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Analysis of historical data to accelerate deletion of Abnormal Toxicity Test requirement for biologicals and vaccines

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ABSTRACT

Abnormal Toxicity Test (ATT) is performed as quality control test by manufacturers and National Control Laboratory (NCL) to ensure safety of biologicals. However, stakeholders are in general consensus that extraneous toxic contaminations are extremely unlikely where Good Manufacturing Practices (GMPs) and consistent production have been established. This test requiring mice and guinea pigs is still a regulatory requirement for the batch release of biologicals in several countries although it has been deleted by some National Regulatory Authorities (NRAs) and Pharmacopoeias while some are still working on its elimination. Therefore, ten years historic data of ATT performed at National Institute of Biologicals (NIB), India on 4813 batches of biologicals including blood and related products, enzymes, hormones and vaccines by using 33637 animals was analyzed. As per Indian Pharmacopeia (IP), 4783 batches of these biologicals passed the test. The test had to be repeated in 30 batches, all of which were blood products and the repetition rate was 0.62 %. Further, after repeat testing, all these 30 batches also passed the test. This data will help the regulatory authorities of countries where ATT is still a requirement, take appropriate decision regarding its deletion. The elimination of ATT from specific monographs of these biologicals will help save many thousands of animals being used globally for this test.

1. Introduction

Biologicals are therapeutic products made from living cells taken from plants, animals or bacteria. They include an extensive range of products such as blood and blood related products, recombinant therapeutic proteins, immunosera, enzymes, hormones, vaccines, tissues, gene therapy and somatic cells to treat various diseases [1]. However, their development requires rigorous safety testing including animal-based Abnormal Toxicity Test (ATT) to ensure their safety for human use. This test is an *in-vivo* quality control test that is performed by National Control Laboratory (NCL) and manufacturers of various countries for batch release testing of biologicals using mice and guinea pigs [2,3]. However, involvement of animal usage in this test underscores the sustainable and ethical practices in manufacturing as well as quality control testing of biologicals [3].

The scientists as well as the regulatory agencies aim to harmonize the 3Rs (Replacement, Reduction and Refinement) in biological regulatory testing standards between regions by replacing animal testing via innovative *in-vitro* methods, by reducing the numbers of animal used and by refining measures to minimize pain and suffering where no alternatives exist. However, the specific initiatives for harmonization and global convergence of 3Rs in regulatory testing of biological products, are still being advanced [4,5]. Consequently, both *in-vivo* and *in-vitro* models are required by the developing as well as the manufacturing enterprises to gain access to all markets globally.

The ATT that is also known as innocuity test or General Safety Test (GST) was developed originally in 1950s and was believed to be an effective tool for detecting nonspecific contaminants and toxins in the

Abbreviations: ATT, Abnormal Toxicity Test; CCSEA, Committee for Control & Supervision of Experiments on Animals; ECBS, Expert Committee on Biological Standardization; FDA, Food and Drug Administration; GLP, Good Laboratory Practices; GMPs, Good Manufacturing Practices; IAEC, Institutional Animal Ethics Committee; IP, Indian Pharmacopeia; IPC, Indian Pharmacopoeia Commission; I/p, Intraperitoneal; I/v, Intravenous; NC3Rs, National Centre for the Replacement, Refinement and Reduction of Animals in Research; NCL, National Control Laboratory; NIB, National Institute of Biologicals; NIID, National Institute of Infectious Diseases; NRA, National Regulatory Authority; QC, Quality Control; SDGs, Sustainable Development Goals; UN, United Nations; WHO, World Health Organization; 3Rs, Replacement, Reduction and Refinement.

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biological products and has remained the standard since then to ensure safety of biologicals before their use in humans. Many nations have made significant progress towards the elimination or reduction of ATT requirements in drug development in the quest of more ethical and scientifically advanced methods. USA, Canada and Europe have already deleted this test from their monographs. Federal Register report (2015) published by the U.S.A. Food and Drug Administration (FDA) amended the biologicals regulations by eliminating the innocuity test requirement for biologics products [6]. In Brazil, Argentina and South Africa also ATT is not required to be performed any longer for release of batches of vaccines. Additionally, in 2018, the WHO Expert Committee on Biological Standardization (WHO-ECBS) announced that it would revoke this test in all its future guidelines and Technical Report Series for biological products [6]. There have been partial deletion of this test or waivers in South Korea and Japan while it is still a regulatory requirement in countries like China and Russia [3]. The Indian Pharmacopoeia Commission (IPC) had earlier recommended that ATT could be omitted for the lot release of certain products including vaccines after establishment of consistency in production to the contentment of the National Regulatory Authority (NRA) and once Good Laboratory Practices (GLP) and Good Manufacturing Practices (GMP) are well established [7]. However, ATT is still a requirement in Indian Pharmacopeia (IP) monographs of some biologicals including blood and blood related products, enzymes and hormones.

In order to comprehend the global efforts for elimination of ATT and to proceed towards complete deletion of this test for all biologicals, there is certainly a requirement of convincing supportive data for each biological product. Therefore, the authors analyzed the last 10 years of historical data of ATT performed on the finished batches of different biologicals that have been tested at National Institute of Biologicals (NIB), India as per their monographs in the IP, before their release into the market for human use. The authors are of the opinion that sharing this historic data will help the regulatory authorities of countries where ATT is still a requirement for these biologicals, take appropriate decision regarding its deletion from the specific monographs.

2. Material and methods

2.1. Biologicals and vaccines tested

The details of the biological products on which the test was performed are provided in Table 1.

2.2. Performance of ATT

The ATT was performed on several biologicals as per the General Test and specific monographs of these products in IP. Five SWISS mice (17–22 g) and two Duncan Hartley guinea pigs (250–350 g) were used for performing the test on each batch of blood and related products and vaccines, while only 5 SWISS mice were used for performing the test for enzymes and hormones. The test for C1 Esterase Inhibitor and Covid-19 vaccine were performed as per manufacturer's protocols. The details of species and number of animals used, dose and route of injection of each biological tested and duration of observation period are provided in Table 1. The animals were observed regularly for any signs of ill health or death during the specified observation period and interpretation was made as per the General Test in IP i.e. The preparation complied with the test if none of the animals died or showed signs of ill health within the specified time interval and did not comply with the test if more than one animal died or showed signs of ill health during the specified time interval. However, in case of death of one animal, the test was repeated and the preparation passed the test if none of the animal in the second group died within the specified time interval.

The animals were provided by the Animal Facility of NIB and all the experiments were performed in the *In-vivo* Bioassay Laboratory. This work was conducted in compliance with relevant regulatory guidelines

Table 1
Details of biological products tested, species and number of animals used, dose and route of injections and observation period.

Biological Products	Animals used	Dose	Route	Observation (days)
	Blood and related products			
Human Albumin	5 mice, 2 guinea pigs	0.5 ml/mice, 5 ml/guinea pig	i/p	7
Human Dried Anti-Hemophilic Fraction (Factor VIII)	5 mice, 2 guinea pigs	1.5 IU/ mice,15 IU/ guinea pig	i/p	7
Tetanus Human Immunoglobulin	5 mice, 2 guinea pigs	0.5 ml/mice, 5 ml/guinea pig	i/p	7
Human Specific Anti-D Immunoglobulin	5 mice, 2 guinea pigs	0.5 ml/mice, 5 ml/guinea pig	i/p	7
Human Normal Immunoglobulin	5 mice, 2 guinea pigs	0.5 ml/mice, 5 ml/guinea pig	i/p	7
Human Plasma Protein fraction	5 mice, 2 guinea pigs	0.5 ml/mice, 5 ml/guinea pig	i/p	7
Human Rabies Immunoglobulin	5 mice, 2 guinea pigs	0.5 ml/mice, 5 ml/guinea pig	i/p	7
Hepatitis B Immunoglobulin	5 mice, 2 guinea pigs	0.5 ml/mice, 5 ml/guinea pig	i/p	7
	Enzymes			
Human Streptokinase	5 mice	50,000 IU in 0.5 ml/mice	i/v	1
Human Urokinase	5 mice	2500 IU in 0.5 ml/mice	i/v	1
C1 Esterase Inhibitor	5 mice, 2 guinea pigs	1.0 ml/mice, 5 ml/guinea pig	i/p	7
	Hormones			
Human Chorionic Gonadotropins	5 mice	1000 IU in 0.5 ml/mice	i/v	2
Human Menopausal Gonadotropins	5 mice	75 IU FSH in 0.5 ml/mice	i/v	1
Granulocyte Colony Stimulating Factor	5 mice	0.5 ml/mice	i/v	7
	Vaccines			
Cell Culture Rabies Vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
Live attenuated Rubella Vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
Live attenuated Measles Mumps and Rubella Vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
Japanese Encephalitis Vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
Live attenuated Measles Vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and	i/p	7

(*continued on next page*)

Table 1 (*continued*)

Biological Products	Animals used	Dose	Route	Observation (days)
		5 ml/guinea pig		
Measles, Rubella Vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
Hemophilus Influenza type b conjugate Vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
Varicella	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
Polysaccharide Typhoid vaccine	5 mice, 2 guinea pigs	One human dose but not more than 1 ml/mice and 5 ml/guinea pig	i/p	7
COVID−19 Vaccine	5 mice, 2 guinea pigs	1 ml/mice, 5 ml/guinea pig	i/p	7

Note: i/p represents intraperitoneal and i/v represents intravenous route of injections, respectively.

provided by Committee for Control & Supervision of Experiments on Animals (CCSEA) and ARRIVE guidelines. The necessary ethical approvals for performance of ATT on all the biological products was granted by the Institutional Animal Ethics Committee (IAEC) of NIB.

2.3. Animal husbandry

The mice and guinea pigs were group housed in polycarbonate and polypropylene cages respectively; room temperature was maintained at 22–24 ± 2 °C and humidity at 50–60 ± 5 %, with a 12-h light/dark cycle. Sterilized balanced pellet diet and RO water were provided *ad libitum* to all animals. Sterilized corn cob was provided as bedding material for the animals.

3. Result and discussion

Although IP has removed ATT from many individual monographs of vaccines for human use, it is still official in the monographs of some biologicals intended for human use. Therefore, in compliance to the IP, ATT was performed to assess the safety of a total of 4813 batches of different biologicals between April 2013 and March 2023. This included 4357 batches of blood and related products, 67 batches of enzymes, 69 batches of hormones and 320 batches of vaccines, details of which are illustrated in Table 2. It was observed that all the batches complied with the requirements of ATT as per the IP. However, in this period of 10 years, the test had to be repeated in 30 batches of biologicals that included 27 batches of human albumin and 1 batch each of dried human antihemophilic fraction (Factor VIII), human plasma protein fraction and human hepatitis B immunoglobulin. The test in all these batches of blood and related products had to be repeated because of death of one guinea pig during the prescribed observation period. It is important to note that the repetition rate of ATT was observed to be only 0.62 %, which is extremely low compared to the total number of batches that were tested. Furthermore, these batches also complied with the test requirements of IP for passing the test after the repeat test was performed. It has also been observed that in the last 10 years period under consideration, none of batches of enzymes, hormones and vaccines required any repeat testing. Further, in the last 3 years period i.e. 2020–2023, there has been no repetition of ATT for any batch of biologicals which might be attributed to consistent manufacturing in well-established GMP facilities in the recent years.

The details of percentage of different category of biologicals tested at NIB are provided in Fig. 1 and it was noted 90.5 % of the batches tested every year in NIB were of blood and related products specifically Human Albumin followed by Dried Human Antihemophilic Fraction (Factor VIII) and various Human Specific Immunoglobulins (Table 2). Further, enzymes, hormones and vaccines comprised 1.4 %, 1.4 % and 6.7 % respectively (Fig. 1).

For performing this *in-vivo* safety test on the 4813 batches of various biologicals, a total of 33637 animals i.e. 24215 mice and 9422 guinea pigs were used during the period from April 2013-March 2023, the year wise details of which are provided in Fig. 2. The authors are of the opinion that sharing such specific product wise data of different biologicals on which ATT was performed, number of batches that complied or did not comply the test and the number of animals used by different countries will help the regulatory authorities in prioritizing taking up deletion of ATT from individual monographs of these products.

Based on a survey performed in 1994–95, Paul-Ehrlich Institute reported performance of about 4367 ATTs for 159 biologicals (human and veterinary sera and vaccinations) utilizing almost 19,000 mice and 8700 guinea pigs with repetitive rate of 1.1 %, and all the batches passed the test [8]. In Japan, approximately 1000 guinea pigs are used to perform more than 500 ATTs per year for releasing an average of 250 lots of vaccines, although since 2008 after review of historical data, waiver of ATT has been granted for several vaccines after the establishment of safety and consistency. In 2020, this waiver was extended to 70–80 % of lot release of vaccines in the country. Further, every year around 400 batches of different blood products are released and on review of their 20 years historic data, National Institute of Infectious Diseases (NIID) Japan has not found any failure of ATT. This data provides evidence that this test is not required for ensuring safety of blood products [3]. Another retrospective analysis of ATT data by Kraemer et al., in which 5896 ATTs were performed using 12420 guinea pigs and 30193 mice, revealed that none of the batches failed in ATT [9,10]. In Russia, 48 vaccines out of 114 required ATT and data available represents that no biological has failed ATT since 2012 though Russian regulatory authority found that in last 5 years, ATT had often failed for cephalosporin antibiotics [3]. This provides an idea of the huge number of animals being utilized by manufacturers as well as NCLs for performing this obsolete and redundant test. An enormous contribution can be made to the 3 Rs by saving these animals, if the efforts for its global deletion are accelerated by all the countries.

Several NRAs and Pharmacopoeias have already eliminated the requirement of ATT, few are working on deleting it, while in some countries this test is still a regulatory requirement for batch release of biologicals and vaccines. The WHO-ECBS in its 69th report has also suggested discontinuation of this test from its subsequent guidelines and recommendations for biological products. The deletion of ATT from these blood and blood related products, recombinant technology-based therapeutics, vaccines and antisera is an initiative towards 3 R's and is certainly desirable [3]. The NC3Rs, UK is also working in collaboration with WHO on how application of 3R principles in quality control testing for batch release of biotherapeutics and vaccines could be made more robust. In 2021, based on a survey they reported that although WHO had removed requirement of ATT but many manufacturers still performed this test in order to comply with national regulations/requirements to market their products in countries which still mandate the ATT in their monographs. This dearth of global harmonization in terms of requirement to perform this test is a significant parameter in its continued use [11].

Table 2
Abnormal Toxicity Test performed for different biological products during the period April 2013- March 2023.

Biological Products	Batches tested (per year)									
	2013–2014	2014–2015	2015–16	2016–17	2017–18	2018–19	2019–20	2020–21	2021–22	2022–23
	Blood and related products									
Human Albumin	283 (6 *)	288 (4 *)	237 (1 *)	212 (5 *)	231	281 (9 *)	287 (2 *)	239	288	251
Dried Human Antihemophilic fraction Factor VIII	43	71	132	82	46	105 (1 *)	155	124	132	125
Tetanus Human Immunoglobulin	30	20	37	41	21	44	30	17	12	17
Anti-D Immunoglobulin	-	3	-	-	25	5	21	16	19	17
Human Normal Immunoglobulin	7	5	8	8	12	15	21	1	-	-
Human Plasma Protein fraction	2	-	2	-	3	4 (1 *)	-	-	1	2
Human Rabies Immunoglobulin	7	4	23	18	10	15	17	6	17	16
Human Hepatitis B Immunoglobulin	9	16	17	16	15	16 (1 *)	7	18	14	18
Total Batches tested/year	**381**	**407**	**456**	**377**	**363**	**485**	**538**	**421**	**483**	**446**
	Enzymes									
Human Streptokinase	1	4	5	3	3	3	15	1	1	9
Human Urokinase	5	3	1	2	-	4	2	1	-	-
C1 Esterase Inhibitor	-	-	-	-	-	-	-	-	1	3
Total Batches tested/year	**6**	**7**	**6**	**5**	**3**	**7**	**17**	**2**	**2**	**12**
	Hormones									
Human Chorionic Gonadotropins	15	2	4	2	2	1	2	4	5	6
Human menopausal Gonadotropins	-	-	-	-	-	-	-	3	2	5
Granulocyte- Colony Stimulating Factor	4	11	1	-	-	-	-	-	-	-
Total Batches tested/year	**19**	**13**	**5**	**2**	**2**	**1**	**2**	**7**	**7**	**11**
	Vaccines									
Cell Culture Rabies Vaccine	13	40	1	-	8	-	-	-	-	-
Live attenuated Rubella Vaccine	30	11	1	3	-	1	-	-	-	-
Live attenuated Measles Mump and Rubella Vaccine	56	4	1	5	-	8	4	-	-	-
Japanese Encephalitis Vaccine	3	2	4	1	-	2	-	-	-	-
Live attenuated Measles Vaccine	59	32	1	4	-	-	-	-	-	-
Measles, Rubella Vaccine	-	-	-	5	-	-	-	-	-	-
Haemophilus Influenza type b conjugate Vaccine	-	2	-	-	10	2	-	2	1	-
Varicella	-	-	-	-	-	-	2	-	-	-
Polysaccharide Typhoid vaccine	1	-	-	-	-	-	-	-	-	-
Novel COVID−19 Vaccine	-	-	-	-	-	-	-	-	1	-
Total Batches tested/year	**162**	**91**	**8**	**18**	**18**	**13**	**6**	**2**	**2**	**0**

*Represents the number of batches for the repeated test as per IP.
- Represents no sample received for the test.

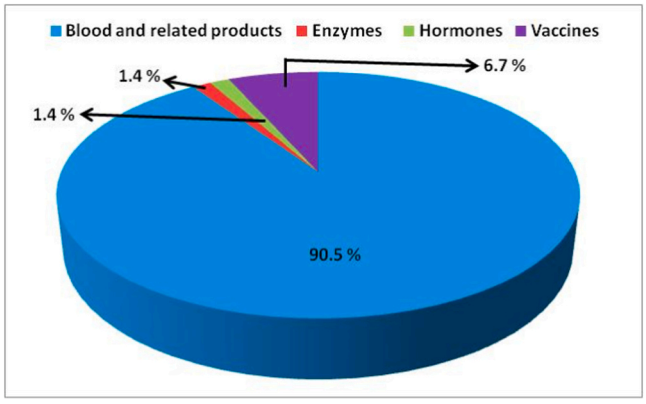

Fig. 1. Percentage of different categories of biologicals tested for Abnormal Toxicity during the period April 2013- March 2023.

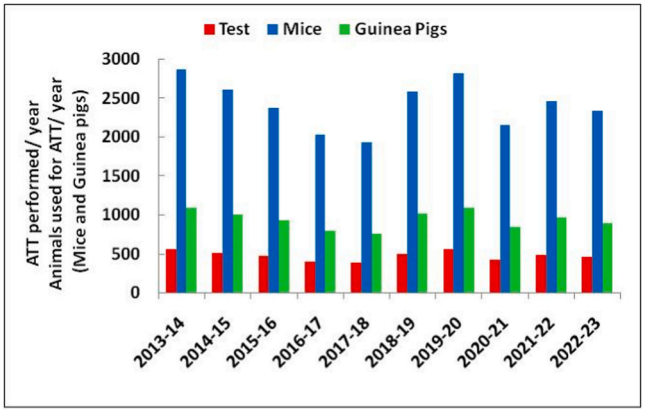

Fig. 2. Data representing the ATT performed/year and animals used/year (mice and guinea pigs) during the period April 2013- March 2023.

The authors also suggest accelerating the efforts for global deletion of ATT but this should be done scientifically on a case-by-case basis. The analysis must be done for specific biological products after obtaining and reviewing sufficient data from various stake holders including NCLs, manufacturers and regulatory authorities in different countries, providing adequate evidence to ensure safety of these specific biological products before use in humans. There should also be very effective post market pharmacovigilance i.e. product related post licensure follow up of the manufacturing process as well as any adverse events. Further, it is suggested that NCL may have the authority to perform ATT on few initial consecutive batches from separate bulk from any new manufacturer till consistency is established. This may be particularly important in developing or underdeveloped countries especially in case of newer or smaller manufacturers who may have limited resources and budgets to

maintain consistency in safety control.

4. Conclusion

The analysis of last 10 years data of ATT performed on several biological products at NIB, India showed that none of the batches failed this test suggesting its discontinuation for the batch release of finished products. In the current time, implementation of GMP and comprehensive QC measures help in ensuring the quality and safety of the biological products and vaccines, thus questioning the requirement of ATT as a safety test. The sharing of such data of ATT performed on specific biological products, exchange of similar information, networking and collaboration between stakeholders and regulatory authorities would help in harmonization and convergence of legislations in different countries. This will further help in accelerating the deletion of ATT globally from the specific monographs wherever it is still a requirement. The deletion of this test would not only help reduce the time and cost of manufacturing these critical therapeutic products but also help save many thousands of animals that are being used globally to perform this test. This will contribute enormously in the implementation of 3Rs in regulatory testing.

CRediT authorship contribution statement

Shikha Yadav: Writing – review & editing, Visualization, Supervision, Project administration, Formal analysis, Conceptualization. **Shachi Yadav:** Investigation, Formal analysis, Data curation. **Anamika Pal:** Investigation, Formal analysis, Data curation. **Harish Chander:** Supervision, Resources, Project administration. **Anup Anvikar:** Supervision, Resources, Project administration. **Priya Sharma:** Investigation, Formal analysis, Data curation. **Lakhan Kumar:** Investigation, Formal analysis, Data curation. **Raveena Kumari:** Investigation, Formal analysis, Data curation. **Mohit Chaudhary:** Investigation, Formal analysis, Data curation. **Suresh Kumar:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Farha Deeba:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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