Path To Remove and Replace Safety Testing In India.

Dr. Pradip Kumar Das
GM-QC
Biological E. Limited
Hyderabad, Telangana INDIA
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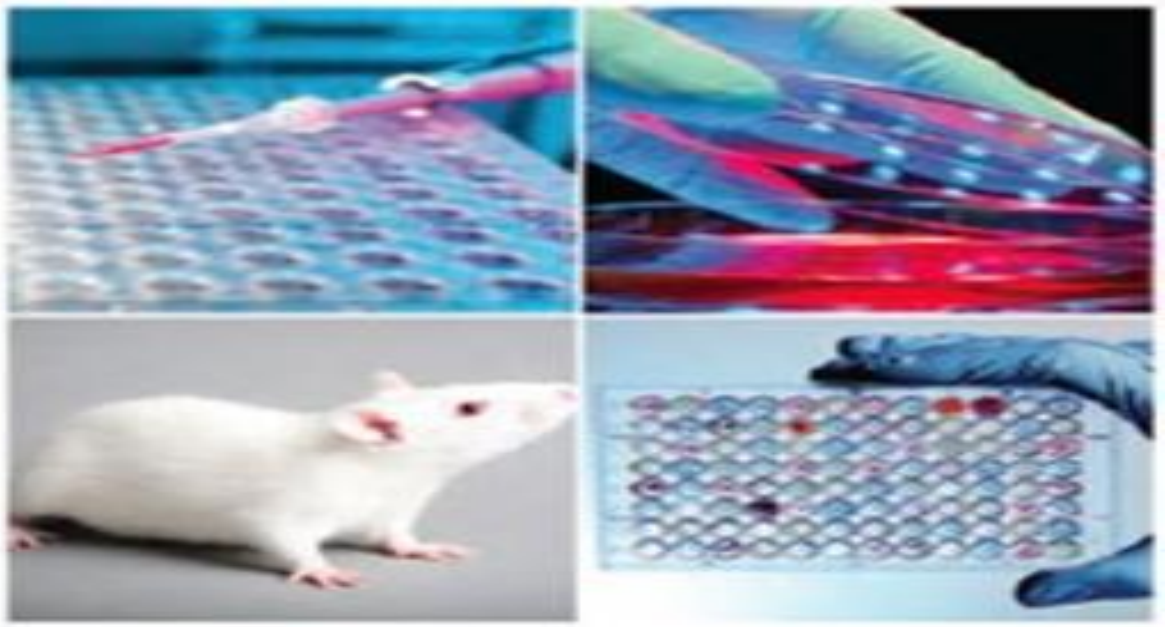

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Introduction

- Advances in vaccine science, analytical technologies, and regulatory frameworks have created a global movement toward replacing traditional **animal-based safety tests** with modern, reliable, and ethical alternatives.
- Historically, safety assurance for vaccines relied on in vivo assays—such as the Abnormal Toxicity Test, Rabbit Pyrogen Test, and Mouse-based potency tests—to detect unexpected toxicity or impurities.
- However, these legacy tests are now recognized as **scientifically limited, poorly reproducible, time-consuming**, and often not predictive of human responses.
- In recent years, international agencies including **WHO, EMA, US FDA, and EDQM**, along with India's **CDSCO and IPC**, have endorsed or implemented the removal of many animal-based safety tests.

- This regulatory shift is supported by the availability of advanced in vitro, molecular, and process-based methods that offer equal or superior sensitivity and specificity. Technologies such as the Monocyte Activation Test (MAT), cell-based functional assays, antigen quantification methods, and robust process analytical controls now provide comprehensive safety assurance without routine animal use.

- The transition from animal tests to modern alternatives represents a scientific and ethical evolution.

- It strengthens product consistency, accelerates batch release, reduces animal use, and aligns vaccine manufacturing with global best practices.

- This modernization is essential for ensuring faster, safer, and more reliable vaccine delivery while upholding the principles of the 3Rs—Replacement, Reduction, and Refinement.

Legacy Animal Safety Tests Targeted for Replacement

Legacy Test	Traditional Animal Model	Status Globally	Replacement Approaches
Abnormal Toxicity Test (ATT/General Safety Test)	Mice + Guinea pigs	Deleted by WHO, EDQM, USP, CDSCO (India)	GMP + process controls
Pyrogen Test (Rabbit Pyrogen Test)	Rabbits	Phasing out globally	In vitro monocyte activation test (MAT) & BET
Test for Residual Pertussis Toxin in aP Vaccines	Mouse Histamine Sensitization Test (HIST)	Being phased out (Europe, Japan)	CHP cell-based assay, LC-MS
Neurovirulence Tests	Monkeys, rodents	Being reduced using genetic, molecular surrogates	Cell-based assays, molecular markers, HTS
Potency Tests (mouse-based)	Mice	Shift towards in vitro and antigen-quantification	ELISA, Luminex, mass spectrometry

Specific toxicity test: Diphtheria

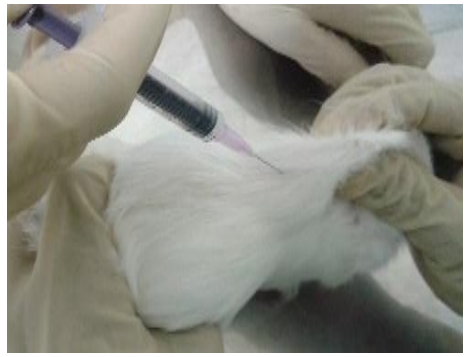
Stage	Test	In vitro Test
Detoxification and purification (Inprocess)	At harvest stage, Detoxification has been shown to be complete by performance of a specific toxicity test either by suitably validated in vivo or in vitro method.	**The Vero cell assay* is highly sensitive** and is considered superior to existing *in vivo* test methods. Once validated, *in vitro* assays should be used for routine batch release.
Specific toxicity (DS)	Each bulk purified toxoid should be tested for the presence of diphtheria toxin. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay	**The Vero cell assay* is highly sensitive** and is considered superior to existing *in vivo* test methods. Once validated, *in vitro* assays should be used for routine batch release.
Reversion to toxicity (DS)	Each bulk purified toxoid should be tested to ensure that reversion to toxicity does not take place during storage. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay, such as the Vero cell assay.	**The Vero cell assay* is highly sensitive** and is considered superior to existing *in-vivo* test methods. Once validated, *in-vitro* assays should be used for routine batch release.
Specific toxicity (FB)	Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHDs, and is observed for 42 days	**Test Removed** based on the satisfaction of NRA

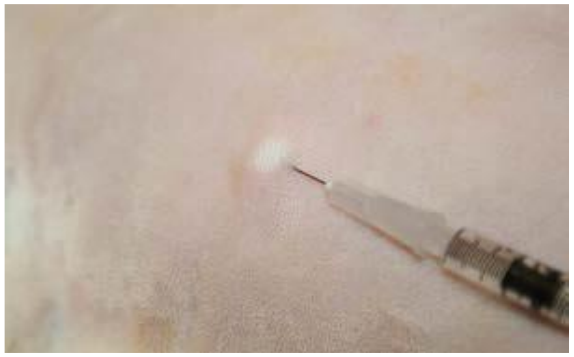
*A detailed procedure Vero cell method is described in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines, WHO IVB 11.11/ EP 11.2/IP 2022.

Absence of Toxin (Specific Toxicity) and Irreversibility of Toxoid - BPDT

In Vivo Test

- Absence of Toxin (Specific Toxicity) is performed in 5 Guinea pigs (250-350g) @500 Lf/mL by subcutaneous route
- Current procedure for Irreversibility test (Reversion to Toxicity) is performed in Guinea pigs (>500g) by Intradermal method @ 50Lf/mL 0.2mL of BPDT 2-8°C and 34-37°C





In vitro Test

- Vero cell assay for specific toxicity (fresh sample) and Irreversibility (2-8 & 34-37°C) in Single 96 well plate.

		1	2	3	4	5	6	7	8	9	10	11	12	
		(Fresh) Lf/mL			(2-8°C) Lf/mL			(34-37°C) Lf/mL						
		100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf	Empty	Control		
Sample Diluted with PBS + Media + Cell	A	●	●	●	●	●	●	●	●	●	○	●	●	Cell control
	B	●	●	●	●	●	●	●	●	●	○	●	●	
	C	●	●	●	●	●	●	●	●	●	○	○	○	Blank
Toxin + Media +Cell	D	○ 1x10^-4	○ 5x10^-5	○ 3x10^-5	● 1x10^-5	● 6x10^-6	● 3x10^-6	● 2x10^-6	● 8x10^-7	● 4*10^-7	○	○	○	
Sample Diluted with PBS + DAT (50 Lf) + Cell	E	●	●	●	●	●	●	●	●	●	○	●	●	DAT Control
	F	●	●	●	●	●	●	●	●	●	○	●	●	
	G	●	●	●	●	●	●	●	●	●	○	●	●	Control Toxoid
Toxin + Media +Cell	H	● 1x10^-4	● 5x10^-5	● 3x10^-5	● 1x10^-5	● 6x10^-6	● 3x10^-6	● 2x10^-6	● 8x10^-7	● 4*10^-7	○	●	●	

Absence of Toxin (Specific Toxicity) and Irreversibility of Toxoid Test : Animal Method Vs. Cell culture method (Vero cell) – Detoxification /BPDT

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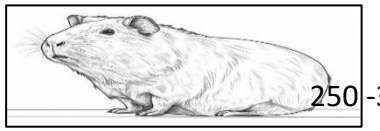
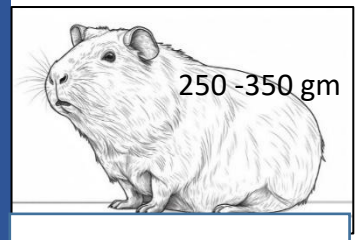
Diphtheria

Specific Toxicity



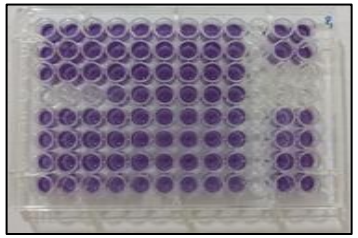
Guinea Pig (Subcutaneous Method)



Sensitivity 0.1 Lf

Complies:
None of animal any symptoms of diphtheria within 42 days or losses weight at the end of the day

Vero Cell



Sensitivity 3X10-5 Lf

The assay is valid if, the sensitivity and specificity of the assay are confirmed.

Irreversibility

Guinea Pig (Subcutaneous Method)

42 Day Incubation (At 2 - 8°C and 37°C)

Sensitivity 0.1 Lf

Fail (If any Animal died)

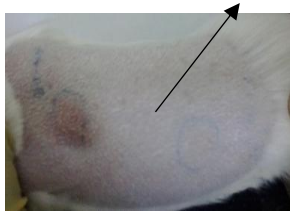
Complies

Complies:
None of animal any symptoms of diphtheria within 42 days or losses weight at the end of the day

Guinea Pig(Intradermal Method)

42 Day incubation (At 2 - 8°C and 37°C) (2 Day test) **Sensitivity 3x10-4 Lf**

Complies



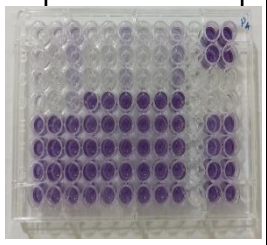
Fail

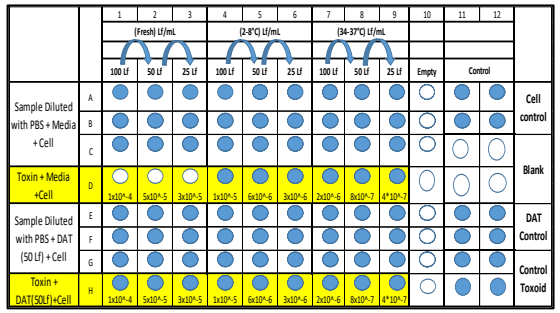
Vero Cell

42 Day Incubation (At 2 - 8°C and 37°C (5 Day Test) **3X10-5 Lf Sensitivity**

Complies

Fail





Conclusion

In Vivo Test	In Vitro (Vero cell assay)
• For specific Toxicity, 5 animals required and observed for 42 days. • For Irreversibility, 42 days incubation and 2 days animal test. • Sensitivity by Subcutaneous method is 0.1Lf/mL, Intradermal method 3x10^-4 Lf/mL. • Result observation by manual.	• For Specific Toxicity, Test duration is 7 days. • For Irreversibility 42 days incubation and 7 days Vero cell test. • Sensitivity by Vero cell method is 3 x10^-5 Lf/mL. **More sensitive and Reliable** • Results observation by Absorbance Readings. no manual intervention is required. • No animals required. • Complete Replacement

Specific toxicity test: Pertussis

Stage	Test	IP Chapter 2.7.20
Specific toxicity	Each final bulk should be tested for toxicity using the mouse weight gain test. The final bulk is considered satisfactory if the following conditions are met: (a) at the end of 72 hours the average weight of the group of vaccinated mice is not less than that preceding the injection, (b) at the end of 7 days the average weight gain per mouse is not less than 60% of that per control mouse, and (c) no deaths occur when 10 mice are used and no more than one death occurs when 20 mice are used.	❖An in-vitro method for detection of residual toxic components should be specific and at least as sensitive as the existing in-vivo method. Where possible, a fully functional in-vitro system should be used (e.g. toxin sensitive cell line).

2.7.20. Substitution of in-vivo Method(s) by in-vitro Method(s) for the Quality Control of Vaccines

CHO cell assay for Detection of Pertussis Toxin in Whole cell Pertussis Bulk antigen and Whole cell pertussis containing adjuvant vaccines (Mouse weight gain test)

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Whole cell pertussis

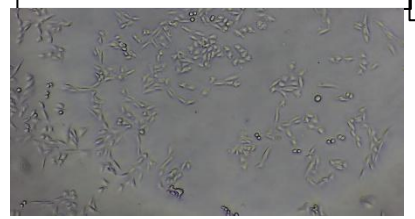
- Specific Toxicity *In vivo*
  - Animal (10 Sample and 10 Control)
  - 7day Observation
  - 0th Day weight
  - 3rd day weight
  - 7th day weight
  - The test is valid if the control group shows no weight loss and no death, at any point over the seven days.
- Pertussis Toxin Detection In vitro
  - CHO cell in 96 well plate
    - 48 hours observation
    - Value should be < 105 IU/SHD
      - Fail
      - Pass

At the end of day 3 the average weight of the group of vaccinated mice is not less than that preceding the injection

At the end of 7 days the average weight gain per mouse is not less than 60 % and not more than 150% of that per control mouse

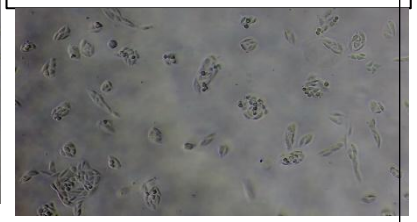
Not more than 5% of the mice die in the sample group. No death should occur when 10 mice are used and not more than one death occurs when 20 mice (retest) are used

Cell control



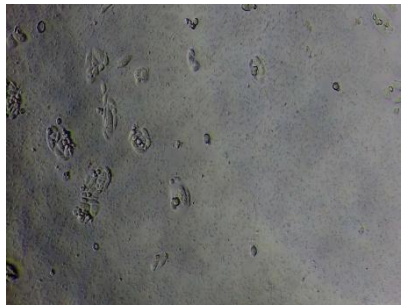
No Clusters

Clusters (Reference PT)



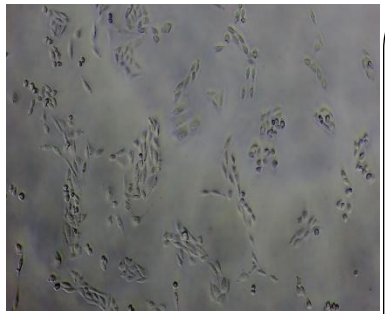
+ After 48 Hours

Cytotoxicity for Adjuvant vaccine



Cytotoxicity After 48 Hours

Vaccine Negative



No Clusters in vaccine after 48 Hours

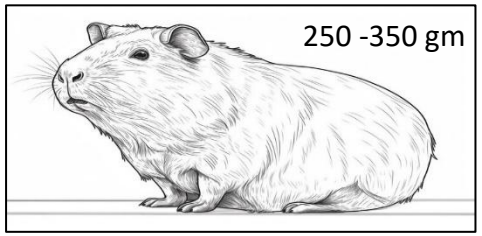
Specific toxicity test: Tetanus

Stage	Test	In vitro test
Detoxification and purification	Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete by performance of a specific toxicity test (..	Amend text to remove reference to the test described in A.3.4.4 or use of an in vivo method. Animal use may be reduced or removed once consistency in production has been demonstrated and NRA approves detoxification test may be omitted from routine release procedures
Specific toxicity	Each bulk purified toxoid should be tested for the presence of tetanus toxin. The test may be performed in vivo using guinea-pigs.	Amend text to remove detail of animal test and preferentially recommend use of validated in vitro assay; also mention validation of the production process to provide assurance of stable detoxification which removes need to perform reversion test
Reversion to toxicity	Each bulk purified toxoid, diluted with the same buffer solution as used in the final vaccine, should be tested for the absence of tetanus toxin in guinea-pigs; the guinea-pigs should each weigh 250–350 g and not previously have been used for experimental purposes.	Remove test – assurance of lack of reversion provided from validation of the production process – not needed for routine batch testing.
A.3.5.2.5 Specific toxicity	Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHDs, and is observed for 21 days	**Remove test**. Test is redundant because a more sensitive toxicity test would have already been performed on the bulk purified toxoid at an earlier production stage.

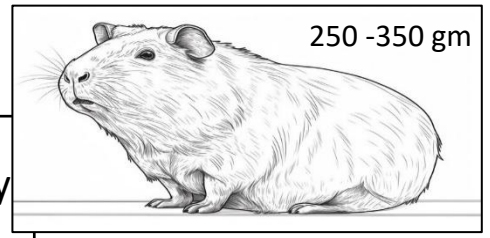
2.7.20. Substitution of in-vivo Method(s) by in-vitro Method(s) for the Quality Control of Vaccines

Absence of Toxin (Specific Toxicity) and Irreversibility of Toxoid Test : Animal Method

Tetanus



- Specific Toxicity
  - Require 5 G. Pig 21 Days
  - After 21 Days
  - Result
- Irreversibility



- 2 -8 Incubation for 28 Days
  - Require 5 G.Pig 21 Days
  - After 21 Days
  - Result
- 2 -8 Incubation for 28 Days
  - Require 5 G.Pig 21 Days
  - After 21 Days
  - Result

❖ **Neurovirulence tests: Refinement & Replacement**

- ✓ The potential danger of live attenuated vaccines is that they might revert to virulence.
- ✓ For oral poliovirus, two alternative assays are available or developed : a transgenic mouse, neurovirulence model and use of HTS technique

❖ **Extraneous agents**

- ✓ The test for extraneous agents is required to detect viral contaminations in virus seed lots, virus harvest, cell culture and control eggs.
- ✓ Only virus seed lots are tested in adult and suckling mice and guinea pigs, other materials are tested in cell cultures. An amount of the seed lot is neutralized by virus-specific antibodies and then injected.
- ✓ The virus seed lot passes the test if no animal shows evidence of infection The nature of the test, detection of unknown contaminations, makes it difficult to develop in vitro alternatives.
- ✓ When the nature of possible viral contaminations in animal cell cultures are known, assays based on PCR are alternatives

❖ **Rabbit Pyrogen test** being replaced with validated BET/MAT- Refinement/Replacement

❖ **Deletion of abnormal toxicity test:** No longer required for batch release.

Scientific approach: What Replaces Animal Tests

Use a risk-based mix of approaches depending on the product and the safety question the animal test was addressing:

- **Process and release controls** — enhanced upstream / in-process controls, validated critical quality attributes (CQAs), and validated removal/detoxification steps (e.g., biochemical assays confirming inactivation).
- **Product-specific in-vitro assays** — cell-based potency, biochemical assays, binding/neutralization assays, molecular assays (e.g., PCR for adventitious agents), and toxoid-specific in-vitro toxicology (toxin-sensitive cell lines or molecular markers).
- **Analytical comparability** — stability, identity, purity, and impurity profiles using orthogonal analytical methods.• Batch record and process mapping — process validation, critical parameter monitoring, and lot-to-lot consistency data.
- **Enhanced GMP & release** testing combined with targeted non-clinical data where needed. WHO/EDQM guidelines describe and support such alternatives and emphasize they must be at least as sensitive and specific as animal tests.

Benefits of Replacing Animal Testing

Scientific

- ❖ More reproducible and sensitive
- ❖ Detects human-relevant contaminants
- ❖ Higher specificity

Operational

- ❖ Faster release timelines
- ❖ Lower batch rejection risk
- ❖ Cost savings

Ethical & Compliance

- ❖ Aligns with 3Rs, CCSEA, WHO norms
- ❖ Avoids unnecessary animal use

Conclusion

- The shift from traditional animal-based safety tests to modern in vitro, molecular, and process-based approaches marks a major advancement in vaccine quality and regulatory science.
- Evidence from global and national regulatory bodies demonstrates that many animal assays—such as the Abnormal Toxicity Test, Rabbit Pyrogen Test, and HIST—are no longer scientifically justified and can be effectively replaced by more precise, reproducible, and human-relevant methods.Adopting these alternatives not only aligns with ethical principles of the 3Rs but also strengthens product consistency, enhances safety assurance, and accelerates batch release timelines.
- With strong validation, risk-based frameworks, and regulatory acceptance, India is well-positioned to complete the transition toward a modern, non-animal safety testing ecosystem.Ultimately, replacing animals in safety testing is not just a regulatory change it is a scientific necessity that supports more reliable vaccines, improves operational efficiency, and promotes responsible and humane biomedical innovation.

The monument commemorates the sacrifice of the mice in genetic research used to understand biological and physiological mechanisms for developing new drugs and curing of diseases

Thank You