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Collaborative Study to Evaluate a Candidate International Reference Reagent for Neutralizing Antibodies Against Ross River Virus

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This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **2 October 2023** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technical Standards and Specifications (TSS). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

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Summary

Ross River fever is a mosquito-borne disease characterized by fever and joint pain which can result in significant morbidity due to long-lived polyarthralgia in patients. Ross River fever is caused by Ross River virus (RRV), an arbovirus and member of the *Alphavirus* genus in the *Togaviridae* family. RRV is endemic to Australia, Papua New Guinea and elsewhere in the Pacific region and has epidemic potential being competent for a wide range of mosquito species, even in the absence of preferred enzootic hosts.

Currently, there are no licensed vaccines available to prevent RRV infections. This collaborative study was undertaken with the aim to assess the suitability of a candidate World Health Organization (WHO) International Reference Reagent (RR) for RRV-specific immunoglobulin (IgG) neutralizing antibodies. The potency of the candidate WHO RR and anti-RRV antibody-positive samples obtained from recovered blood donors were evaluated using a range of virus neutralization and immunoassays with the aim of assigning an internationally agreed unitage to the candidate WHO RR. The candidate RR (1500/19) consisted of a lyophilized anti-RRV plasma preparation comprising a pool of five donations from anti-RRV IgG-positive blood donors.

Six additional samples were included in the study: three individual anti-RRV-antibody positive plasma samples, a duplicate of 1500/19 as well as two further samples to investigate specificity – a negative plasma control sample and an anti-chikungunya virus (CHIKV) positive plasma sample; RRV and CHIKV are both members of the Semliki Forest complex of *Alphaviruses* and serological cross-reactivity within the complex has been described.

The collaborative study materials were distributed to 11 laboratories from 5 different countries. The samples were assayed on three separate days and the data were collated and analysed at the Paul-Ehrlich-Institut (PEI). Data were returned by 10 of the participating laboratories. The assays used consisted of a mixture of different types of virus neutralization assays (using RRV or pseudoviruses), binding assays (commercially available, developed in-house) such as enzyme-linked immunosorbent assays, immunofluorescence tests, microsphere-based assays or and haemagglutination-inhibition assays. Laboratories performing neutralization assays used their own virus stocks and in-house developed methods.

The results showed that the candidate RR was well detected by the majority of participants. Intra-assay variation was considerably lower than inter-assay variation for 1500/19. The candidate RR was investigated for its ability to harmonize results and assay variability was substantially reduced, when titres from the panel of anti-RRV positive samples were expressed relative to 1500/19. Harmonization by the candidate RR also applied to the anti-CHIKV antibody positive sample, albeit to a lesser extent.

The candidate RR is stable under recommended conditions of storage, i.e. at or below -20ºC, and is therefore suitable for long term use. On-going real-time and accelerated stability studies of the candidate RR are in progress. It is proposed that 1500/19 be established as the 1st WHO International RR for anti-RRV neutralizing antibodies (IgG) with an assigned unitage of 1,000 units per ml.

Introduction

Ross River virus (RRV), the causative agent of epidemic polyarthritis or Ross River fever, is member of the *Alphavirus* genus in the *Togaviridae* family and is transmitted by a variety of mosquito vectors (Chen *et al*., 2018; Yuen *et al*., 2021). The virus is enveloped, with a positive sense, single-stranded RNA genome (~11.8 kb) that encodes non-structural and structural proteins in two separate open reading frames (Chen *et al*., 2018).

Outbreaks of epidemic polyarthritis were reported in several states in Australia as well as Papua New Guinea in the first half of the twentieth century. It was not until 1959, that RRV was first isolated near the Ross River, Queensland from an *Aedes vigilax* mosquito and the link made to cases of epidemic polyarthritis in Queensland following serological testing (Doherty *et al*., 1963). Isolation of RRV from a human was first reported in 1972 from a child in Northern Queensland with a rash (Doherty *et al*., 1972) and subsequently from a horse (Pascoe *et al*., 1978). There are a large number of potential vertebrate hosts of RRV including both domestic and wild animals. Macropods such as kangaroos and wallabies seem to be the most important reservoirs of RRV, however, infections have been identified in cats, dogs, horses, bats and possums (Clafin and Webb, 2015). Evidence suggests that although marsupials are important RRV hosts, they are not essential for virus circulation, since RRV infections occur where marsupials are absent on islands such as French Polynesia, Fiji and American Samoa in the Pacific (Lau *et al*., 2017; Togami *et al*., 2020). In some instances, because of the high level of viraemia in human cases of RRV infection, there is evidence of human–mosquito–human transmission (Harley *et al*., 2001).

More than 40 species of mosquitoes in Australia are known to act as reservoirs of RRV and of these, at least 10 species have been shown to be competent as vectors based upon laboratory experiments (Clafin and Webb, 2015). The mosquito vectors belong to *Aedes* and *Culex* species found in coastal mosquitoes (*Aedes camptorhynchus* and *Aedes vigilax*), freshwater (*Culex annulirostris*) as well as urban environments (*Aedes notoscriptus*) in Australia and beyond (Clafin and Webb, 2015; Walker *et al*., 2018).

Ross River fever is the most prevalent arboviral disease in Australia and RRV infections are notifiable with approximately 5,000 cases reported each year throughout Australia, although this is likely to be an underestimate (https://www.health.gov.au/diseases/ross-river-virus-infection). However, RRV infections have been found beyond Australia and infections occur in Papua New Guinea. In 1979-1980, the largest ever outbreak occurred with tens of thousands of infections in the Pacific Island Countries and Territories where RRV infections had not been previously reported (Lau *et al*., 2017). RRV infections have been identified not only in Australia and Papua New Guinea where the infection is endemic, but also in the Solomon Islands, Fiji, the Cook Islands, American Samoa, New Caledonia, Wallis and Futuna and French Polynesia. One recent study identified RRV sequences in mosquitoes captured in Yunnan province, China, which borders Myanmar, Laos and Vietnam (Feng *et al*., 2022).

Occasionally, RRV infections occur in travellers returning from endemic regions, including Australia as well as the Pacific islands. Examples of countries where RRV has been imported include Germany (Pröll *et al*., 1999; Tappe *et al*., 2009; Cramer *et al*., 2011; Schleenvoigt *et al*., 2015), Singapore (Hossain *et al*., 2009), New Zealand (Lau *et al*., 2012), Japan (Tochitani *et al*., 2014), and the Netherlands (Reusken *et al*., 2015). Such importations may pose a potential risk of RRV becoming established elsewhere (Shanks, 2019), similar to other emerging arboviruses such as Zika virus (ZIKV) and chikungunya virus (CHIKV), where one or two mutations in the viral

envelope enabled CHIKV to infect *Ae. albopictus* mosquitoes and widening the geographic range of the virus (Tsetsarkin and Weaver, 2011).

Symptoms of Ross River fever usually develop within 3 to 11 days after the bite of an infected mosquito. The symptoms typically include pain and stiffness that can affect multiple joints, myalgia, weakness, fatigue, fever, rash, headache as well as swollen lymph nodes (Mackenzie *et al*., 2017; Yuen *et al*., 2021). Similar to closely related virus infections such as chikungunya fever, polyarthralgia following RRV infection may last months or even years causing significant morbidity. Approximately 50-75% of RRV infections are asymptomatic (Farmer and Suhrbier; 2019). Viraemia coincides with the onset of symptoms of Ross River fever, with IgM responses appearing ~ 4 days after the start of the illness and lasting ~1-3 months. Specific anti-RRV IgG antibodies appear within ~2 weeks after the appearance of IgM antibodies. The levels of IgG decline over time, however, they are long-lived. Confirmation of infection by RRV is performed by paired serological testing with a >4-fold increase in titres by enzyme-linked immunosorbent assay (ELISA), in combination with disease presentation, although results may be affected by false positive/negative results particularly for IgM (Farmer and Suhrbier; 2019). Very few commercial diagnostics tests are available; diagnosis outside of endemic regions is performed by specialist reference laboratories.

As no specific treatments are approved for Ross River fever, patients are usually given supportive care and prescribed analgesics and anti-inflammatory drugs to treat symptoms. Prevention of RRV infections relies on mosquito control, prevention of bites and avoidance of high-risk areas and the use of insect repellents and bed nets and screens (Walker *et al*., 2018).

There are currently no licensed vaccines available for human use. Both the RRV E1 and E2 envelope glycoproteins are targets for neutralizing antibodies, in particular the A and B domains of E2 (Powell *et al*., 2020a; Powell *et al*., 2020b). The E1 and E2/E3 proteins form heterodimers, after proteolytic cleavage of the E3 protein, E1/E2 are expressed as a trimeric spike on the surface of the virus that facilitates Mxra8 arthritogenic alphavirus receptor binding (Zhang *et al*., 2018). One candidate vaccine against RRV has been investigated in clinical trials. The candidate is an aluminium adjuvanted formalin- and UV-inactivated whole virus vaccine, prepared by infection of Vero cells with RRV. In an early phase study, the candidate vaccine was well tolerated and showed good immunogenicity in human volunteers not previously exposed to RRV (Aichinger *et al*., 2011). In passive transfer experiments, human vaccinee sera protected adult mice from viraemia and young mice from arthritic disease following challenge with RRV (Holzer *et al*., 2011). In a phase III clinical trial 1,755 adult volunteers aged 16 to 59 years and 209 older adult volunteers aged > 60 years, received three doses of 2.5 µg of the adjuvanted candidate vaccine on day 0, day 21 and day 180. There were few adverse events, including no arthritic signs. Although not an efficacy trial, neutralizing antibodies (with titers of > 1:10) were present in sera from 91.5% of the younger group and 76% of the older age group. It was also reported that 89.1% of the younger age group and 70.9% of the older age group achieved titres of > 11 "PanBio units" using the PanBio, commercially available ELISA (Wressnigg *et al*., 2015). In the earlier clinical trial, it was established that the PanBio cut-off of ≥ 11 "PanBio units" is equivalent to a neutralization titre of 1:5.7 (Holzer *et al*., 2011). Although, it is unclear which specific RRV protein is used as the target antigen of the PanBio ELISA since the package insert simply states "RRV antigen". However, it should be noted that for serological assays such as ELISAs, changes in composition of reagent lots may be a source of variation between different batches of the final product. The use of a well-characterized reference preparation or standard, would be of value both for monitoring of batch-to-batch consistency and for result reporting in a common "*unit of measure*" by different assays determining the same analyte(s) (Baylis *et al*., 2021). However, it should be noted that for serological assays such as ELISAs,

changes in reagent lots are the greatest source of normal variation and the use of a standard will be of value for monitoring purposes (Baylis *et al*., 2021).

The aim of this study was to develop a suitable antibody reference material to be able to compare results of neutralization assays for anti-RRV antibodies, including those generated during natural infection as well as those produced in response to different candidate vaccines. This project was endorsed by the World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) in March 2023. It is hoped, that the use of such a reference material will be useful in determining antibody titres that better define protection against RRV, especially where clinical trials designed to evaluate efficacy are not feasible. Clinical diagnostic testing for anti-RRV antibodies also lacks standardization. Reference material for anti-RRV antibodies will be useful for serological assay standardization, mainly as controls for assay performance e.g. batch testing. More standardization will result in better understanding of RRV (sero-)epidemiology in Australia and beyond.

The Paul-Ehrlich-Institut (PEI), Federal Institute for Vaccines and Biomedicines, is a WHO Collaborating Centre for both the quality assurance of blood products and *in vitro* diagnostic devices and for the standardization and evaluation of vaccines. The PEI has developed a candidate anti-RRV antibody preparation for testing and comparison across assays and laboratories to evaluate its suitability as a WHO RR. This study evaluated the potency of the proposed candidate WHO International Reference Reagent (RR) for anti-RRV antibodies in parallel with other antibody preparations obtained from anti-RRV antibody positive blood donors, using assays in routine use in the participants' laboratories. The aim was to demonstrate assay harmonization using the candidate RR and agree on an assigned unitage for the candidate standard following statistical analysis of the study data at the PEI.

Study materials

Candidate International Reference Reagent– 1500/19

The candidate RR (1500/19) was prepared using a pool of 5 anti-RRV antibody-positive plasma donations each with a volume of 320 ml. The individual plasma donations were collected in Townsville, Australia during 2017. The donations were screened for the presence of anti-RRV IgG antibodies initially by enzyme-linked immunosorbent assays (ELISAs) and confirmation using haemagglutination inhibition assays and microneutralization. The individual plasma donations tested positive for anti-RRV antibodies, but were negative for other *Alphavirus* antibodies and anti-DENV and anti-ZIKV antibodies using a mixture of ELISAs and viral pseudotyping assays (Henss *et al*., 2019).

The plasma samples from the donor were tested by NAT to ensure the absence of Human Immunodeficiency Virus Type 1 (HIV-1) Group M RNA, HIV-1 Group O RNA, Human Immunodeficiency Virus Type 2 (HIV-2) RNA, Hepatitis C Virus (HCV) RNA, and Hepatitis B Virus (HBV) DNA using the cobas TaqScreen MPX Test, v2.0 (Roche Diagnostics GmbH, Mannheim, Germany). No HIV-1/2 RNA, HCV RNA or HBV DNA were detected. The donations were tested for RRV RNA using an in-house NAT assay. Extraction of RNA was performed from 200 µL of plasma using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH, Hilden, Germany). Elution of the viral nucleic acid was performed using 70 µL of elution buffer, and 5 µL of the eluate was used for the RT-PCR. RRV RNA was detected using reverse-transcription real time PCR and

the following primers: 5'-GGAAGAAGGGATTGAGTACCA and 3'-TCGTCAGTTGCGCCCA and probe 5'-6 FAM-AACAACCCGCCGGTCCG-BBQ. Amplification reactions were performed using the Multiplex RNA Virus Master Kit (Roche Diagnostics GmbH, Mannheim, Germany). Detection of RRV RNA was performed using the LightCycler 480 (Roche Applied Science GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. None of the donations were reactive for RRV RNA. In addition, the plasma was tested for the presence of CHIKV RNA, DENV RNA and ZIKV RNA using the ExiPrep™ Dx Viral RNA Kit (Bioneer Corporation, Daejeon, Republic of Korea) on the ExiPrep™ 16 Dx platform incorporating an internal control. The entire eluate was analyzed by PCR; set-up was performed using the ExiSpin™ device (Bioneer Corporation) and amplification/detection reactions using the Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer Corporation) using the AccuPower® ZIKV(DENV,CHIKV) Multiplex Real-Time RT-PCR Kit (Bioneer Corporation). The plasma samples were negative for CHIKV RNA, DENV RNA and ZIKV RNA.

Collection and use of the plasma for development of the candidate RR was approved by the Australian Red Cross Blood Service Human Research Ethics Committee (Reference number: HFaddy 90914).

For the lyophilization the pool of plasma donations was diluted 1:1 with cell culture grade water. Processing was performed during January 2019. For the processing, 1.0 ml volumes were dispensed into 7 ml amber glass vials. In total, 3170 vials were prepared. After completion of the freeze-drying procedure, the vacuum was broken by the introduction of nitrogen gas and the vials sealed and capped with Flip Off Tear Off caps. All the operations were performed in a qualified class A clean room (for filling equipment and in front of the freeze dryer). Residual moisture was determined by Karl Fischer analysis. The number of filled vials, coefficient of variation of the filled volume and residual moisture content of the vials is shown in the production summary (Table 1); all parameters are in compliance within WHO guidance (2004). The filling and lyophilization was performed at the Division of Reference Standards European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France. Vials of the candidate standard are intended for reconstitution in 0.5 ml of cell culture grade water.

Vials of the candidate standard are held at the Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany. The vials are kept at -20°C with continuous temperature monitoring.

All manufacturing records are held by PEI and are available on request by the WHO Expert Committee on Biological Standardization.

Additional samples

Several anti-RRV-positive samples from anonymous Australian blood donors were included in the study to evaluate, in a limited way, commutability of the candidate RR. The 1st WHO International Standard for anti-chikungunya immunoglobulin G (1502/19) was prepared from three plasma donations from a German patient who contracted chikungunya fever whilst travelling in Brazil in 2016 (Baylis *et al*., 2022). The anti-CHIKV plasma was included in order to control for specificity since RRV, like CHIKV is also an *Alphavirus* and both belong to the Semliki Forest complex where antigenic cross-reactivity is well known. A pool of 15 plasma donations, sourced from the United States of America, were included as a negative control, obtained from healthy blood donors from the United States of America; all donations were tested and found negative for the presence of HBV, HCV and HIV-1/2 using the cobas® TaqScreen MPX Test v 2.0.

The candidate RR (provided in duplicate), the other anti-RRV-positive samples and the specificity controls are shown in Table 2; all samples have been given a code number S1-S7. Samples were dispensed into volumes suitable for the different assays used by each participating laboratory and stored as liquid/frozen materials. Samples were provided in triplicate. One sample, the pool of anti-CHIKV antibody-positive plasma donations (1502/19), was lyophilized – donation testing and processing was performed as described above.

Collaborative Study

Eleven laboratories from 5 different countries volunteered to participate in the study. In total, 10 laboratories returned results and are listed in Appendix 1. Laboratories from 5 different countries returned results: Australia (3), Austria (1), Germany (3), Malaysia (1), and the United States of America (2). One laboratory from Australia was unable to return results due to the onset of the Coronavirus disease 2019 (COVID-19) pandemic. The participating laboratories included specialist arbovirus laboratories, vaccine manufacturers, clinical virology laboratories, as well as developers of *in vitro* diagnostic devices. For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing in Appendix 1.

All collaborative study materials were shipped to participating laboratories on dry ice and participants were requested to store the materials at or below -20°C until use. The samples included in the panel are described above and listed in Table 2. Participants were asked to test the panel using their routine assay for anti-RRV IgG antibodies, testing the panel of samples in three separate assay runs, using fresh vials of each sample for each run and performing two independent dilution series where possible. The study protocol is outlined in Appendix 2. For the preparation of dilutions, participants were requested to use their usual diluent.

Several lyophilized preparations were evaluated in the study and these were reconstituted before use by participants using cell culture grade water. Samples S1, S4 and S5 which were all lyophilized were reconstituted in 0.5 ml of water. All other samples were provided as liquid/frozen materials.

Statistical Methods

The evaluation of raw data was performed with CombiStats version 6.1 (European Directorate for the Quality of Medicines and HealthCare/Council of Europe, Strasbourg, France) - using a sigmoid, 4-parametric dose-response model (quantitative data) and a quantal response model (qualitative data - probit-transformed). Both methods are described in detail in the European Pharmacopoeia, chapter 5.3 (Council of Europe, 2021). With both models, the 50% plaque reduction neutralization or neutralization titres ($PRNT_{50}$ or NT_{50}, respectively) or EC_{50} titres (other assays) were estimated (i.e. the dilution/titre at which 50% of the maximum signal could be observed, or, for qualitative data, the cut-off between positive and negative signals).

Further statistical analysis (i.e. estimation of a consensus value for all combined datasets) was performed with SAS®/STAT software, version 9.4, SAS System for Windows (SAS Institute, Inc., Cary, NC, USA). Individual estimates (both $PRNT_{50}/NT_{50}$ and EC_{50} and potencies relative to samples S1 or S5) were combined using a mixed linear model with random factor 'assay type' (neutralization or binding/other assay) and 'participant'. Combined estimates were accompanied with 95% confidence intervals. The relative potencies of the panel of samples were estimated relative to the candidate standard i.e. sample S1 (1500/19) with an assigned potency of 1,000 units (U)/ml.

The coefficient of variation was used to describe the relative variability of the measurements. The influence of relevant factors (as participant, assay type, sample) on the intermediate precision as well as the intra-assay precision (repeatability) was evaluated by means of a mixed linear model (an analysis of variance, ANOVA, using fixed and random factors) using log transformed EC_{50} estimates of the individual results. This method uses (restricted) maximum likelihood estimates, which may lead to a small difference between the estimated variance and the usual variance estimator. The inter-assay precision (intermediate precision) was estimated as sum of the variances of the relevant factors and is described by the coefficient of variation. For the intra-assay precision (repeatability) the residual variance was used. The measurement uncertainty is described as the estimated total variance from the ANOVA (sum of variances from relevant factors plus residual variance), also denoted as coefficient of variation.

Stability Studies

Stability of the candidate standard is under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the candidate standard have been stored at -20°C (the recommended storage temperature) as well as baseline samples stored at -80°C. For the accelerated thermal degradation, vials were incubated at +4°C, +20°C, +37°C and +45°C for 2 weeks, 1 month, 3 months, 6 months, 12 months and 36 months. Following incubation at the respective temperature, the vials were stored at -80°C until analysis. For analysis, the contents of the vials (one vial per time point/temperature) were reconstituted in 0.5 ml of cell culture grade water and tested for anti-RRV IgG antibodies using an in-house ELISA developed at the PEI.

Stocks of the RRV T48 prototype strain were generated using an infectious clone containing the full-length virus sequence (a kind gift from Richard Kuhn, Purdue University), as described previously (Kuhn *et al.*, 1991). For the ELISA, Nunc-Immuno 96-well MaxiSorp ELISA plates (Thermo Fisher Scientific, Darmstadt, Germany) were coated with 10^6 plaque forming units of RRV per well in 100 µL PBS at 4°C overnight. After blocking for 1 h with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), the samples for testing were diluted 1:200 (samples were diluted in PBS containing 0.05% Tween 20 and 1% BSA) and added to the plates in triplicate, and incubated for 1 h at 37°C. A goat anti-human IgG secondary horse radish peroxidase (HRP)-coupled antibody (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:2,500 was then added for 1 h at room temperature. For detection, 3,3′,5,5′-tetramethylbenzidine substrate (Merck Millipore, Darmstadt, Germany) was added, and the reaction was stopped after 15 min with 1 M H_2SO_4. Absorbance was measured at 450 nm (reference wavelength 620 nm) with a Tecan spark reader (Tecan, Männedorf, Switzerland). The titres of the samples were expressed relative to the baseline samples stored at -80°C.

Results

Data received – collaborative study

Data were received from a total of 10 of the 11 participating laboratories. Overall, 12 datasets were returned all of which could be further evaluated. Virus neutralization data were determined by 5 laboratories (live virus (n=4) and pseudovirus (n=1)). Data from ELISA methods were returned by 3 laboratories representing both commercial (n=1) and in-house developed assays (n=2). Further immunoassays include a microsphere-based assay, haemagglutination inhibition assay as well as

two indirect immunofluorescence assays (IFAs – one commercial assay and one developed in-house).

Some laboratories reported results for more than one type of assay. Where a laboratory performed more than one assay method, the results from the different methods were analysed independently, as if from separate laboratories, and coded, for example, laboratory 9A and laboratory 9B.

The types of methods used by the participants are listed in Table 3.

Neutralization assays

The combined means for the $PRNT_{50}$ and NT_{50} titres from the different laboratories are shown in Table 4 and in histogram form in Figures 1-7, upper panel (neutralization data shaded light blue) and by box and whisker plot in Figure 8 (upper panel). The titres were determined by PEI based upon the data provided by the participants. For all the neutralization assays, laboratories used their own virus stocks and in-house developed methods (Table 3). Consistent detection of all positive anti-RRV antibodies was reported by the participating laboratories using neutralization assays. Laboratories 1, 2, 3 and 9A all performed neutralization using RRV stocks, in the case of Laboratory 10A, neutralization was performed using a pseudotyped lentiviral vector, however, reported potencies of the pseudotyped assay were in a similar range compared to the potencies determined by the conventional neutralization assays.

The mean titres were within ~0.8 log_{10} range for sample S1 (1500/19) and its replicate S5. A similar range of values was observed for the remaining anti-RRV antibody positive samples included in the study.

Sample S4 which contained anti-CHIKV antibodies, was detected by all laboratories, albeit with low titres with the exception of Laboratory 9A where it was not possible to estimate a result. With the exception of laboratory 10A, all laboratories reported sample S6 as negative (Table 4).

Relative Potencies – Neutralization assays

On the basis of the combined data from the neutralization assays, the mean neutralizing titres were expressed relative to sample S1 (1500/19) with an assumed potency of 1,000 U/ml. The relative potencies are shown in Table 5. Figures 2-7, lower panels show the relative potency data for all assays (virus neutralization data is shaded blue) in histogram form and by box and whisker plots (Figure 8 lower panel). Expression of the relative potencies for all the anti-RRV antibody positive samples resulted in a reduction in the variation between assays (with the range varying from ~0.17-0.3 log_{10}) when potencies were compared to S1 (1500/19). There was a modest reduction (with the range varying by ~1.13 log_{10}) in the variation when S4 (anti-CHIKV) was expressed relative to S1; however, this was not as marked as for the anti-RRV samples.

Figure 8 shows the relative potency data across the range of samples for the neutralization methods; the upper panel shows the mean potencies and the lower panel shows the mean potencies relative to S1 showing significant reduction in variation. The relative potency data provide some evidence for commutability of the candidate RR with the donor samples included in the study.

Binding assays

Several antibody binding assays were evaluated in the study; for the analysis, the following immunoassays have been grouped together: ELISAs, assays based in microspheres, haemagglutination inhibition or IFAs. The mean potencies of the anti-RRV-antibody titres determined by the immunoassays are shown in Table 6. Figures 1-7 (upper panels) illustrate, in histogram form, these mean potencies (shaded in violet). Across the assays, sample S1 (the candidate RR – 1500/19) and its' replicate S5, show almost identical potencies for the respective assays (Table 6). Overall, the range of potencies varies by up to ~ 3 $\log_{10}$. If the haemagglutination inhibition assay data are excluded, the range of potencies is 0.8-1.1 $\log_{10}$.

Laboratory 4 used the only commercially available ELISA for evaluation of the study samples. All the anti-RRV antibody-positive samples were found to be reactive. In the case of sample S4 positive for anti-CHIKV antibodies, the sample was reactive at a 1:100 dilution; in the case of sample S6, the negative control, reactivity was only observed at the 1:10 dilution.

Laboratory 9B used an ELISA developed in-house. The laboratory reported that a high background was observed for some of the samples (S1, S5, S6 and S7); however, although the assay validity criteria were met, the reasons for the background are unclear. All other samples gave acceptable results i.e. S2 and S3 were found positive whilst S4, positive for anti-CHIKV antibodies was found positive as a 1:100 dilution in two of the three assay runs; the third assay run was non-reactive.

Laboratory 10B used an in-house ELISA and all the anti-RRV antibody-positive samples were found to be reactive; no reactivity was observed for either S4 or S6.

Laboratory 6 used an in-house developed multiplexed microsphere immunoassay able to detect anti-RRV antibodies in a panel of target *Alphaviruses* which included CHIKV, Barmah Forest virus (BFV), Sindbis virus (SINV) as well as RRV. Laboratory 6 correctly identified that sample S4 contained anti-CHIKV antibodies as well as correctly identifying all the anti-RRV antibody samples; no reactivity was observed with the negative control sample (S6).

Laboratory 8 tested the panel of samples by haemagglutination inhibition using inactivated RRV. All the anti-RRV antibody-positive samples included in the panel were correctly reported as reactive with differing titres. Both the anti-CHIKV antibody-positive sample (S4) and the negative plasma control (S6) were found non-reactive. The haemagglutination inhibition assay gave consistently lower potencies than the other assays for all the anti-RRV antibody-positive samples.

Levels of anti-RRV binding antibodies were determined by IFA by Laboratory 5 using a commercially available assay and by Laboratory 7 using an in-house developed method. In the case of both laboratories, all anti-RRV antibody-positive samples were reported as reactive as well as the anti-CHIKV antibody-positive sample (S4), albeit at a lower titre; 1:100 in the case of Laboratory 5 and 1:320 in the case of Laboratory 7. The finding that both laboratories using IFAs found that sample S4 which contains anti-CHIKV antibodies was reactive is because, CHIKV, like RRV, belongs to the Semliki Forest complex of *Alphaviruses* where such cross-reactivity is well known (Henss *et al*. 2019; Nguen *et al*., 2020). The negative control sample (S6) was found non-reactive in both of the IFAs.

Relative potencies – Binding assays

On the basis of the combined data from the binding assays (immunoassays including the IFAs), the mean titres were expressed relative to samples S1 (1500/19) the candidate RR. The assumed potency was 1,000 U/ml for S1. The relative potency data are shown in Table 7 (relative to S1 – 1500/19).

Figures 1-7 (lower panels) show the relative potency data for all assays (the immunoassay data are shown in violet) in histogram form and by box and whisker plot in Figure 9, lower panel.

Expression of the relative potencies for all the anti-RRV antibody positive samples resulted in a reduction in the variation between assays (with the range varying from ~0.11-0.34 $\log_{10}$) when potencies were compared to S1 (1500/19). Whilst the haemagglutination inhibition assay gave lower potencies for the anti-RRV samples, the relative potency analysis resulted in much better agreement with the other data sets. The harmonization applied, in a more limited way (~0.7 $\log_{10}$), to the anti-CHIKV antibody-positive sample where it was detected.

Determination of Overall Laboratory Means – Combined Results

The overall mean values and the range of estimates (for all types of assay i.e. virus neutralization as well as the other types of immunoassay) for the candidate RR S1 (1500/19) and the other samples is shown in Table 8. For the data presented in Table 8, it is clear that sample S1 and the replicate sample S5 are very close in value i.e. 2.82 $\log_{10}$ and 2.84 $\log_{10}$, respectively. The overall relative potencies against sample S1 (1500/19) are shown in Tables 9. Comparing the 95% confidence intervals for the panel of samples in Table 8 with those shown in Tables 9 (relative potency data), for each anti-RRV sample there is a clear reduction in the 95% confidence interval when data is expressed relative to the candidate RR i.e. S1 (1500/19) further demonstrating the value of using a standard to facilitate harmonization of results.

Inter- and intra-assay variation

It was possible to compare inter-and intra-assay variability for the replicate candidate RR preparation S1/S5 - the data are shown in Table 10. As expected, inter-assay variability (208 %) far exceeds intra-assay variability for the replicate samples (29 % for samples S1/S5).

Results of Stability Studies

Accelerated thermal degradation studies have been performed by incubation of vials of 1500/19 at higher temperature i.e. +4°C, +20°C, +37°C and +45C° and compared to vials stored at -20°C, the normal storage temperature, and the baseline samples stored at -80°C (Table 11). There was a slight drop in titres at the higher temperatures e.g. after 3 months incubation at +37°C the antibody titre had fallen by approximately half.

After 3 years, there was no evidence of loss of titre of the candidate RR when stored at -20°C the normal storage temperature, moreover storage of the vials at +4°C for 3 years did not result in a loss of potency. Collectively, these data indicate acceptable stability of the candidate RR.

Conclusions

In this study, a range of virus neutralization protocols and immunoassays were used to evaluate a candidate RR for anti-RRV IgG antibodies. In general, the panel of samples, including the candidate RR, were well detected by the participating laboratories, however, data demonstrated wide variations in potencies of the candidate RR and the other study samples which is not unexpected and

regularly observed in similar collaborative studies. However, the aim of such studies is to investigate whether candidate standards can be used to harmonize data from different assays. Indeed, it was found that normalization of results using candidate RR (1500/19) was able to harmonize data between the virus neutralization assays as well as the immunoassays. Furthermore, the relative potency data provide some evidence for commutability of the candidate RR for evaluation of individual donor samples included in the study.

With the RRV neutralization assays, laboratories used their own virus stocks. Laboratory 2 used a RRV isolate (SW2089) recovered from an *Anopheles annulipes* mosquito captured in Mandurah, Western Australia in 1988 (Sammels *et al*., 1995). Laboratory 3 used the RRV candidate vaccine virus that was isolated from a patient with symptoms of epidemic polyarthritis in 1990 in North East Australia (Yu and Aaskov, 1994). Laboratory 10A, using a lentiviral vector pseudotyped with the T48 prototype RRV strain. T48 was originally isolated near the Ross River, Queensland in 1959 from an *Aedes vigilax* mosquito (Doherty *et al*., 1963). In the case of Laboratories 1 and 9A, the RRV isolate was not disclosed. Four genotypes of RRV have been identified (Michie *et al*., 2020) T48, the prototype RRV strain belongs to genotype 1 and SW2089 belongs to genotype 3. Sequence data are not available publicly for the RRV candidate vaccine strain. Regarding the potency data, in the case of Laboratory 3, where the RRV candidate vaccine virus was used in the neutralization assays, the potency of the samples was always lower than the other laboratories performing neutralizations. Laboratories 2 and 10A using SW2089 and T48, respectively reported similar potencies, irrespective of the RRV genotype. For all neutralization assays, relative potency analysis significantly improved the agreement between laboratories demonstrating the importance of using a standard.

There were a very small number of cases where sample S6 was reported as sporadically reactive for anti-RRV antibodies and titres were very low, demonstrating, in general very good specificity across the assays used in the study, irrespective of the format.

In addition, in order to investigate cross-reactivity with other *Alphaviruses* a plasma pool of anti-CHIKV was included in the study. The individual plasma samples in the anti-RRV plasma pool had been previously characterized using a range of assays (including ELISAs and viral pseudotyping – Henss *et al*., 2019) to ensure that they were specific for anti-RRV and that antibodies to other *Alphaviruses*, that might be expected to be found in Australia, were not present. Across all assays, ~67% found sample S4 reactive for anti-CHIKV antibodies. This observation is not unexpected, because CHIKV, like RRV, belongs to the Semliki Forest complex where such cross-reactivity is well known (Henss *et al*. 2019; Nguen *et al*., 2020). Relative potency analysis of sample S4 (anti-CHIKV pooled plasma) against sample S1 demonstrated harmonization of the reported potencies. This observation would suggest it might be possible to use S1 for standardization of anti-CHIKV assays, however, the 1st WHO International Standard for anti-CHIKV (sample S4) is more appropriate for this purpose being established in 2022 (Baylis *et al*., 2022).

For the relative potency analysis, candidate RR anti-RRV antibody sample S1 (1500/19) was assigned an arbitrary unitage of 1,000 U/ml. We propose that 1500/19 be established as the 1st World Health Organization International Reference Reagent for anti-RRV antibodies (immunoglobulin G) with a unitage of 1,000 units (U)/ml for neutralizing activity following reconstitution in 0.5 ml of cell culture grade water. Clearly, the RR will be valuable for harmonization of anti-RRV antibody neutralization assays. Although harmonization of assays determining binding antibodies was demonstrated during the study, in this context, the RR should be used rather more cautiously i.e. simply as a control reagent assuring assay performance (Baylis *et al*., 2021) – with no unitage being defined for this purpose and being applicable for the detection of

binding antibodies of defined specificity e.g. anti-RRV E2 IgG, the material should not yet be used to compare between groups of assays with different specificities. Use of a standard will be important in the context of vaccine studies, to better define potential correlates of protection.

Real-time stability studies have indicated that the candidate RR is stable under normal conditions of storage, i.e. at -20°C or below for 3 years and therefore suitable for long term use as well as at elevated temperatures, i.e. after 1 year incubation at +20°C there was no significant reduction in anti-RRV antibodies which would support shipment at ambient temperature. On-going studies on the real-time stability under normal storage conditions as well as studies concerning thermal degradation are in progress.

In conclusion, with the epidemic potential of RRV and the difficulties in determining efficacy in clinical trials, the establishment of a standard is an important step forward in providing a reference material with a traceable common reporting unit to be able to compare results obtained in different laboratories and better define protective levels of antibodies. Better standardization is essential in order to facilitate a better understanding of RRV (sero-) epidemiology in Australia and the surrounding areas including Papua New Guinea, islands in the Pacific region and beyond, and the similar clinical presentation with viruses such as BFV and CHIKV mean that accurate diagnostic testing is essential.

Recommendations

Based upon the results of the collaborative study, it is proposed that the pooled plasma sample from anti-Ross River virus-positive blood donors, code number 1500/19, should be established as the 1st World Health Organization International Reference Reagent for anti-Ross River virus neutralizing antibodies (IgG) with a unitage of 1,000 units (U)/ml. Potential correlation between neutralizing antibodies and (certain types of) binding antibodies needs to be established by further studies. The custodian laboratory is the Paul-Ehrlich-Institut.

Approximately 2,900 vials are available for distribution (containing 0.5 ml of lyophilized plasma residue per vial).

Comments from participants

After circulation of the draft report for comment, replies were received from eight participants. The majority of the comments were editorial in nature and the report has been amended accordingly. All responding participants were in agreement with the conclusions of the report.

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Table 1. Production summary for the candidate RR 1500/19

Code	1500/19
Name	Anti-RRV
No. vials	3170
Presentation	7 ml glass vials, flip off-tear off seals
Nominal fill volume*	1 ml
Mean fill mass (g)	1.0084 (n=34)
CV of fill weight (%)	0.05 %
Mean residual moisture (%)	0.95 % (n=8)
CV of residual moisture (%)	6.38 %

*Vial contents to be reconstituted in 0.5 ml of cell culture grade water
CV - coefficient of variation; n - number of vials tested
Fill mass was determined at regular intervals throughout the fill

Table 2. Collaborative study materials

Study code number (PEI code number)	Presentation	Description of preparation
S1 (1500/19)	Lyophilized	Pool of five anti-RRV antibody-positive Australian plasma donations
S2	Liquid/frozen	Single anti-RRV antibody-positive plasma donation
S3	Liquid/frozen	Single anti-RRV antibody-positive plasma donation
S4 (1502/19)	Lyophilized	Pool of three plasma donations from a German convalescent patient infected with CHIKV in Brazil (Baylis *et al.*, 2022)
S5 (1500/19)	Lyophilized	Pool of five anti-RRV antibody-positive Australian plasma donations
S6	Liquid/frozen	Pool of fifteen negative American plasma donations
S7	Liquid/frozen	Single anti-RRV antibody-positive plasma donation

Table 3. Methods used by study participants

Lab #	Assay method	Analyte/Strain	Read out
1	Virus neutralization assay	RRV	$PRNT_{50}$
2	Virus neutralization assay	RRV SW2089	$PRNT_{50}$
3	Virus neutralization assay	RRV vaccine virus (Yu and Aaskov, 1994).	NT_{50}
4	Commercial indirect ELISA (IgG)	RRV antigen	OD/End-point dilution
5	Commercial IFA (IgG)	RRV-infected cells	End-point dilution (pos./neg.)
6	In-house multiplexed microsphere immunoassay (IgM and IgG)	CHIKV, RRV, BFV, SINV	Median fluorescence intensity
7	In-house IFA (IgG)	RRV-infected cells	End-point dilution (pos./neg.)
8	Haemagglutination inhibition	Inactivated RRV	End-point dilution (pos./neg.)
9A	Virus neutralization assay	RRV	$PRNT_{80}$
9B	In-house capture ELISA (IgG)	RRV-infected cells	End-point dilution (pos./neg.)
10A	Pseudovirus neutralization assay	Lentivirus vector/RRV T48 (E3-E1)	NT_{50} - RLU
10B	In-house ELISA	RRV-infected cells	End-point dilution (pos./neg.)

Chikungunya virus (CHIKV), Ross River virus (RRV), Barmah Forest virus (BFV), Sindbis virus (SINV); Plaque reduction neutralization titre 50 or 80 ($PRNT_{50/80}$); enzyme-linked immunosorbent assay (ELISA); immunofluorescence assay (IFA); optical density (OD); positive (pos.); negative (neg.); relative light units (RLU). Virus neutralization assays are highlighted in blue.

Table 4. Neutralization titres - combined geometric means $PRNT_{50}/NT_{50}$ ($\log_{10}$), combination of each independent assay run per laboratory

Lab code	Sample						
	S1	S2	S3	S4	S5	S6	S7
1	3.17	3.45	3.19	1.30	3.29	-	3.21
2	2.99	3.12	2.86	1.26	2.90	-	2.99
3	2.35	2.53	2.45	0.59	2.41	-	2.39
9A*	2.98	3.02	2.99	-	2.96	-	3.04
10A	2.93	3.07	2.96	2.26	3.23	1.91	3.23

*Laboratory 9A reported data as $PRNT_{80}$ values; these were re-calculated as $PRNT_{50.}$ No neutralization (-).

Table 5. Neutralization titres calculated relative to the candidate RR - sample S1 – 1500/19 (1,000 (3 log$_{10}$) units (U)/ml)

Lab code	Sample					
	S2	**S3**	**S4**	**S5**	**S6**	**S7**
1	3.28	3.03	1.11	3.12	-	3.05
2	3.13	2.86	1.26	2.91	-	3.00
3	3.19	3.10	1.18	3.07	-	3.04
9A	3.05	3.02	-	2.98	-	3.06
10A	3.14	3.03	2.24	3.21	2.09	3.30

No neutralization (-).

Table 6. Immunoassays - combined geometric means EC$_{50}$ (log$_{10}$), combination of each independent assay run per laboratory

Assay type	Lab code	Sample						
		S1	S2	S3	S4	S5	S6	S7
IA	4	3.12	3.44	3.44	2.42	3.13	1.30	3.44
IFA	5	3.50	3.67	3.67	2.50	3.50	-	3.50
IA	6	3.06	3.22	3.15	2.08	3.05	-	3.08
IFA	7	2.96	2.96	2.96	2.66	2.96	-	2.96
HI	8	1.45	1.65	1.55	-	1.35	-	1.55
IA	9B	#	2.80	2.40	2.30	#	#	#
IA	10B	2.55	2.72	2.61	-	2.51	-	2.53

(Enzyme) immunoassay/immunoassay (IA); haemagglutination inhibition assay (HI); indirect immunofluorescence assay (IFA). #In the case of Laboratory 9B, for some samples assay results could not be interpreted due to high background. Laboratory 10B only performed a single run for samples S1-S7. Non-reactive (-).

Table 7. Immunoassay titres calculated relative to the candidate RR - sample S1 – 1500/19 (1,000 (3 $\log_{10}$) units/ml)

Assay type	Lab code	Sample					
		S2	S3	S4	S5	S6	S7
IA	4	3.32	3.32	2.29	3.01	1.18	3.32
IFA	5	3.17	3.17	2.00	3.00	-	3.00
IA	6	3.15	3.08	2.05	2.99	-	3.01
IFA	7	3.00	3.00	2.70	3.00	-	3.00
HI	8	3.20	3.10	-	2.90	-	3.10
IA	9B	#	#	#	#	#	#
IA	10B	3.17	3.05	-	2.95	-	2.98

#It was not possible to determine relative potencies for data returned by Laboratory 9B. Non-reactive (-).

Table 8. Overall combined means for samples S1-S7

Sample	N_1	N_2	Mean ($\log_{10}$)	95% CI	
S1	11	30	2.82	0.72	4.93
S2	12	33	2.97	1.03	4.91
S3	12	33	2.85	0.81	4.89
S4	9	20	1.87	-4.77	8.52
S5	11	28	2.84	0.58	5.11
S6	2	3	1.60		
S7	11	30	2.90	0.75	5.05

N_1 – number of participants with results; N_2 =number of assays overall participants; Mean ($\log_{10}$) – consensus mean estimated across assays; 95% CI - 95% confidence interval for mean estimate

Table 9. Overall potencies relative to candidate RR - sample S1 (1500/19) with an assumed unit age of 1,000 (3 $\log_{10}$) U/ml.

Sample	N_1	N_2	Mean ($\log_{10}$)	95% CI	
S2	11	30	3.16	2.80	3.51
S3	11	30	3.06	2.37	3.76
S4	8	18	1.85	-3.40	7.10
S5	11	28	3.00	2.63	3.38
S6	2	3	1.61		
S7	11	30	3.08	2.64	3.52

N_1 – number of participants with results; N_2 - total number of results analyzed per sample; Mean ($\log_{10}$) – consensus mean estimated across assays; 95% CI - 95% confidence interval for mean estimate

Table 10. Analysis of variance - inter-assay variability and intra-assay variability for S1 and S5

Factor	CV
Sample (S1 or S5)	*
Participant	122%
Assay type (qualitative or quantitative)	106%
Inter-assay variability	208%
Intra-assay variability	29%
Measurement uncertainty (overall variability)	218%

CV - coefficient of variation; *Factor - assay type was not estimable

Table 11. Stability of candidate IS sample S1 (1500/19)

Incubation temperature	Incubation time					
	2 weeks	4 weeks	3 months	6 months	12 months	36 months
-20ºC	0.94	1.01	0.90	1.10	1.17	1.02
+4ºC	1.02	1.05	0.93	1.20	1.09	1.02
+20ºC	1.03	1.05	0.79	1.18	1.14	0.67
+37ºC	1.01	0.93	0.41	N.T.	N.T.	N.T.
+45ºC	0.84	0.59	N.T.	N.T.	N.T.	N.T.

N.T. Not tested – heat-treated lyophilized residue was insoluble. Potency expressed relative to -80ºC baseline samples of 1500/19.

Figure 1-7. Histograms showing mean potencies for samples S1-S7 and potencies relative to candidate RR (sample S1 - 1500/19)

Upper panels - 1-7 show the mean $PRNT_{50}/NT_{50}$ or EC_{50} for each laboratory for each sample (S1-S7) as log_{10} dilution (violet background - immunoassays (ELISAs, microsphere assays, haemagglutination inhibition, indirect immunofluorescence assays); light blue background - virus neutralization assays).

Lower panels - 2-7 show the mean potency (log_{10} units (U)/ml, relative to S1 with assumed potency of 1,000 U/ml) for each laboratory for each sample (S2-S7).

Figure 8. Box and whisker plots showing data harmonization by reporting of potencies of samples relative to the candidate standard S1 (1500/19) – virus neutralization assays.

Top panel - mean potencies for each sample for each participating laboratory (where data could be analysed; lower panel - mean potencies (log_{10} U/ml, relative to sample S1 (with assumed potency of 1,000 U/ml) for each laboratory and each sample (S2-S7). Boxes indicate interquartile range; horizontal lines within each box indicate median; whiskers indicate the ranges from 5% to 95% percentiles.

Figure 9. Box and whisker plots showing data harmonization by reporting of potencies of samples relative to the candidate standard S1 (1500/19) – immunoassays (including (ELISAs, microsphere and haemagglutionation inhibition, immunofluorescence assays).

Top panel - mean potencies for each sample for each participating laboratory (where data could be analysed; lower panel - mean potencies (log_{10} U/ml, relative to sample S1 (with assumed potency of 1,000 U/ml) for each laboratory and each sample (S2-S7). Boxes indicate interquartile range; horizontal lines within each box indicate median; whiskers indicate the ranges from 5% to 95% percentiles.
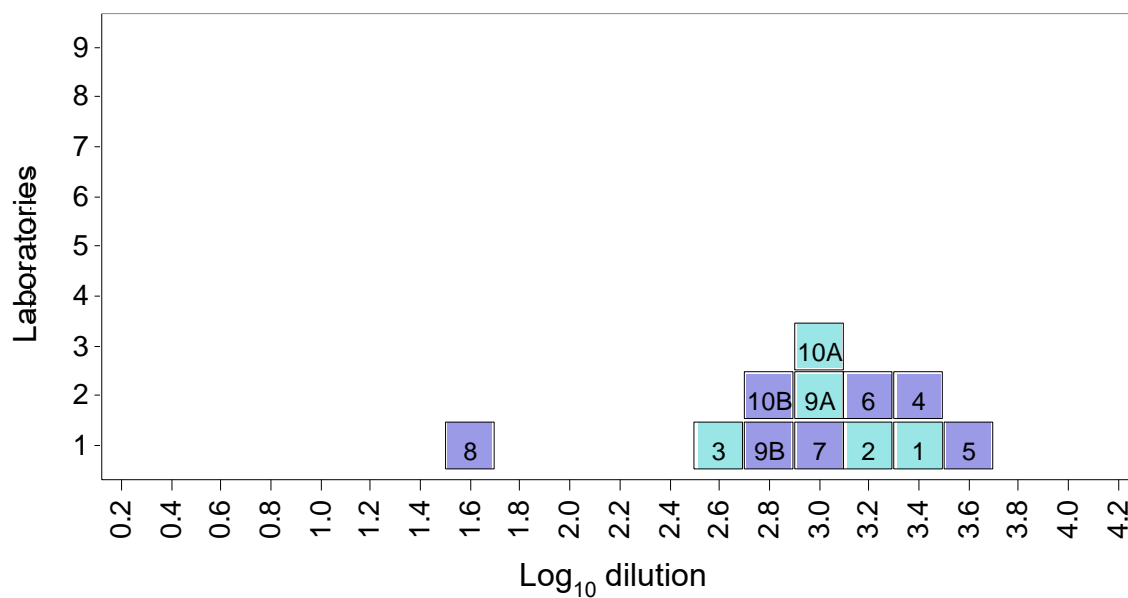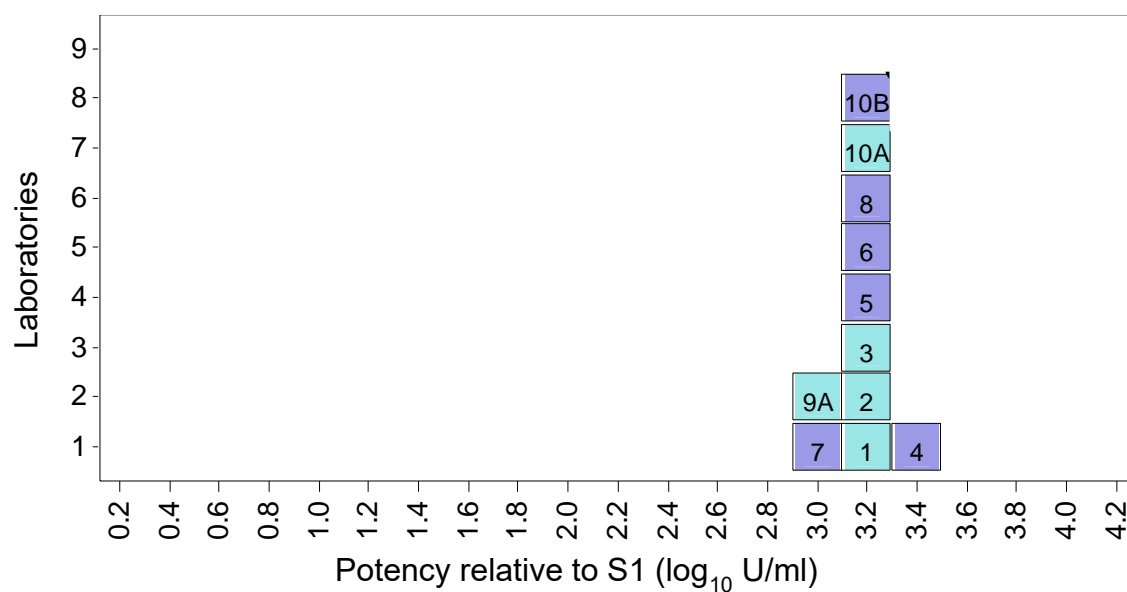
Figure 1A. Geometric mean potencies of sample S1
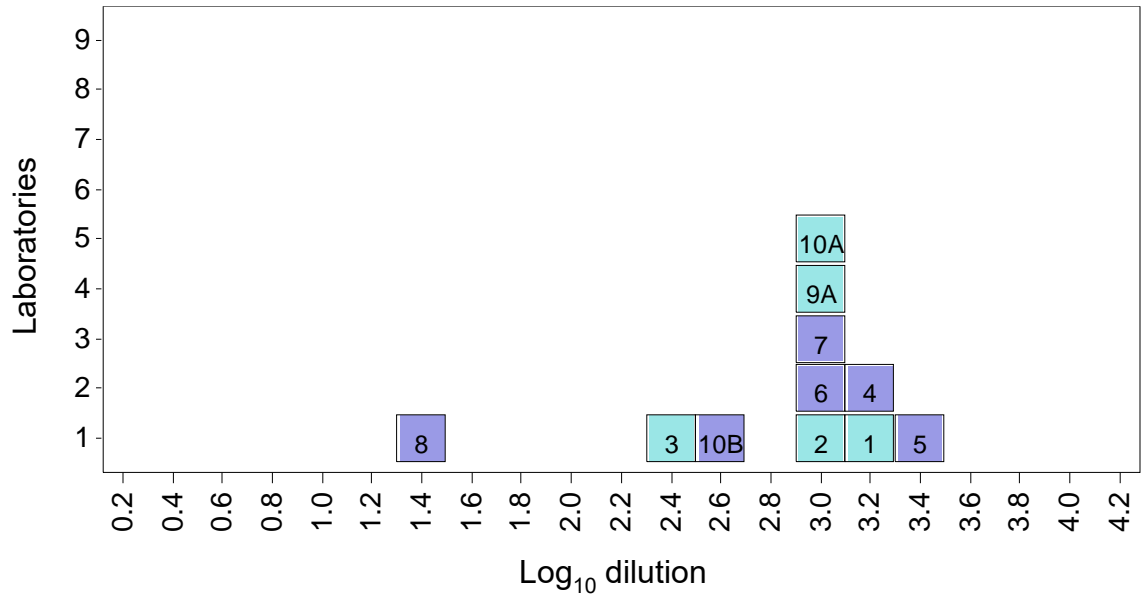
Figure 2A. Geometric mean potencies of sample S2

Figure 2B. Relative potencies for sample S2

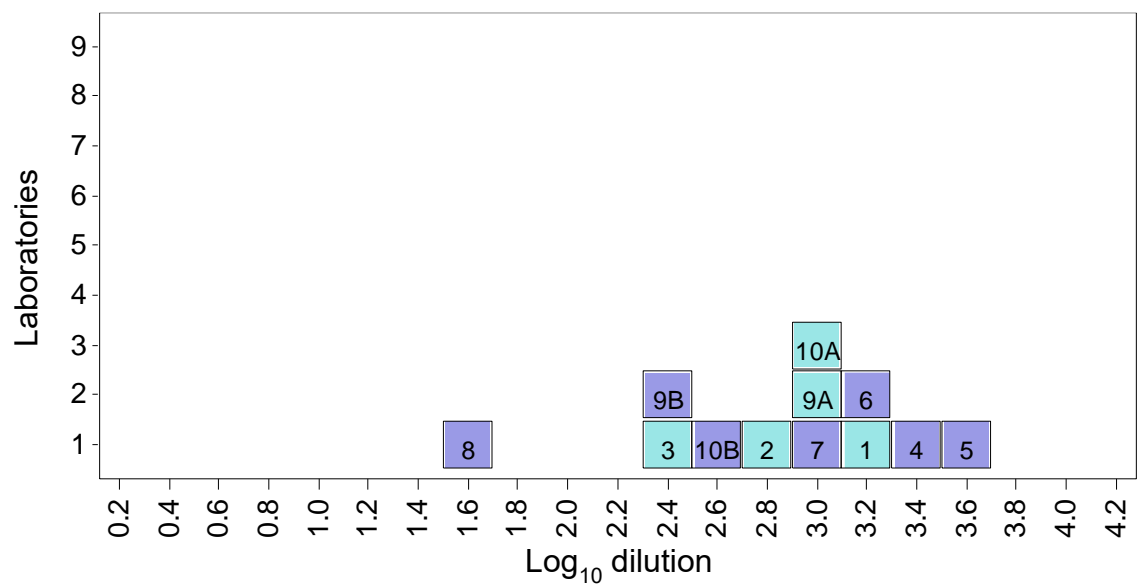**Figure 3A.** Geometric mean potencies of sample S3
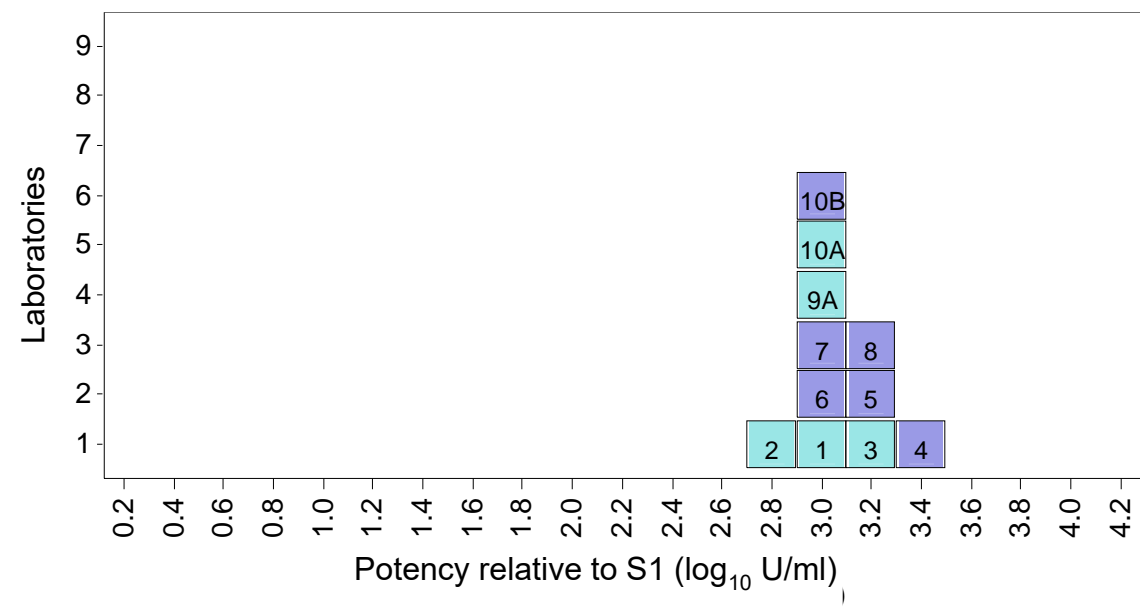

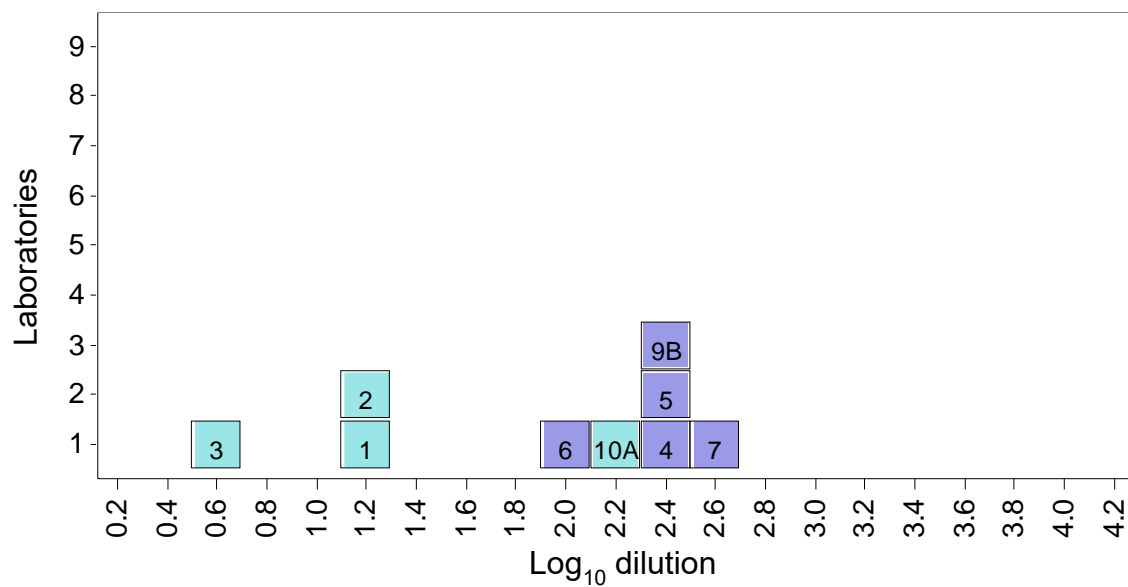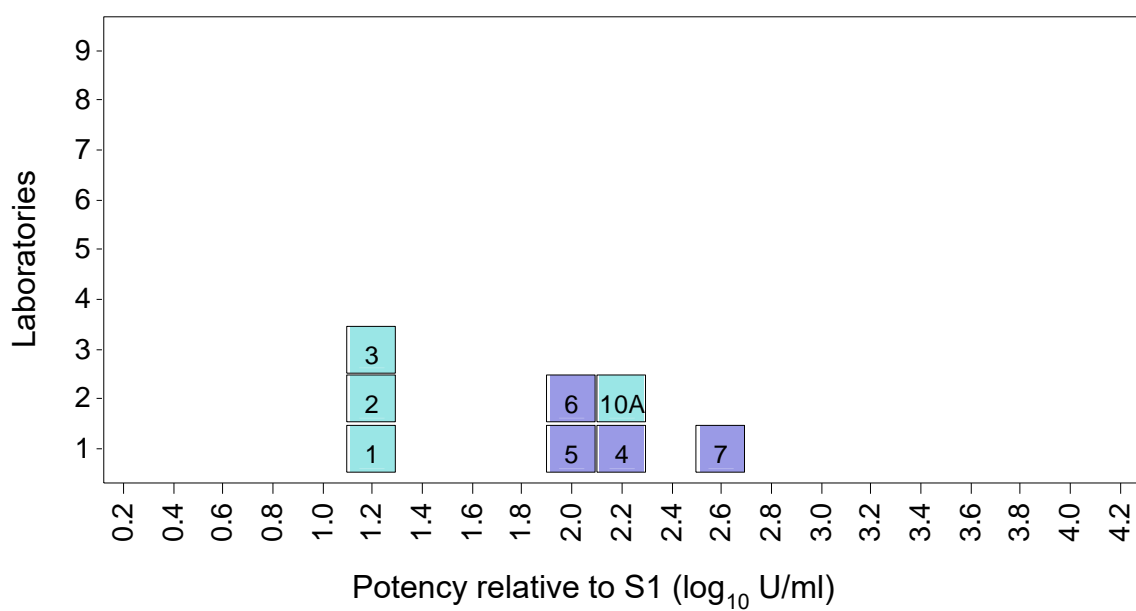**Figure 3B.** Relative potencies for sample S3

Figure 4A. Geometric mean potencies of sample S4
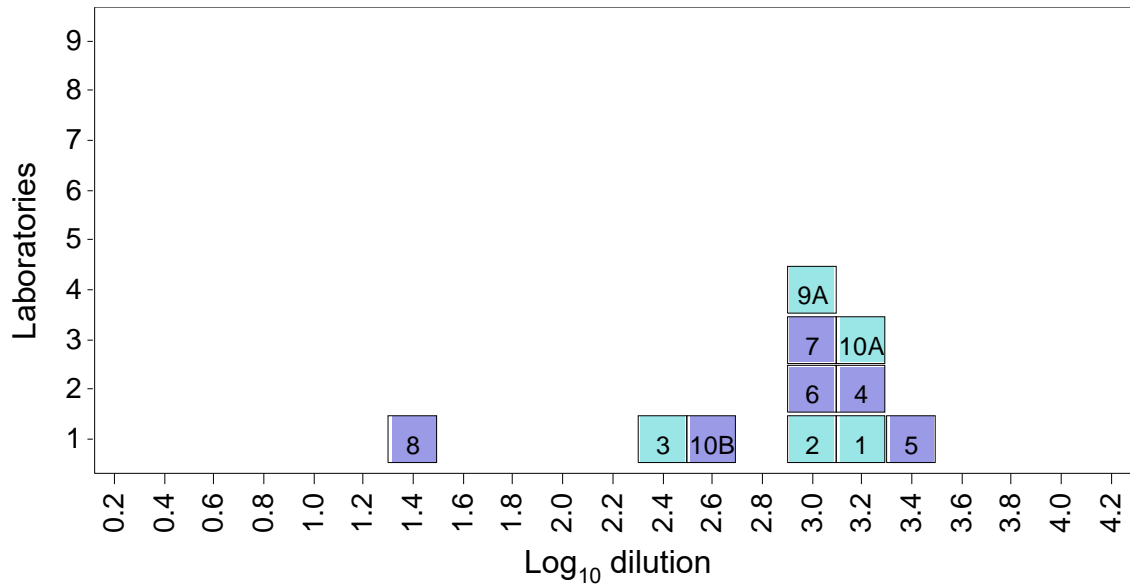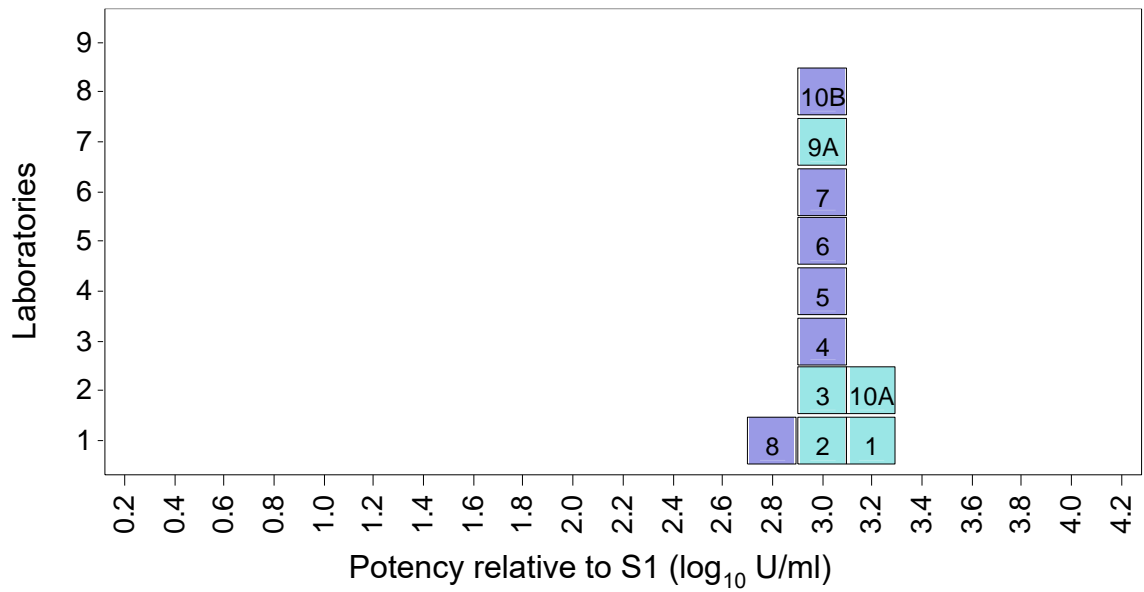

Figure 4B. Relative potencies for sample S4

Figure 5A. Geometric mean potencies of sample S5

Figure 5B. Relative potencies for sample S5
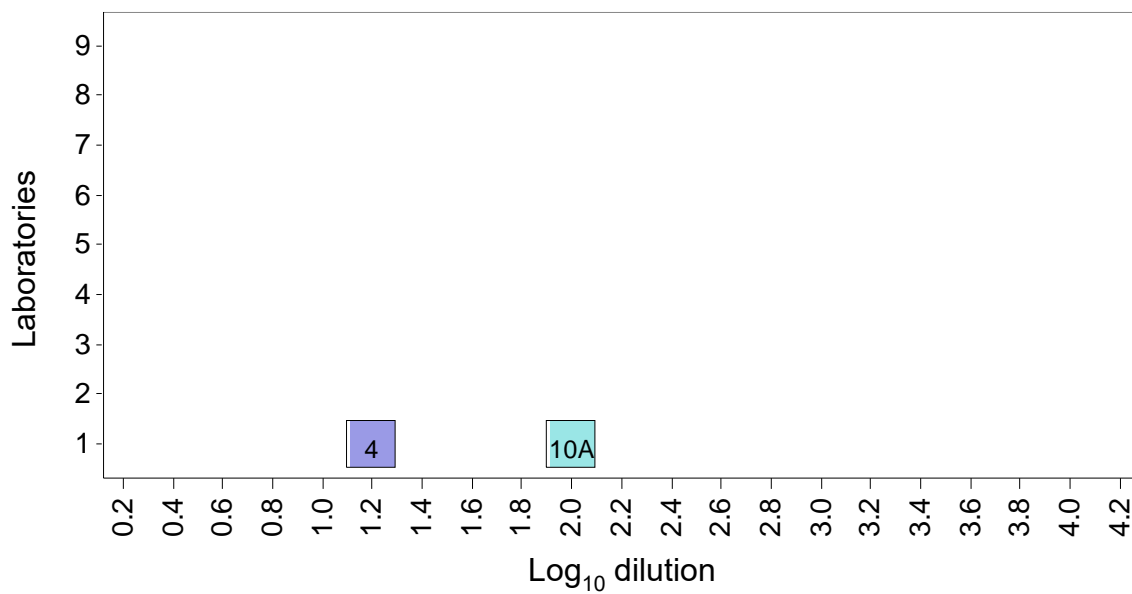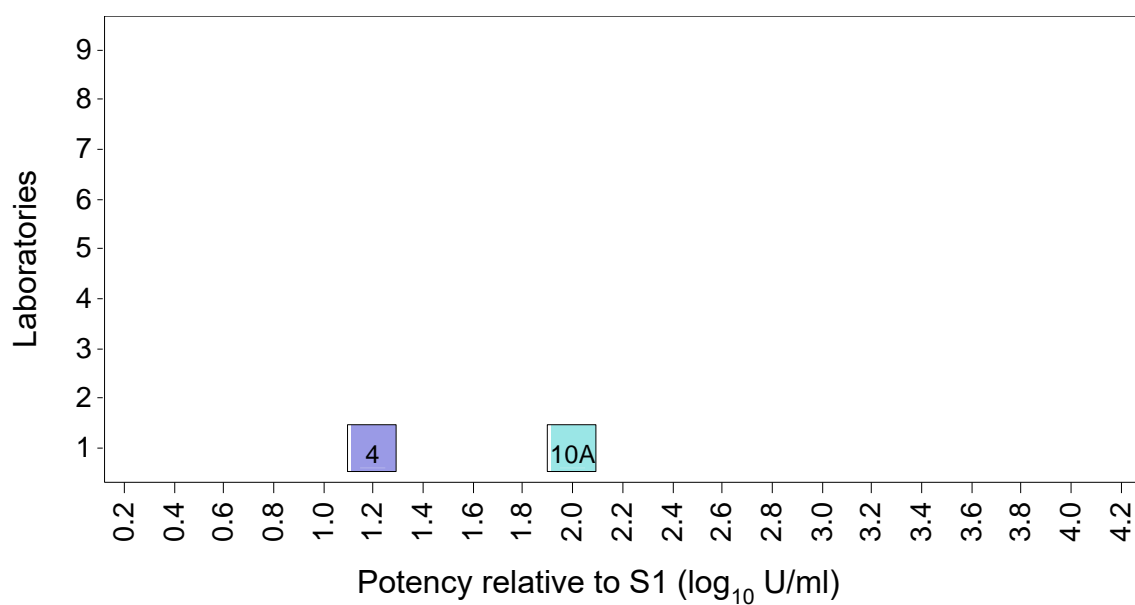
Figure 6A. Geometric mean potencies of sample S6

Figure 6B. Relative potencies for sample S6

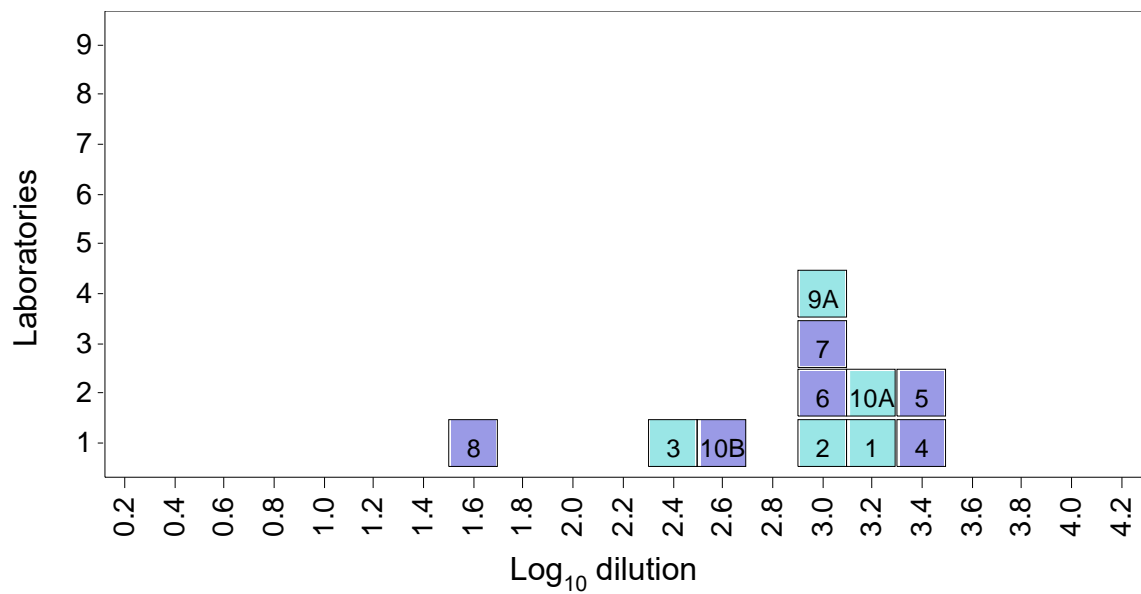**Figure 7A.** Geometric mean potencies of sample S7

Figure 7B. Relative potencies for sample S7

Figure 8. Box and whisker plots showing data harmonization by reporting of potencies of samples relative to the candidate standard S1 (1500/19) – virus neutralization assays

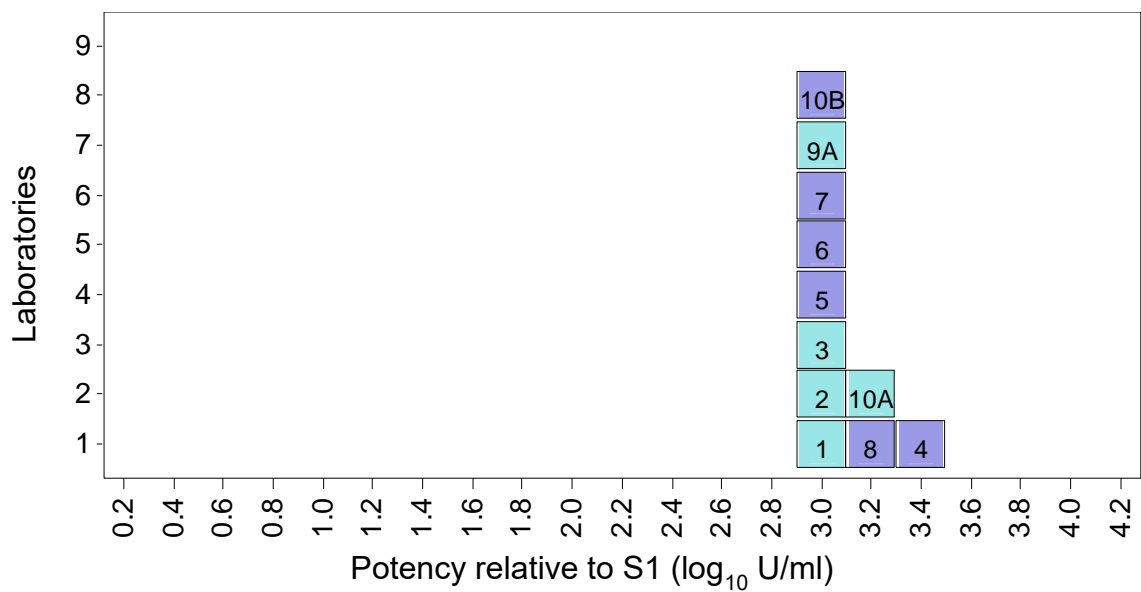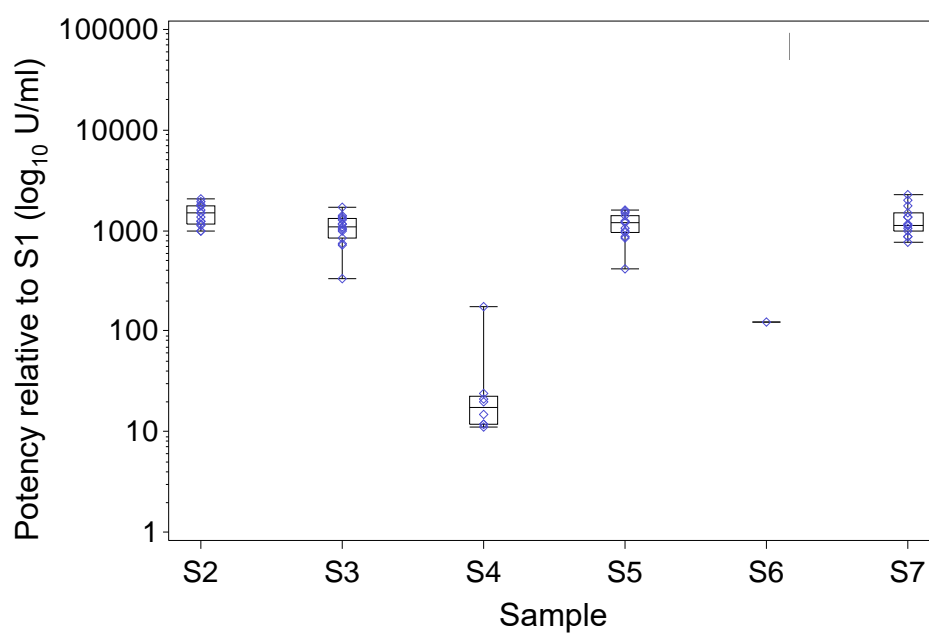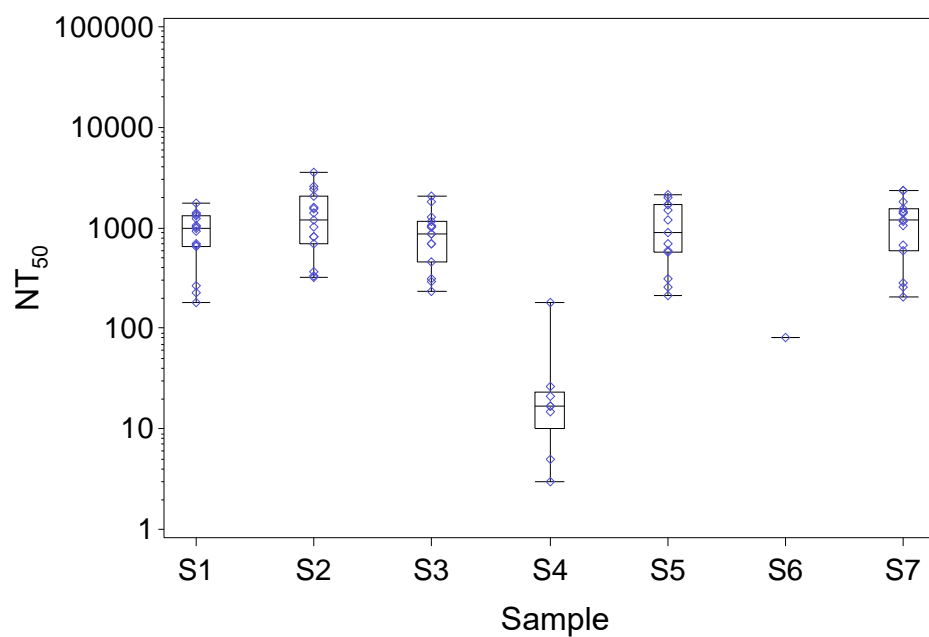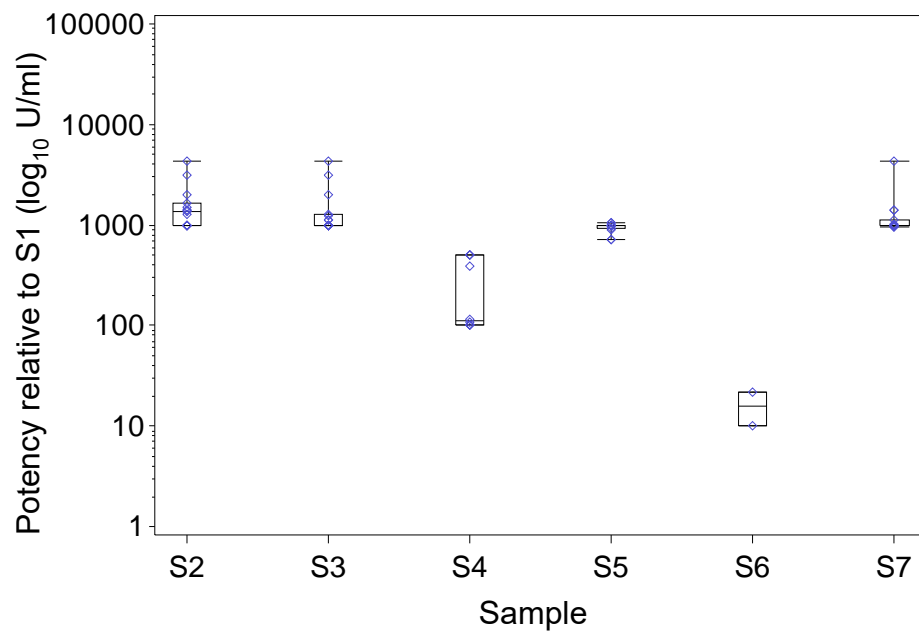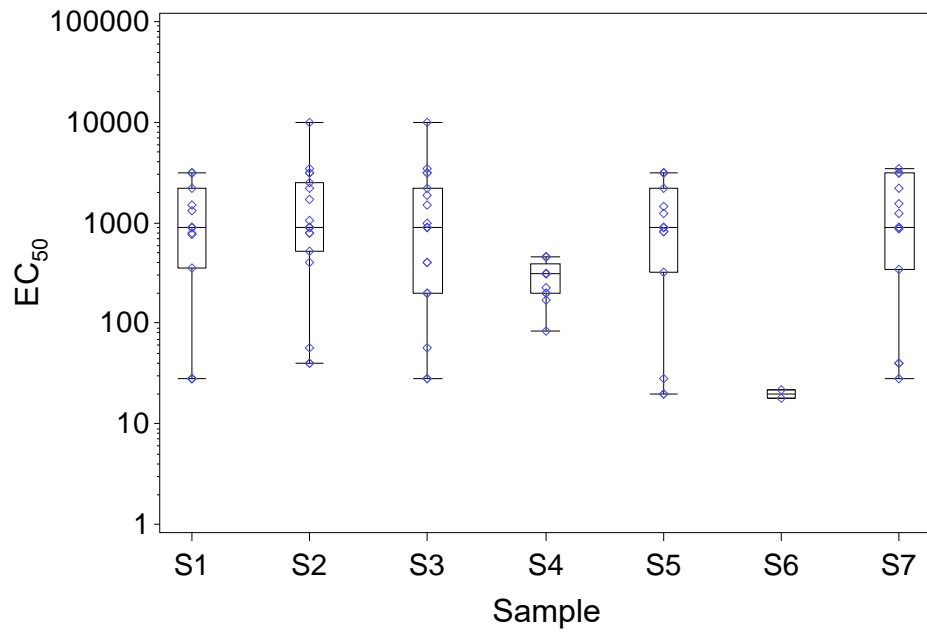**Figure 9.** Box and whisker plots showing data harmonization by reporting of potencies of samples relative to the candidate standard S1 (1500/19) –binding assays

Appendix 1. List of participating laboratories that returned data (alphabetically according to country/affiliation)

Scientist(s)	Affiliation
Helen Faddy, Chiara Carnevali	Australian Red Cross Lifeblood, Brisbane, Australia
David Smith, Suzi McCarthy	PathWest Laboratory Medicine WA, Perth, Australia
Carmel Taylor, Peter Moore	Queensland Health Forensic and Scientific Services, Coopers Plains, Australia
Maria Farcet, Thomas Kreil	Takeda Manufacturing Austria AG, Vienna, Austria
Petra Emmerich#, Jonas Schmidt-Chanasit, Ronald von Possel#	Bernhard-Nocht-Institut für Tropenmedizin, Hamburg, Germany; #University of Rostock, Rostock, Germany
Lisa Henß, Barbara Schnierle	Paul-Ehrlich-Institut, Langen, Germany
Konstanze Stiba, Erik Lattwein, Marleen Janku	EUROIMMUN AG, Lübeck, Germany
Jamal I-Ching Sam, Chong Long Chua, Athirah Shafiqah Abu Bakar, Yoke Fun Chan	University of Malaya, Kuala Lumpur, Malaysia
Ann Powers, Jeremy Ledermann	Centers for Disease Control and Prevention, Fort Collins, United States of America
Scott Weaver, Grace Rafael	University of Texas Medical Branch, Galveston, United States of America

Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

Paul-Ehrlich-Institut

Study Protocol

Collaborative Study to Evaluate a Candidate Standard for Ross River Virus Antibodies

Background

Ross River virus (RRV) belongs to the *Alphavirus* genus in the *Togaviridae* family of viruses. Similar to chikungunya virus, RRV causes polyarthritis, myalgia, and rash with some patients developing chronic arthralgia. RRV occurs in Australia, where it is the most widespread arbovirus and it is found in the South Pacific region.

There are currently no international reference materials available for the determination of the potency of antibodies developed following infection with RRV. Anti-RRV antibody potency determination is important to determine the analytical sensitivity of serological assays, to enable discrimination of cross-reactive RRV antibodies from other anti-*Alphavirus* antibodies, to attempt to define clinical parameters such as protective levels of antibody and to in general achieve better comparability of results obtained in different laboratories.

The Paul-Ehrlich-Institute (PEI), Federal Institute for Vaccines and Biomedicines developed an anti-RRV antibody candidate preparation for testing and comparison across assays and laboratories to evaluate its suitability as a standard for RRV antibodies.

Objective

The study will evaluate the potency of the proposed candidate material for anti-RRV antibodies in parallel with other antibody preparations using assays in routine use in the participants laboratories. The aim is to select the most suitable candidate standard for assay harmonization and agree an potency for the candidate standard following statistical analysis of the study data at the PEI.

Samples

The candidate material consists of a Plasma pool obtained from Australian blood donors with a past RRV infection. The candidate material has been lyophilized.

Additional liquid/frozen plasma samples are provided to test for commutability as well as specificity of the assays used in the participating laboratories.

None of the lyophilized samples or the liquid/frozen plasma samples have been heat-inactivated. Assays requiring heat inactivation should ensure that all samples are appropriately heat-inactivated.

The materials are not for administration to humans or animals. They are not for *in vitro* diagnostic use; they are for evaluation purposes only and should not be used to determine the validity of assays for RRV antibodies. Seven samples are provided and coded (S1 to S7). Three sets of samples are provided per assay – sufficient for three assay runs. A new set of samples should be used for each assay run. Laboratories that have indicated that they perform more than one type of assay have been sent additional samples for each respective assay.

Shipment and Storage of Samples

All samples are shipped on dry ice and should be stored at ≤ -20°C, upon receipt.
Participants are asked to confirm receipt of samples and to report any anomaly on the "Acknowledgment of Receipt" form accompanying the plasma samples.

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Federal Institute for Vaccines and Biomedicines

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Study Protocol

Participants are requested to use their preferred methods indicated in the PEI questionnaire for the detection of anti-RRV antibodies according to the listed requirements:

- **Please note, that the provided liquid/frozen plasma materials were <u>NOT</u> heat-inactivated**
- Please perform three independent assays for anti-RRV antibodies on three different days. Each Laboratory will receive 3 sets of samples for 3 independent test repeats. If more than one method is to be performed, additional sets of samples have been provided.
- **The lyophilized samples should be reconstituted in 0.5 ml sterile, molecular-grade water shortly before use and mixed by gentle agitation for ~ 10 minutes. Use a fresh vial for each assay.**
- Include all samples in each assay to allow for comparison of the antibody potency. If this is not feasible, please record which samples were tested concurrently.
- Please prepare a series of dilutions for samples S1 – S7 in each independent assay. If possible, at least two independent replicate series of dilutions (not two samplings from a single dilution series) should be assayed.
- For the dilution series it is important to cover the range of quantifiable antibody concentrations (at least 4 steps) including at least one step beyond the endpoint dilution. For experiment 1, we suggested a starting dilution which is noted in the reporting sheet. Please adjust dilutions in the subsequent assays if needed and record the change in the Excel reporting sheet.
- **Please only perform testing for IgG.**
- An Excel reporting sheet is provided for recording all essential information. Only use the reporting sheet for recording the results to help facilitate the statistical analysis of the data at PEI.
- Please use the Excel reporting sheet to document the results for each dilution. We will use the raw data for statistical analysis. For neutralization assays, please calculate the neutralization titer, if possible. However, please also provide the raw values.
- Note the cut-off value in the designated field and include for each sample dilution tested if it is considered positive or negative according to the assay specifications.
- For better comparability, please specify in the reporting sheet the conditions of the assay used (diluent type, virus strain and titer, incubation time, cell seeding conditions used for the specific assay) under "Remarks"
- Please note all deviations from the assay protocol in the Excel reporting sheets.

Reporting of Results

Participants are requested to report the results as soon as possible after receipt of the plasma samples, latest on **30th April 2020**. All completed forms (Participant Questionnaire and Results Reporting form) should be returned by e-mail to Sally.Baylis@pei.de and Hanna.Roth@pei.de.

Data Analysis

The confidentiality of each laboratory will be ensured with each participant being anonymous to the other laboratories. All data resulting from the collaborative study will be analyzed at PEI by an experienced biometrician. The analysis will assess the potency of all provided materials relative to each other and the sensitivities of the different assay methods.

Participants will receive a copy of the drafted study report including data analysis and proposed conclusions. If the study has a successful outcome and the analysis suggest the use of the candidate standard helps to harmonize agreement between laboratories, the results of the report will be communicated to the World Health Organization for consideration as an international standard or reference reagent.

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Participation in the collaborative study is further dependent on the following conditions:

- Data obtained in the assays are not to be published or cited before completion of the study (including report drafting) without permission of the study organizer.
- The participants are not allowed to use the study materials for purposes other than conducting the collaborative study.
- Individual participant's data will be coded and reported "blind" to other participants during the preparation of the study report, and also subsequent publications.
- Participants accept responsibility for safe handling and disposal of the materials provided.

Appendix 3. Draft instructions for use

Paul-Ehrlich-Institut
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

A WHO Collaborating Centre
for Quality Assurance of Blood Products and
In vitro Diagnostic Devices

World Health Organization International Reference Reagent for Anti-Ross River Virus Neutralizing Antibodies (IgG)

PEI code 1500/19

(Version 1.0, April 2023)

1. INTENDED USE
The World Health Organization (WHO) International Reference Reagent for anti-Ross River virus (RRV) neutralizing antibodies (IgG) was developed from a pool of five plasma donations from blood donors from Townsville, Australia and evaluated in an international collaborative study. The principal use of the Reference Reagent is for the calibration and harmonization of serological assays for the quantification of anti-RRV neutralizing IgG. The standard can be used as reagent for control for immunoassay performance. Further details of the collaborative study are available in the report (1).

2. UNITAGE
The Reference Reagent has been assigned a unitage of 1,000 units (U)/ml after reconstitution in 0.5 ml sterile, cell culture grade water. The unitage relates to antibody (IgG) neutralization activity for virus neutralization assays.
For other types of immunoassay, the reference reagent may be used as a control reagent (with no assigned unitage) following dilution (dilution to be determined by the user and is assay dependent). The application of the standard is applicable to detection of specific RRV antigenic targets such as envelope proteins, whole virus – it should not be used to compare between groups of assays of different specificity.

3. CONTENTS
Each vial contains the freeze-dried residue of 0.5 ml of human plasma.

4. CAUTION
THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.
As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. The plasma has been for negative for hepatitis B virus, hepatitis C virus as well as human immunodeficiency virus by NAT testing.
It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

5. USE OF MATERIAL
No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.
The material is supplied lyophilized and should be stored at or below –20°C. Each vial should be reconstituted in 0.5 ml of sterile nuclease-free water. The product should be reconstituted just prior to use. For virus neutralization assays, the reconstituted material should be heat-inactivated prior to use.

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Paul-Ehrlich-Str. 51-59
63225 Langen, Germany

6. STABILITY
As the stability studies with accelerated conditions indicate high stability of the lyophilized reference material under the recommended storage conditions (at or below –20°C), there is no expiry date assigned to the International Standard. This approach complies with the recommendations for the preparation, characterization and establishment of international and other biological reference standards (1). The reference material is held at the Paul-Ehrlich-Institut (PEI) within assured, temperature-controlled storage facilities. During its life cycle the stability is monitored at regular intervals. The international standard remains valid with the assigned potency and status until withdrawn or amended.

Reference materials should be stored on receipt as indicated on the label. Once, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact PEI.

7. REFERENCES
1. Collaborative study to evaluate a candidate International Reference Reagent for neutralizing antibodies against Ross River virus. 2023. WHO Expert Committee on Biological Standardization.

2. World Health Organization. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). WHO Technical Report Series 2006. 932, 73-131.

8. ACKNOWLEDGEMENTS
We are grateful to the anonymous donor who provided plasma and to all collaborative study participants.

9. FURTHER INFORMATION
Further information for this material can be obtained as follows: whocivd@pei.de or
WHO Biological Reference Preparations:
http://www.who.int/biologicals/en/

10. CUSTOMER FEEDBACK
Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to whocivd@pei.de

11. CITATION
In any circumstance where the recipient publishes a reference to PEI materials, it is important that the correct name of the preparation, the PEI code number, the name and the address of PEI are cited correctly.

Email: whocivd@pei.de
Web: http://www.pei.de

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12. MATERIAL SAFETY SHEET

Physical properties (at room temperature)	
Physical appearance	Lyophilized powder
Fire hazard	None
Chemical properties	

Stable	Yes	Corrosive: No
Hygroscopic	No	Oxidising: No
Flammable	No	Irritant: No

Other (specify)	Material of human origin
Handling:	See caution, section 4

Toxicological properties	
Effects of inhalation:	Not established - avoid
Effects of ingestion:	Not established - avoid
Effects of skin absorption:	Not established - avoid

Suggested First Aid	
Inhalation	Seek medical advice
Ingestion	Seek medical advice
Contact with eyes	Wash thoroughly with water. Seek medical advice
Contact with skin	Wash thoroughly with water. Seek medical advice

Action on Spillage and Method of Disposal
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

13. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of agreement or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

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If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

Email: whoccivd@pei.de
Web: http://www.pei.de