessment of the suitability of the serology assay for calibration of test vaccines and transferability of the IU when the serology assay (second assay) reacts to interfering components (WHO /IBV/11.11, V.6.3)



- Thus, a vaccine of similar composition of the test vaccine and calibrated against the IS should be included in the serology assay as in-house reference vaccine.

- A minimum of 5 and up to 10 individual serology potency tests should be used in assigning the value to the in-house reference vaccine.

- Data monitoring should be performed with the in-house reference vaccine by monitoring the geometric mean titre at each immunising dose, or by calculating the ED50 of the dose-response.

- Stability of the in-house reference vaccine must be established and the data obtained must be used in support of establishing the shelf-life and any replacement strategy.

Validation of an alternative potency methods

CHMP adopted on the November 9, 2017, *Guidance for individual laboratories for transfer of quality control methods validated in collaborative trials with a view to implementing 3Rs* (EMA/CHMP/CVMP/3Rs/94436/2014)

"In order to limit the use of animals and to avoid duplication of work, labs are encouraged, wherever possible, to maximise the use of data and information available from other sources in a rationalised strategy.

Supporting data can come from a number of sources, including accumulation of product data, data published from individual laboratories, and published study reports from collaborative trials.

A lab's own data from participation in a given collaborative study can also be used to support final product specific validation for regulatory acceptance"

Case	Scenario	Action
1	The laboratory participated in the collaborative study and intends to test a product that was included in that study.	No additional method validation is normally needed provided the method procedure is aligned with the method used in the collaborative study and the results from the laboratory were satisfactory. Supporting documentation demonstrating the transfer should be provided. The laboratory's data from the collaborative study may be used as part of the supporting documentation.
2	The laboratory participated in the collaborative study and intends to test a product included in that study but one or more changes have been introduced to the test protocol compared to the one used in the collaborative study.	
3.1	The laboratory participated in the collaborative study and intends to test an active substance in a product related to one that was included in that study (for example a product using the same manufacturing process that may contain fewer or additional antigens, a different adjuvant or excipients).	
3.2	The laboratory participated in the collaborative study and intends to test a related active substance in a product from a different manufacturer or manufacturing process, or newly developed product.	
4	The laboratory did not participate in the study and intends to test a product that was included in the study.	
5	The laboratory did not participate in the collaborative study and intends to test a product that was not included in the study.	

Serology implementation by NCLS

The same scenario of the manufacturer applies

Minimisation of animal use may be achieved by using the same animal model, protocol standard and specifications as the Manufacturers.

Potency for D or T

by single dilution assay

to reduce the number of animal

(Reduction)

Single dilution assay - 1

In this procedure (Reduction), one group of animals is treated with a single dilution of test vaccine and a comparable group is treated with a single dilution of the reference vaccine.

This procedure does not permit a check of assay validity by testing linearity and parallelism of dose-response lines and does not provide an estimate of potency.

Single dilution assay shows only that a vaccine meets a defined minimal (or maximal) specification.

Thus, this procedure cannot be applied if an actual estimate of potency is required or if both upper and lower limits are required for the potency of the vaccine.

Single dilution assay- 2

The simplified single dilution method can be applied only when the following conditions have been satisfied with the multiple dilution assay:

- the potency of the test vaccine consistently and significantly exceeds the minimum requirements (lower 95% limit >40 IU/human dose).
- a significant regression has been demonstrated for the test vaccine over time.
- parallelism between test and reference vaccine has been demonstrated over time.

It is recommended that data from a series of 10 to 20 recent and consecutive multiple-dilution assays should be available for study and confirmation of the above conditions.

Different products will require separate evidence that these conditions are met. Following the introduction of changes in the vaccine production process (e.g. purification, adjuvant, formulation) or in the testing method, evidence that the conditions are met must be provided.

Single dilution assay- 3
Selection of appropriate dilutions

- For the reference vaccine, historical data are used to select a dilution containing a number of IU known to elicit an immune response in the lower part of the dose – response curve. For a quantal response, about 10-20% protection is considered acceptable.

- For the vaccine under test, all test products are assumed to contain the minimum required potency (e.g. 30 IU per single human dose of diphtheria vaccine). Based on this assumption, a dilution of the test vaccine is made which hypothetically contains the same number of IU as the reference vaccine.

Single dilution assay - 4

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 serological assays, suitable indicators to monitor consistency are:

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

Summary of 3Rs application in the quality control of D & T vaccines

Test	Refinement	Reduction	Replacement
Diphtheria potency	- Intradermal challenge - Serology	Single dilution assay	--
Tetanus potency	- Paralysis (humane end-point) - Serology	Single dilution assay	---
Diphtheria &Tetanus potency in combined vaccines	Serology	Serology for DT in one set of animals	----

Consistency approach in lot release testing of established vaccines

Current approach	Consistency approach
Each lot is unique	Each lot is one of a series
Emphasis on Quality Control on final lot	Emphasis on every step of production (from the seed lot up to the final product)
Potency read out is ≥ IU/ dose	Read out : no deviation from consistency
Use of International Reference Preparations	Benchmarking to clinical or historical lot

Absence of tetanus/diphtheria toxin and reversion to toxicity, specific toxicity in guinea pigs

Specific toxicity – tetanus

	WHO	Ph.Eur
Absence of toxin	Toxoid bulk	Toxoid bulk
Reversion to toxicity	Toxoid bulk	Toxoid bulk
Specific toxicity	Vaccine Final lot	Can be omitted on the final vaccine lot as part of routine lot release subject to process validation

Absence and specific toxicity for Tetanus vaccines

	Absence of tetanus toxin	Tetanus Specific toxicity
No. of guinea pigs	5	5
Weight of guinea pigs	250-350 gr	250-350 gr
Injection site	sc	sc
Stage of production	Toxoid bulk	Final lot
Quantity of tetanus toxoid injected	At least 500 Lf	5 shd stated on the label The content in Lf varies if the vaccine is intended for pediatric or adult use
Observation period for the guinea pigs	21 days	21 days
Test acceptance criteria	The bulk purified toxoid complies with the test if during the 21 days following the injection no animal shows signs of, or dies from tetanus. If more than 1 animal dies from non-specific causes, repeat the test; if more than 1 animal dies in the second test, the toxoid does not comply with the test.	If within 21 days of the injection any of the animals shows signs of, or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

Further action

The Ph. Eur. will remove the test " specific toxicity" for tetanus from the General Provision of the monographs of tetanus single /combined vaccines.

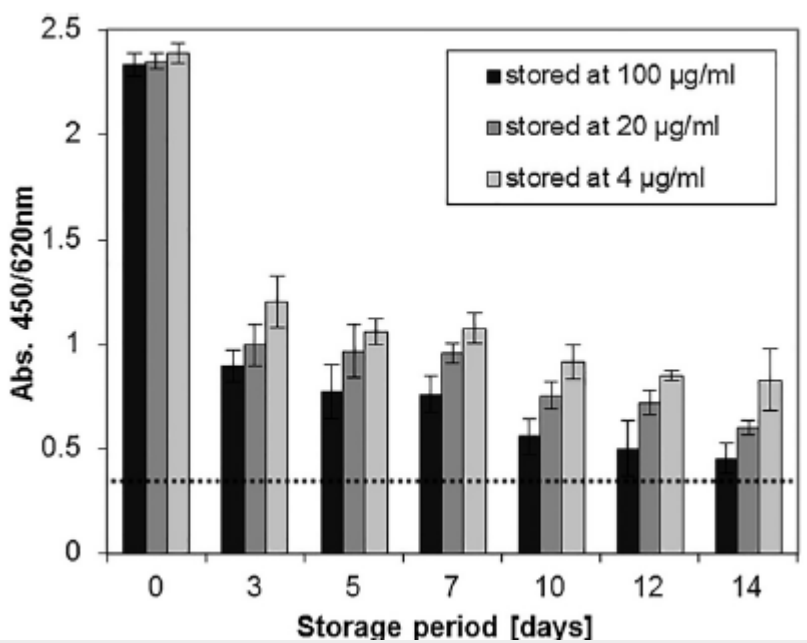
Only the test of Absence of toxicity will be kept and performed on the toxoid bulk.

Reversion to toxicity in guinea pigs

Tetanus toxoids are subjected to toxicity testing after storage at 37 °C and 5 °C for 42 days.

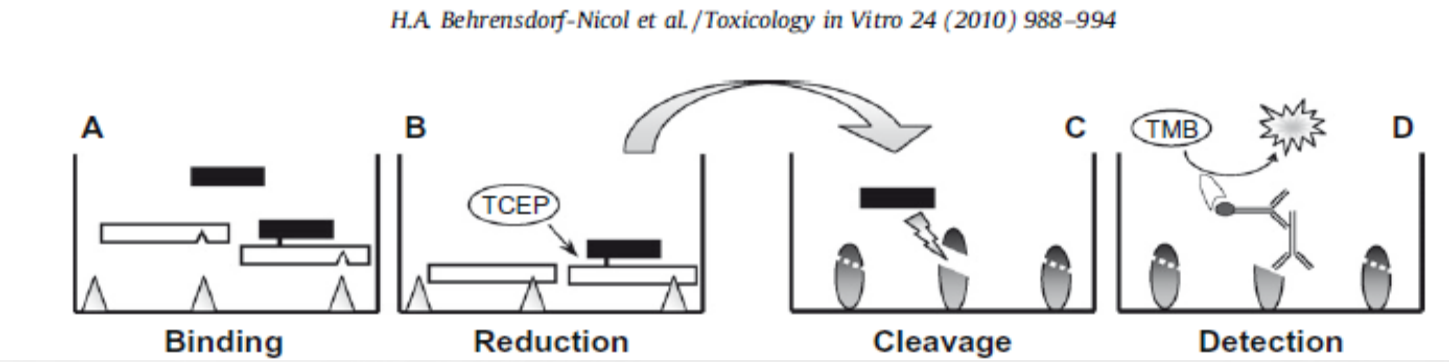
It was shown that tetanus toxin loses its toxicity rapidly during 37 °C storage.

The 37 °C storage test for human and veterinary tetanus vaccines lacks relevance.



H.A. Behrensdorf-Nicol, B. Kraemer. Is the test for irreversibility of tetanus toxoids still relevant ? Vaccine 2019, 37:1721-1724

In vitro assay for the absence of tetanus toxin- BINACLE



Coat with Ganglioside GT1b (receptor of TeNT)
TeNT binds via H chain, the addition of reducing agent release and activate the L toxin chain
The sup is transferred to a 2 plate

active L chain cleave the Synaptobrevin-2;
The cleavage site is detected by polyclonal Ab, followed by biotinylated secondary Ab, streptavidine conjugated peroxidase and peroxidase substrate

- *H.A. Behrensdorf-Nicol, et al.* Binding and Cleavage (BINACLE) assay for the functional *in vitro* detection of tetanus toxin.....Vaccine 2013,31:6247 ;
- *H.A. Behrensdorf-Nicol, et al.* Results of an international transferability study of the BINACLE (binding and Cleavage) assay for *in vitro* detection of tetanus toxicity. Biologicals 2014, 42: 199
- BSP collaborative study

Future Ph.Eur. status for specific toxicity of tetanus toxoid

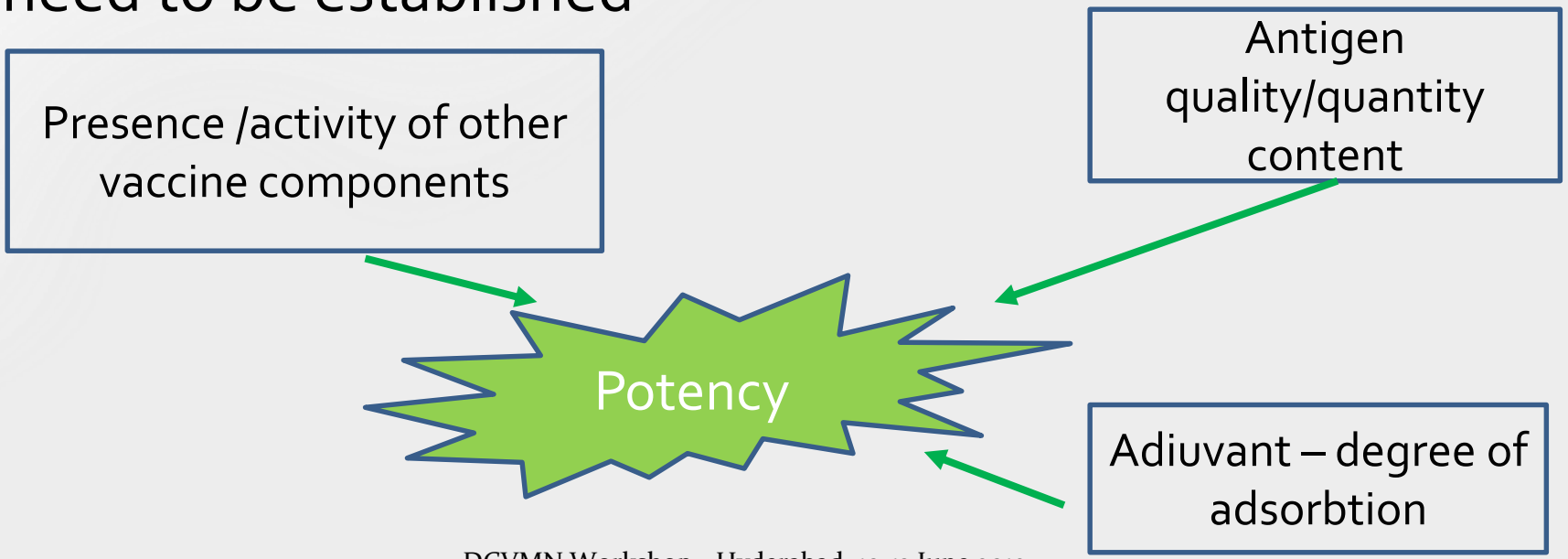
Test	Refinement	Reduction	Replacement	Deletion
Tetanus toxoid reversion to toxicity in gp	------	------	-------	Yes,will be deleted
Tetanus specific toxicity in gp	-------	Yes , only Absence on the toxoid bulk	*In vitro* test (BINACLE) under evaluation by Ph. Eur.	-------

Specific toxicity – diphtheria

	WHO	Ph.Eur
Absence of toxin	Toxoid bulk (500 Lf/ gp)	Toxoid bulk (100 Lf /Vero cells)
Reversion to toxicity	Toxoid bulk (Diluted to the same concentration as in final lot , then 10 shd/gp)	Toxoid bulk (100 Lf /Vero cells)
Specific toxicity	Vaccine Final lot (5 shd/gp)	Can be omitted on the final vaccine lot as part of routine lot release subject to process validation (5 shd/gp)

Replacement of animal tests by *in vitro* tests for potency - 1

In vitro assays should focus on quality attributes relevant for the biological function of the vaccine;
In vitro assays can be more powerful in detecting quality differences, but the meaning of the difference in potency need to be established



Replacement of animal tests by *in vitro* tests for potency - 2

- Quality attributes capable to detecting changes in the product characteristics , thus relevant for immune protection
- Be stability indicating
- Correlation/concordance between *in vivo* and *in vitro* assays should not be expected

Conclusions

There has been much activity in recent years aimed at simplifying the current potency tests for D & T, reducing the number of animals used and refining the end-point used in potency testing;

Some studies have also shown the possibility of using the same animals to test the potency of several antigens;

Conclusions - 2

- The alternative tests should be implemented by
- Manufacturers :

- during development of a new vaccine in order to gain sufficient information to replace the *in vivo* test (in parallel with the *in vivo test* – may be*)*

- as routine release test of established produced vaccines

- NCLs : for batch release

Thank you for your attention