Biologicals 44 (2016) 556–566

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Collaborative study for the calibration of a replacement International Standard for diphtheria toxoid for use in flocculation test

Laura Coombes [a, *], Peter Rigsby [b], Dorothea Sesardic [a], Paul Stickings [a]

[a] Division of Bacteriology, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK
[b] Biostatistics Section, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK

ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form
22 June 2016
Accepted 29 July 2016
Available online 30 September 2016

Keywords:
International standard
Flocculation
Diphtheria toxoid
Antigenicity

ABSTRACT

We present the results of a collaborative study for the establishment of a replacement International Standard (IS) for diphtheria toxoid for use in flocculation test and its calibration in Lf units. Calibration was performed using Ramon flocculation method, standardized using the 2nd IS. The candidate standard was assigned a unitage of 1870 Lf/ampoule based on results from 25 laboratories in 15 different countries and was established as the 3rd IS for diphtheria toxoid for use in flocculation test by the WHO Expert Committee on Biological Standardization (ECBS) in October 2015.

The study also assessed the use of alternative methods for measuring Lf. Participants were asked to determine the Lf value of the candidate standard using an Enzyme Linked Immunosorbent Assay (ELISA) established at NIBSC, or other suitable in-house method. 10 laboratories performed ELISA according to the NIBSC protocol, 1 laboratory performed flocculation using laser-light scattering according to an in-house protocol, and 1 laboratory performed another in-house ELISA. Results suggest these methods may provide suitable alternatives to the Ramon flocculation test, subject to validation, and that the new standard could act as a suitable reference preparation in these methods.

1. Introduction

Diphtheria is caused by exotoxin-producing strains of the bacterium Corynebacterium diphtheriae. Active immunization against diphtheria is based on the use of diphtheria toxoid (DTxd), a chemically detoxified preparation of diphtheria toxin, to induce protective antibody responses. Diphtheria vaccines form an essential component of the primary immunization schedule of children and have been part of the WHO Expanded Programme on Immunization (together with tetanus and pertussis components) since its inception in 1974 [1]. The bulk toxoid intermediates of diphtheria vaccines can also be used as carrier proteins in polysaccharide conjugate vaccines against invasive bacterial infections caused by Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae [2].

DTxd is produced by growing a toxigenic strain of C. diphtheriae in liquid media and converting the toxin to inactive toxoid by treatment with formaldehyde [1]. Antigenic strength and purity of the bulk toxoid is evaluated by measurement of 'limit of flocculation' (Lf) units. DTxd for use in production of vaccines for human use must meet minimum requirements for purity (Lf units per milligram of protein nitrogen). The current WHO minimum requirement for antigenic purity of DTxd has been set as not less than 1500 Lf/mg of protein nitrogen [3]. Measurement of antigen content in Lf also serves as a good indicator of the consistency of production, and testing of the crude toxin prior to inactivation is recommended for monitoring purposes [3].

Flocculation is an *in vitro* method based on the observation that antigen and antibody aggregate and form visible floccules when mixed in certain proportions in solution. The precipitate develops more rapidly when equivalent amounts of antigen and antitoxin are present than when an excess of either is available [4]. In the original Ramon flocculation method [5–7] the antigen

Abbreviations used: IS, International Standard; ECBS, Expert Committee on Biological Standardization; ELISA, Enzyme Linked Immunosorbent Assay; DTxd, Diphtheria Toxoid; Kf, Flocculation rate (time for first tube to flocculate); Lf-eq, Lf equivalent; RS, Reference Standard.

* Corresponding author. Fax: +44 (0) 1707 641054.
E-mail address: laura.coombes@nibsc.org (L. Coombes).

http://dx.doi.org/10.1016/j.biologicals.2016.07.005

concentration is kept constant and different amounts of antitoxin are added to a series of tubes. The antitoxin content of the first tube to flocculate can be used to calculate the Lf value of the sample. The time in minutes for the first tube to flocculate is known as the Kf value, and is a useful indicator of the quality of the antigen and antitoxin used.

The flocculation unit was originally a relative unit defined as the amount of toxin (or toxoid) equivalent to one IU of antitoxin in the flocculation test (Ramon version) [8]. In 1970 the WHO Expert Committee on Biological Standardization decided to examine the feasibility of defining the Lf unit internationally by means of a reference toxoid preparation calibrated in Lf, rather than by means of an antitoxin preparation [9]. Subsequently the 1st International Reference Reagent of Diphtheria Toxoid for Flocculation Test (DIFT) was established in 1989, calibrated using the fifth International Reference Preparation of Diphtheria Antitoxin for the Flocculation Test (DIF), carrying the valid Lf definition at the time [10,11]. DIFT was replaced with the 2nd International Standard (IS) for Diphtheria toxoid for use in Flocculation test (02/176) in 2007 following depletion of stocks [12].

Due to its simplicity, speed and economy, flocculation remains the primary method used by vaccine manufacturers to evaluate toxin and toxoid concentrations in Lf. Most laboratories use the toxoid IS to calibrate a suitable antitoxin in Lf-equivalent (Lf-eq) units. This antitoxin can then be used routinely to estimate Lf values of unknown toxin or toxoid samples. Various modifications of the original Ramon flocculation test exist, for example, keeping antitoxin concentration constant and adding different amounts of antigen (Dean-Webb method [13]), or varying the concentrations of both components simultaneously (Levine-Wyman method [14]). However different versions of the method give different equivalence amounts, therefore it is important that the same version of the test is used for calibration of the local reference antitoxin and for routine use [11].

Stocks of the 2nd IS (02/176) are in limited supply and a project was initiated to calibrate and establish a replacement standard. Candidate material for the replacement standard was provided to NIBSC for formulation and filling prior to freeze-drying. A similar material from another manufacturer was also provided to NIBSC, and prepared in the same way, for a proposed new Pharmacopoeial Reference Standard (RS) for Diphtheria Toxoid. Collaborative study (NIBSC code CS509) was initiated with the primary aim of calibrating these materials in Lf units using Ramon flocculation test standardized against the 2nd IS. 26 laboratories in 15 countries (Argentina, Belgium, Canada, China, Croatia, Cuba, Denmark, France, Hungary, India, Indonesia, Japan, Republic of Korea, The Netherlands and United Kingdom) participated in the collaborative study and 25 of these performed flocculation assays used for calibration of the candidate standards. A secondary aim of the collaborative study was to assess the suitability of alternative antigen detection methods for measuring Lf of diphtheria toxoid. 10 laboratories performed a capture ELISA assay developed at NIBSC. In addition 1 laboratory returned results from another in-house ELISA and 1 laboratory performed an alternative flocculation method using laser light scattering to obtain a more objective detection of antigen–antibody complexes. The participating laboratories are listed in the Appendix and are referred to throughout this report by a code number, allocated at random, and not related to the order of listing.

2. Materials and methods

2.1. Bulk material and processing

Purified diphtheria toxoids were provided by two different manufacturers and were identified as Toxoid A (candidate replacement IS) and Toxoid B (candidate RS). Both materials complied with the required quality control tests for bulk purified toxoids including safety, sterility and antigenic purity (>1500 Lf/mg PN). Full details of the two materials are summarized in Table 1. Each batch of diphtheria toxoid was stabilized by the addition of 0.1 M sodium chloride and 1% trehalose before freeze-drying. Filling (1 ml per ampoule) was performed within NIBSC's Standard Processing Division on 21st November 2013 (Toxoid A) and 15th May 2014 (Toxoid B) using an automated filling line (Bausch & Stroebel, Ilshofen, Germany). The material was stirred constantly during filling and the temperature was maintained between +4 and 8 °C. The filled ampoules were freeze-dried using a Serail CS100 freeze-dryer (Le Coudray St Germer, France). The freeze-drying programme was initiated on the day of filling and set as follows: The product was frozen over 90 min to −50 °C and held for 4 h before applying vacuum. Primary drying was at −40 °C shelf temperature and 30 μbar vacuum for 40 h. This was followed by a 15 h ramp to +30 °C and a secondary drying at that temperature for a further 20 h. Ampoules were back-filled with dry nitrogen to atmospheric pressure, stoppered in-situ, followed by flame sealing. The finished products were coded 13/212 (Toxoid A) and 14/132 (Toxoid B) and were stored at −20 °C in the dark at NIBSC. The freeze-dried candidate standards 13/212 and 14/132 were labelled as Preparation A and Preparation B respectively for the collaborative study and are referred to as such hereafter.

2.2. Post fill characterization of candidate standards

Freeze-dried candidate toxoids were examined for appearance, residual moisture content, oxygen head space and total antigen

Table 1
Details of bulk material and filled products.

Details of bulk material	**Toxoid A**	**Toxoid B**
Lot number	D-70-01	292202 (diluted)
Antigenic purity	2141 Lf/mg PN	2197 Lf/mg PN
Lf content (manufacturer)	1840 Lf/ml	750 Lf/ml
Lf content (NIBSC ELISA)	1942 Lf/ml	743 Lf/ml
Details of filled product	**Preparation A**	**Preparation B**
NIBSC code	13/212	14/132
No. Ampoules filled	5109	6810
Appearance	Robust homogenous cake	Robust homogenous cake
Mean fill mass	1.01 g (CV 0.21%) ($n = 179$)	1.01 g (CV 0.14%) ($n = 232$)
Mean dry weight	0.03 g (CV 0.62%) ($n = 6$)	0.03 g (CV 2.04%) ($n = 6$)
Mean residual moisture	0.38% (CV 15.27) ($n = 12$)	0.58% (CV 21.70) ($n = 12$)
Mean oxygen head space	0.64% (CV 16.72) ($n = 12$)	0.30% (CV 36.06) ($n = 12$)
Lf content (NIBSC ELISA)	1847 Lf/ampoule	686 Lf/ampoule
Lf content (NIBSC flocculation)	1840 Lf/ampoule	675 Lf/ampoule