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PROPOSED SECOND WHO INTERNATIONAL STANDARD FOR CLOSTRIDIA PERFRINGENS BETA AND EPSILON ANTITOXINS

Central Veterinary Laboratory, Weybridge, U.K.

The first WHO International Standards for Cl.perfringens Beta and Epsilon Antitoxins were prepared in 1954. Freeze-drying facilities available at that time limited batch sizes to 1100 ampoules.

By 1982 it was obvious that, although existing stocks of the Beta standard would suffice for another ten years at the existing rate of demand, the stock of the Epsilon standard would be exhausted in 1984. Production of replacement standards began in October 1982.

Material

Bulk sera were kindly donated by The Wellcome Research Laboratories, U.K., and processed by the Central Veterinary Laboratory. 1.0 ml volumes were freeze-dried in glass ampoules and sealed under an atomsphere of dry nitrogen by fusion of the glass. 3608 ampoules of the beta antitoxin, coded 2Cp beta At, and 3690 ampoules of the epsilon antitoxin, coded 2Cp epsilon At, were placed in cold storage at -20°C.

Characteristics

	Cl.perfringens beta antitoxin	Cl.perfringens epsilon antitoxin
Potency before freeze drying*	4963 IU/ml	1034 IU/ml
Precision of fill		
Mean wet volume per ampoule	1.027 ml	1.025 ml
Standard deviation	0.013 ml	0.0026 ml
Vacuum before back filling with dry nitrogen	0.466 mb	0.4 mb
Sterility tests (5 ampoules)	Pass	Pass

Collaborative Calibration Study

Six laboratories were invited to take part in a collaborative exercise to calibrate the proposed second standards against the existing ones. Four laboratories accepted of whom three completed the full exercise based on the previously agreed protocol described in Appendix 1. The names and addresses of the collaborators are given in Appendix 2, in an order unrelated to the laboratory serial numbers. The toxins used in the assays were also kindly donated by Wellcome Research Laboratories, U.K.

* Values provided by Wellcome Research Laboratories

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Results obtained are summarized for individual laboratories in Tables 1 to 4. The values shown are the 50% end-point titres calculated as the reciprocal of the volume of antitoxin per mouse needed to protect 50% of the mice. Potency ratios were calculated, wherever possible, from the results for the proposed standard and the existing standard on each day, and are shown in the tables.

Accelerated degradation

Ampoules of the proposed second standards were stored at -20°C, +4°C, room temperature and 37°C (dry) and compared with each other and the existing standards by toxin neutralisation tests in mice using two mice per dose level. Results are summarized in Table 5.

There were no significant changes in titre over the 392 days at any of the storage temperatures.

Minor components

The titres of minor antitoxic components of the proposed second standards were determined by single assays. The results are summarized in Table 6.

Summary and Conclusions

Table 7 contains the combined geometric mean potency values obtained for Laboratories 1-3, for both proposed standards, together with the 95% confidence limits where it was possible to calculate these. The results are expressed in Units per ampoule.

Since the differences between the results obtained by each laboratory are not statistically significant, an overall unweighted geometric mean potency value has been calculated for each proposed standard, based on these results and the relative potency values obtained in the accelerated degradation tests with ampoules of proposed and existing standards stored at -20°C and assayed on a particular day. The overall values are given as unweighted mean values because the between-laboratory variations were not significantly greater than the within-laboratory variations. Laboratory 4 was unable to carry out the full programme and because its results would introduce a significant bias, particularly for the proposed standard for *Cl.perfringens* beta antitoxin, it has not been included in the overall assessment.

The two preparations 2Cp beta At and 2Cp epsilon At are therefore presented as proposed replacements for the existing WHO International Standards for *Cl.perfringens* Beta and Epsilon Antitoxins. Stocks of the latter are actually exhausted apart from a small reserve which must be retained for the time being. It has therefore become necessary to issue ampoules of 2Cp epsilon At with a provisional unitage of 1049 IU per ampoule (the value obtained from preliminary analysis of the the results) to fulfill current requests.

From the final analysis of the results it is suggested that the unitage to be assigned to the preparations are:

Proposed Second International Standard for *Clostridium perfringens* Beta

Antitoxin: 4770 IU per ampoule

Proposed Second International Standard for *Clostridium perfringens*

Epsilon Antitoxin: 1020 IU per ampoule

It is recommended that preparation 2Cp epsilon At be established as soon as possible but that preparation 2Cp beta At be held in reserve for establishment at a future date when stocks of the existing standard have been further depleted.

TABLE 1: 50% end-point titres obtained by Laboratory 1

	BETA STANDARDS					EPSILON STANDARDS				
Day	Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio	Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio
1	A	1	3790			A	1	1110		
	1	1		<3450		1	1		1110	1.00
	1	1		4170	1.10	1	1		>1250	
	1	1		4170	1.10	1	1		1150	1.04
2	A	2	3900			A	2	1180		
	2	2		3750	0.96	2	2		I/S	
	2	2		I/S		2	2		>1250	
	2	2		<3620		2	2		1212	1.03
3	A	3	4560			A	3	1140		
	3	3		4770	1.05	3	3		I/S	
	3	3		4350	0.95	3	3		I/S	
	3	3		4540	1.00	3	3		1050	0.92
4	B	4	<3450							
	4	4		3850						
	4	4		<3330						
	4	4		4170						
5	B	5	4560							
	5	5		3450	0.76					
	5	5		3450	0.76					
	5	5		4170	0.91					

I/S = Inadequate slope

Table 2: 50% end-point titres obtained by Laboratory 2

Day	BETA STANDARDS: Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio	EPSILON STANDARDS: Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio
1	A	1	6250			A	1	1120		
	1	1		>5880		1	1		1030	0.92
	1	1		5360	0.86	1	1		1070	0.96
	1	1		I/S		1	1		1110	0.99
	A	2	4560			A	2	1120		
2	2	2		5720	1.25	2	2		1050	0.94
	2	2		5270	1.16	2	2		>1110	
	2	2		4770	1.05	2	2		>1110	
	A	3	5590			A	3	790		
3	3	3		5320	0.95	3	3		< 970	
	3	3		5730	1.03	3	3		< 970	
	3	3		5460	0.98	3	3		< 970	

I/S = Inadequate slope

TABLE 3: 50% end-point titres obtained by Laboratory 3

Day	BETA STANDARDS					EPSILON STANDARDS				
	Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio	Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio
	A	1	5590			A	1	910		
1	1	1		5270	0.94	1	1		910	1.0
	1	1		4840	0.87	1	1		950	1.04
	1	1		5560	0.99	1	1		950	1.04
	A	2	5360			A	2	910		
2	2	2		4770	0.89	2	2		950	1.04
	2	2		5170	0.96	2	2		970	1.07
	2	2		4840	0.90	2	2		950	1.04
	A	3	5590			A	3	910		
3	3	3		5000	0.89	3	3		950	1.04
	3	3		5270	0.94	3	3		1050	1.15
	3	3		5170	0.92	3	3		1000	1.10

TABLE 4: 50% end-point titres obtained by Laboratory 4

BETA STANDARDS

Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio
A	1	3790		
1	1		4564	1.2

EPSILON STANDARDS

Antitoxin Ampoule Number	Toxin Vial Number	Existing Standard	Proposed Standard	Potency Ratio
A	1	1118		
1	1		1118	1.0

TABLE 5 Accelerated Degradation Tests - 50% end-point titres

Time (days)	Temp (°C)	Existing Standard	Proposed Standard
BETA STANDARD			
70	-20		4540
70	+4		4540
70	RT		I/S
70	+37		5000
392	-20	6250	
392	-20		5000
392	+4		5000
392	RT		5000
392	+37		5000
EPSILON STANDARD			
104	-20		1380
104	+40		1380
104	RT		1310
104	+37		1540
147	-20		>1380
147	+4		>1380
147	RT		>1380
147	+37		>1380
286	-20	1160	
286	-20		1210
286-8	+4		1400
286-8	RT		1400
286-8	+37		1400
392	-20	910	
392	-20		980
392	+4		980
392	RT		980
392	+37		980

TABLE 6: Content of selected minor components of the proposed standards

Antitoxin	COMPONENT: anti-Cl. perfringens alpha toxin	COMPONENT: anti-Cl.perfirngens beta toxin	COMPONENT: anti-Cl.perfringens epsilon toxin
Proposed beta standard	10.7 - 13.0 units per ampoule	4770 units per ampoule	24 units per ampoule
Proposed epsilon standard	less than 2.5 units per ampoule	less than 2.0 units per ampoule	1020 units per ampoule

Table 7: Geometric mean potency values (and 95% confidence limits) for tests carried out by 3 Laboratories.

Laboratory	BETA STANDARD		EPSILON STANDARD	
	Number of comparisons	Units per ampoule	Number of comparisons	Units per ampoule
1	9	4730(4250-5270)	4	1000
2	7	5170(4600-5800)	4	950
3	9	4610(4460-4760)	9	1060(1020-1090)
Overall †	26	4770(4550-5000)	19	1020(990-1050)

† including accelarated degradation test results

Appendix 1

Collaborative assay of proposed Second International Standards for Cl.perfringens beta and epsilon antitoxins

Materials Supplied

2 ampoules of the existing Cl.perfringens type B antitoxin (CwBat)
5 ampoules of the proposed new standard antitoxin (2Cp beta At)
5 ampoules of a dried preparation of Cl.perfringens type B toxin

2 ampoules of the existing Cl.perfringens type D antitoxin (CwDAt)
5 ampoules of the proposed new standard antitoxin (2Cp epsilon At)
5 ampoules of a dried preparation of Cl.perfringens type D toxin
Supply of report forms (2 kinds)

Materials required but not supplied

Distilled Water
Peptone broth
White mice, body weight 18-22 grams
Borate buffered saline

Assay programme

The intention of the assay is that collaborators should use their own test methods as far as is practicable, but follow the programme on page 2 exactly. Before embarking on the main programme it will be necessary to carry out preliminary tests to calibrate reagents against the existing standards. Examples of such tests are described on pages 4-9.

We require three separate tests to be performed, on 3 separate days at one week intervals, and on each day 3 separate series of dilutions prepared from the same master solutions. The results from Day 1 to be taken into account when setting up the test for Day 2.

Day 1

	Dilution Series 1, Assay 1
Toxin vial 1 master solution)	Dilution Series 2, Assay 2
CwBat ampoule 1 master solution)	Dilution Series 3, Assay 3
2Cp beta At ampoule 1 master solution)	Toxin calibration 1
	Back titration 1

1 week after day 1,

Day 2

	Dilution Series 1, Assay 4
Toxin vial 2 master solution)	Dilution Series 2, Assay 5
CwBat ampoule 1 master solution)	Dilution Series 3, Assay 6
2Cp beta At ampoule at master solution)	Toxin calibration 2
	Back titration 2

1 week afer day 2,

Day 3

	Dilution Series 1, Assay 7
Toxin vial 3 master solution)	Dilution Series 2, Assay 8
CwBat ampoule 1 master solution)	Dilution Series 3, Assay 9
2Cp beta At ampoule 3 master solution)	Toxin calibration 3
	Back titration 3

Participants may also test the proposed new standard antisera exactly in parallel with the main (compulsory) assays but using their own methods and reagents, particularly their own toxins. Details of all tests to be recorded on the report sheets provided. A separate short form is included for recurring items. The results of all preliminary assays should be included on the report sheets, as well as those of the main assays.

Suggested methods of assay

Beta standard

The existing standard anti Cl. perfringens type B serum (CwBat) contains 5000 International units of antibody per ampoule, by definition.

Reconstitute the entire contents of an ampoule of freeze-dried serum (CwBat) with 1.0ml distilled water. Allow the ampoule to stand for 1 hour after adding the distilled water and wash out with borate buffered saline pH 8.4 (BBS) and add 66% glycerol. It is suggested that the standard for Cl. perfringens beta antitoxin be made up to a total of 50 ml with BBS/glycerol as master solution, incorporating all the washings from the ampoule. Subsequent dilutions are all made in BBS alone. Take care to mix well before use to disperse the glycerol evenly throughout. Make dilutions of CwBat so that 1.0 ml solution contains 10 units of antibody.

Reconstitute the contents of an ampoule of beta toxin (NBX 982) in 20 ml peptone broth. For use dilute a further 1 in 4.7 in broth. Toxin solutions should not be stored in liquid form before use. They should be reconstituted on the day of assay and calibrated on that day. Please indicate on the report form the procedure used. The toxin supplied should suffice for the assays required, although a small reserve will be held at Weybridge for contingencies.

Borate buffered saline (BBS)

Make a stock mixture of:	Sodium pyroborate (Sodium tetraborate)	57 grams
	Boric acid	84 grams
	Sodium chloride	99 grams

Mix dry in an electric blender

For use, mix:	Stock buffer salts	74 grams
	Sodium chloride	15 grams
	Distilled water	5,000 ml
	Final pH 8.4	

Toxin calibration

Prepare the mixtures:

BBS	2.01 ml	1.92	1.8	1.68	1.53
Standard serum (CwBat) (10 i.u. per ml)	0.6	0.6	0.6	0.6	0.6
Toxin solution	0.39	0.48	0.6	0.72	0.87

Mix by inversion. Allow to stand at room temperature for 30 minutes and then inject 0.5 ml intravenously into each of 4 mice. Observe for 5 days and record numbers of dead and survivors at the end of each 24 hour period on the report forms provided. Record the times of deaths that occur in the first few hours after injection.

Also perform a 'back titration' simultaneously with the toxin calibration assay, as follows:-

BBS	2.01 ml	1.92	1.8	1.68	1.53
Standard serum (CwBat) (10 i.u. per ml)	0.39	0.48	0.6	0.72	0.87
Toxin solution	0.6	0.6	0.6	0.6	0.6

Then, if the selected dilution of toxin (e.g. 1/4.7) does not contain one L+ dose in 1.0 ml of solution but still falls within the range of the back titration it will be possible to adjsut the results accordingly. On the assumption that 0.1 ml of the selected toxin dilution contains one L+ dose the first ranging assay of the proposed new standard and the two toxin calibration assays can be carried out simultaneously.

First range to test proposed new Standard antiserum (2 Cp beta At)

Reconstitute the entire contents of an ampoule of 2Cp beta At in the same way as the existing Standard CwBat and prepare a master solution as before.

Mix the master solution of 2Cp beta At well to disperse the glycerol evenly throughout, then make a working solution by diluting 1/10 in BBS. Prepare the range:-

BBS	2.01	1.92	1.8	1.68	1.53
Serum (1 ampoule in 500 ml)	0.39	0.48	0.6	0.72	0.87
Toxin	0.6	0.6	0.6	0.6	0.6

Mix by inversion, leave at room temperature 30 minutes, inject 4 mice and observe as before.

Units per ampoule tested for	7692	6250	5000	4167	3448

Second range (examples)

Once an approximate value has been obtained construct a range at 10% steps and proceed as before (e.g. if value lies between 4167 and 5000 try the range 3750-5555).

BBS	1.86	1.8	1.74	1.68	1.6
Standard serum (10 i.u./ml)	0.54	0.6	0.66	0.72	0.8
Toxin solution	0.6	0.6	0.6	0.6	0.6
Units per ampoules test	5555	5000	4545	4167	3750

Epsilon Standard

The existing standard anti-Cl. perfringens type D serum (CwDat) contains 1000 International units of antibody per ampoule, by definition.

Reconstitute the entire contents of an ampoule of freeze-dried serum with 1.0 ml of distilled water. Allow the ampoule to stand for 1 hour after adding the distilled water and wash out with borate buffered saline pH 8.4 (BBS) and add 66% glycerol. Take care to mix well before use to disperse the glycerol evenly throughout.

It is suggested that the standards for Cl. perfringens epsilon antitoxin be made up to a total of 10 ml with BBS/glycerol as master solution, incorporating all the

washings from the ampoule. Subsequent dilutuions are all made in BBS alone. Make dilutions of CwDat so that 1.0 ml of solution contains 10 units of antibody.

Reconstitute the contents of an ampoule of epsilon toxin (NDX 986) in 2.0 ml peptone broth. For use dilute a further 1 in 25 in broth. Toxin solutions should not be stored in liquid form before use. Dry toxin should be reconstituted on the day of assay and calibrated on that day. Please indicate on the report form the procedure used. The toxin supplied should suffice for the assays required, although a small reserve will be held at Weybridge for contingencies.

Toxin calibration

Prepare the mixtures:

BBS	2.01	1.92	1.8	1.68	1.53
Standard serum (10 i.u./ml)	0.6	0.6	0.6	0.6	0.6
Toxin solution	0.39	0.48	0.6	0.72	0.87

Mix by inversion, allow to stand at room temperature for 30 minutes and then inject 0.5 ml, of each mixture intravenously into each of 4 mice.

Observe for 5 days and record numbers of dead and survivors at the end of each 24 hour period on the report forms provided. Record the times of deaths that occur in the first few hours after injection.,

Simultaneous with this assay perform a back titration of the toxin.

BBS	2.01	1.92	1.8	1.68	1.53
Standard serum (10 i.u./ml)	0.39	0.48	0.6	0.72	0.87
Toxin solution	0.6	0.6	0.6	0.6	0.6

This assay makes allowance for slight deviations in the potency of the test toxin and allows the toxin calibration and first ranging assay of the proposed new standard to be carried out on the same day .

First range to test proposed new standard antiserum (2Cp epsilon At)

Reconstitute the entire contents of an ampoule of 2Cp epsilon At in the same way as the existing Standard CwDat and prepare a master solution as before.

Mix the master solution of 2Cp epsilon At well to disperse the glycerol evenly throughout, then make a working solution by diluting 1/10 in BBS.

Prepare the range:

BBS	1.92	1.86	1.8	1.74	1.68
Standard serum (1 ampoule in 100 ml)	0.48	0.54	0.6	0.66	0.72
Toxin solution	0.6	0.6	0.6	0.6	0.6

Mix by inversion, leave to stand at room temperature for 30 minutes and then inject 0.5 ml of each mixture intravenously into each of 4 mice. Observe for 5 days and record reactions.

Units per ampoule tested for	1250	1111	1000	909	833

Short Report For, recurring Items

Make diluent (used to standardize mixture volumes): i.e. BBS pH 8.4 or your own diluent.

Mice Breed Six Weight range

Toxin/antitoxin mixture incubation temperature
incubation time
volume injected

Appendix 2

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