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Establishment of an equine tetanus antitoxin reference standard for veterinary use in Japan

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ABSTRACT

To establish the first National Veterinary Assay Laboratory (NVAL) equine tetanus antitoxin reference standard for veterinary use, we manufactured vials of a candidate antitoxin. These were quality tested for moisture content, vacuum, colour, clarity, and the presence of foreign objects. Ultimately, 115 quality-controlled vials were prepared. To estimate the antitoxin potency of the candidate standard, three different laboratories conducted parallel line assays alongside the existing antitoxin standard. These potency estimates ranged from 38 to 42 IU. This activity was maintained for two years after manufacture, as compared with a fresh vial. No statistically significant non-linearity or non-parallelism of the regression lines was observed ($p > 0.05$). Statistical assessment of inter- and intra-laboratory variability revealed acceptable coefficients of variation of 3.2% and 2.4–3.1%, respectively. Based on these results, the potency of the potential reference standard was calculated at 40 units of antitoxin activity per 1-mL vial. Vials of this preparation were distributed for use as the first equine tetanus antitoxin reference standard for veterinary use in September 2015.

1. Introduction

Reference materials are exceedingly important in the quality control of biologicals for veterinary use. To ensure the quality of veterinary biologicals in Japan, some are standardised and distributed as national standard reference materials by the National Veterinary Assay Laboratory (NVAL). National reference standards for tetanus toxoid and tetanus toxin have been established and distributed to veterinarians for use as quality controls [1]. However, NVAL has not yet established a national reference standard for tetanus antitoxin and the currently available reference standard for veterinary use is provided by the National Institute of Infectious Diseases (NIID). There is a limited stock of this reference standard and the establishment of a new national standard for veterinary use is therefore required.

Passive immunization with tetanus antitoxin is the classical method of treating tetanus in human and animals. Several commercial tetanus antitoxin products have been distributed and used in Japan. Prior to distribution, all products are assessed using a toxin neutralization test, where their potencies are expressed relative to that of a national tetanus antitoxin reference standard; the activity of this standard is expressed in IU, a measure that is based on the activity of an international standard [2,3]. According to the minimum requirements for veterinary biological products in Japan, commercial products must include more than 330 IU/mL, calibrated to the reference standard distributed by NIID using a parallel line assay [3–5].

The manufacturers of veterinary tetanus antitoxin products and NVAL need to determine the potencies of these products using an appropriate tetanus antitoxin reference standard. The products are derived from horse and an equine reference standard would therefore be ideal.

Previous reports have described changing international standards from horse serum to human immunoglobulin [7] and

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between different human immunoglobulin standards [8]. These replacements involved collaborative assays that were performed by a number of laboratories and the present study therefore used this approach. We first manufactured a new candidate horse-derived tetanus antitoxin reference standard and then tested its quality. Three different laboratories participated in collaborative assays to quantify the activity of this new reference standard in relation to the current reference standard.

2. Materials and methods

2.1. Preparation of the new candidate reference standard

Vials of tetanus antitoxin were prepared (Fig. 1). This 'Hokken' antitoxin stock serum was manufactured in accordance with the minimum requirements for veterinary biological products in Japan [3]. Briefly, serum was obtained from horses that had been immunised subcutaneously with the tetanus toxin antigen [3]. Throughout the manufacturing process, the activity of the horse serum and stock solutions (IU) were calculated using a mouse neutralisation test (see Section 2.3). Fifty millilitres of horse serum containing 500 IU of antitoxin activity per mL was adjusted to pH 4.5 at room temperature using sodium acetate (50 mM). Caprylic acid (3.3 mL) was added gradually to this solution and mixed well for 30 min at room temperature prior to centrifugation at 1460 × g for 10 min at 4 °C. NaOH (0.1 M) was added to the supernatant to adjust it to pH 6.0 at room temperature. The solution was dialyzed three times against saline for 3 h at 4 °C using a membrane with a molecular weight cut-off of 14,000 and then filtered through a 0.45-μm filter. The solution was adjusted to 41 IU/mL using horse serum from non-immunised animals. This solution was divided into 124 aliquots of 1 mL per vial and freeze-dried for 42 h. The potency, degree of vacuum, moisture content, colour, clarity and the presence of any foreign object was studied for 11, 126, 5, 126, 4 and 4 vials, respectively, as described below.

2.2. Quality control tests

The moisture content of each tested vial was determined and the residual moisture was calculated [3]. The degree of vacuum was determined using a Tesla coil in a darkroom. Each vial was tested for colour, clarity, and the presence of foreign objects. The activity (IU) was determined using the mouse neutralisation test; this testing was conducted on the prepared stock serum, fresh stock solution and one fresh aliquot of the reference standard by Kitasato Daiichi Sankyo Vaccine Co., Ltd., and the stored aliquots were tested again 2 years later by the collaborating laboratories (Section 2.3).

2.3. Mouse neutralisation test

Mouse neutralisation testing was conducted in accordance with the minimum requirements for biological products [2]. The candidate tetanus antitoxin standard was adjusted to approximately 1 IU/mL with 0.017 M phosphate-buffered saline solution (pH 7.0) containing 0.2 w/v% gelatine (PBS-G) prior to testing. The NIID laboratory prepared vials of the current equine tetanus antitoxin standard (Lot 4) containing 41 IU and diluted these to 1 IU/mL with PBS-G. Standardized vials of freeze-dried tetanus toxin (NVAL, Tokyo, Japan) were used for this test. Each vial contained 4.1×10^6 median lethal doses (LD_{50}). The vials were adjusted to 1.0×10^6 LD_{50} with PBS-G, the test toxin dilution. The diluted test toxin was stored at −80 °C until use, within 2 months of dilution.

The current and test equine antitoxin standards were diluted to produce five final concentrations (0.4, 0.45, 0.5, 0.55 and 0.6 IU). The diluted test toxin was further diluted to a concentration of five test doses per 1.0 mL. Two millilitres of each of the current or new antitoxin reference dilutions were combined with 2 mL toxin dilution and mixed well. Each mixture was allowed to stand for 1 h before subcutaneously injecting 0.4 mL into the inguino crural regions of four 4-week-old mice. The mortality rates of the mice were determined daily for 5 days after injection.

In total, 13 mouse neutralisation tests were performed, including those using stock serum, stock solution, the contents of a

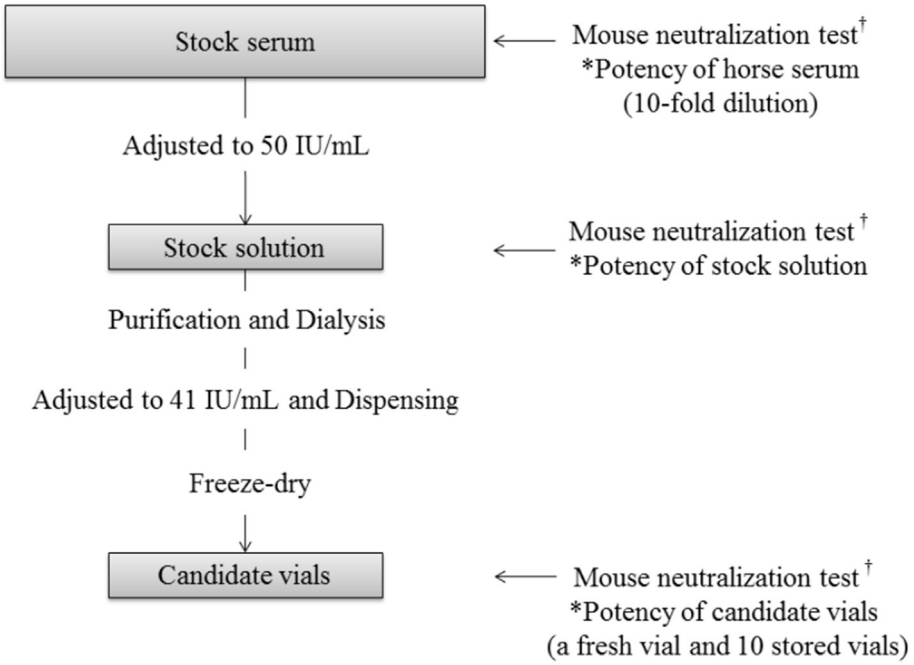

Fig. 1. Manufacturing procedure and potency test points of the new reference standard candidate. †Calibrated using the current equine reference standard.