邦の状況に即した、よりよい国家検定制度
を検討してゆくことが重要である。

F. 研究発表

1) 誌上発表

なし

2) 学会発表

なし

G. 知的財産権の出願・登録状況

なし

アンケート調査票

Questionnaire about vaccine lot release system

The WHO ECBS adopted "Guidelines for Independent Lot Release of Vaccines by Regulatory Authorities" in 2010, and we have recognized anew the importance of having a well-established and globally harmonized lot release system. We are now discussing how our vaccine lot release system can be improved. The information about the lot release system of your country obtained through this questionnaire will make a useful contribution to our discussion. We would like to express our sincere appreciation for your cooperation in advance.

Please answer the following questions by placing a check next to the response(s) that apply to your country's lot release system. For some questions, depending on the particular product, lot and so on, several different responses might apply to the same question. In that case, please indicate all the responses which apply in the various cases.

[Question about biological products in general]

Q1. What kinds of drugs are subject to independent lot release?

☐ Vaccines
☐ Anti-sera
☐ Plasma derivatives
☐ Biotherapeutics
☐ Other (Please specify in the column below.)

Comment:

[Questions about lot release system for vaccines]

Q2. What procedures are required for the lot release of vaccines?

☐ Review of manufacturers' summary protocol only
☐ Independent testing only
☐ Review of summary protocol plus independent testing
☐ Recognition/acceptance of lot release certificates from the NRA/NCL of another country
☐ Other (Please specify in the column below.)

Comment:

Q3. Which classes of lots are subject to lot release?

☐ Lots which are produced domestically and marketed domestically

☐ Lots which are produced domestically but are for export only

☐ Lots which are imported or procured and marketed domestically

☐ Other (Please specify in the column below.)

Comment:

[Questions about independent testing for lot release of vaccines]

Q4. What percentage of lots is tested?

☐ Every lot (100% of lots)

☐ Less than 100% of lots

☐ Zero % of lots

☐ Other (Please specify in the column below.)

Comment:

Q5. What items are tested when independent testing of measles vaccine is performed?

☐ Appearance

☐ Potency (Virus concentration)

☐ Thermal stability

☐ Sterility

☐ General safety test (Abnormal toxicity test, Innocuity)

☐ Residual moisture

☐ Test for freedom from extraneous viruses

☐ Test for neurovirulence

☐ Identity

☐ Other (Please specify in the column below.)

Comment:

Q6. What items are tested when independent testing of split influenza vaccine is performed?

☐ Appearance
☐ Haemagglutinin content (Potency)
☐ Purity
☐ Fractionation test
☐ Protein content
☐ Endotoxin
☐ Sterility
☐ General safety test (Abnormal toxicity test, Innocuity)
☐ Test for leukopenic toxicity
☐ Test for Freedom from ether
☐ Identity
☐ Other (Please specify in the column below.)

Comment:

Q7. What items are tested when independent testing of acellular pertussis vaccine is performed?

☐ Appearance
☐ Potency (Mouse immunogenicity test)
☐ Potency (Modified intracerebral challenge assay)
☐ Residual activity of pertussis toxin (eg. HIST)
☐ Accelerated reversion test
☐ Protein content
☐ Endotoxin
☐ Sterility
☐ General safety test (Abnormal toxicity test, Innocuity)
☐ Adjuvant content
☐ Preservative content
☐ Content of residual detoxifying agent
☐ pH
☐ Identity
☐ Other (Please specify in the column below.)

Comment:

Q8. What items are tested when independent testing of human papillomavirus (r-DNA) vaccine is performed?

☐ Appearance
☐ Sterility
☐ Endotoxin
☐ General safety test (Abnormal toxicity test, Innocuity)
☐ Protein content
☐ Adjuvant content (adjuvant:)
☐ Degree of absorption of each type
☐ Degree of absorption of Monophosphoryl Lipid A (if applicable)
☐ Potency (in vitro relative potency assay)
☐ Potency (in vivo assay)
☐ pH
☐ Extractable volume
☐ Identity of each type
☐ L1 purity (for intermediate stages)
☐ Degree of intact L1 monomer (for intermediate stages)
☐ Other (Please specify in the column below.)

Comment:

Q9. For a new product, at what stage do you usually start a collaboration/discussion with a manufacturer regarding development and/or transfer of testing methodology and capability?

☐ Early pre-application stage, e.g. non-clinical trial stage
☐ Late pre-application stage, e.g. clinical trial stage
☐ Immediately before application
☐ Post-application stage
☐ Post-approval stage
☐ No collaboration/discussion
☐ Other (Please specify in the column below.)

Comment:

Q10. What specifications/standards are used to judge the test results of independent testing?

☐ Specifications approved in the marketing authorization dossier
☐ Standards prescribed by the official compendium such as pharmacopoeia
☐ Standards described in the international guidelines such as WHO recommendations
☐ Other (Please specify in the column below.)

Comment:

Q11. Do you have any routine system to disclose the data obtained by lot release testing to the relevant manufacturer?

☐ Yes
☐ No

Comment:

Q12. Do you have any legal or formal system to disclose the data obtained by lot release testing to a third party if detailed data are requested?

☐ Yes
☐ No

Comment:

Q13. Have you stored samples for future reference?

☐ Yes
☐ No

Comment:

Q14. If you have some useful Websites offering information about the lot release system of your country, e.g. information about vaccines and their corresponding testing items, please indicate the URLs in the column below.

Comment:

[Information sharing about this questionnaire]

Q15. Do you permit your answers to this questionnaire to be shared with relevant persons of other countries' NCL/NRA or WHO?

☐ Yes

☐ Yes, however, conceal the name of the country

☐ No

Comment:

Q16. Do you wish to receive the compiled results of this questionnaire? If "yes", we will send it to you later.

☐ Yes

☐ No

Comment:

表　ロットリリース制度に関するアンケート結果

	Canada	China	EU	Japan	Korea	Taiwan	A	B
Q1. What kinds of drugs are subject to independent lot release?	Vaccines Anti-sera Plasma derivatives Biotherapeutics	Vaccines Plasma derivatives Other (IVD for blood screening)	Vaccines Plasma derivatives	Vaccines Anti-sera Plasma derivatives Others(Tuberculin(PPD), Varicella antigen, BCG for intravesical injection)	Vaccines Anti-sera (Antivenom(Equine)) Plasma derivatives Others(Tuberculin(PPD), Botulinum toxin,)	Vaccines Anti-sera Plasma derivatives Others(Tuberculin(PPD), Botulium toxin)	Vaccines Plasma derivatives Others *<Comment #1>*	Vaccines
Q2. What procedures are required for the lot release of vaccines?	Protocol review only Protocol review + testing *<Comment #2>*	Protocol review + testing	Protocol review + testing Recognition/acceptance of EU OCABR Certificates	Protocol review + testing	Protocol review + testing	Protocol review + testing (?) Recognition/acceptance of lot release certificates Others *<Comment #3>*	Protocol review + testing	Protocol review + testing Recognition/acceptance of lot release certificates Others *<Comment #4>*
Q3. Which classes of lots are subject to lot release?	Produced and marketed domestically Produced domestically for export only Imported or procured and marketed domestically *<Comment #5>*	Produced and marketed domestically Imported or procured and marketed domestically *<Comment #6>*	Produced and marketed domestically Imported or procured and marketed domestically	Produced and marketed domestically Imported or procured and marketed domestically *<Comment #7>*	Produced and marketed domestically Imported or procured and marketed domestically	Produced and marketed domestically Imported or procured and marketed domestically	Produced and marketed domestically Produced domestically for export only Imported or procured and marketed domestically	Produced and marketed domestically Produced domestically for export only Imported or procured and marketed domestically
Q4. What percentage of lots is tested?	Less than 100%	100%	100%	100%	Less than 100% *<Comment #8>*	100% Other *<Comment #9>*	Less than 100% *<Comment #10>*	Less than 100% Other *<Comment #11>*

表　ロットリリース制度に関するアンケート結果（つづき）

	Canada	China	EU	Japan	Korea	Taiwan	A	B
Q5. Test items; measles	Potency (Virus concentration)	Potency (Virus concentration) Sterility General safety test Identity *<Comment #12>*	Appearance Potency (Virus concentration) Thermal stability Identity	Potency (Virus concentration) Residual moisture Test for freedom from extraneous viruses (For bulk product) Test for neurovirulence (First 5 consecutive lots)	Appearance Potency (Virus concentration) Sterility General safety test Residual moisture Identity *<Comment #13>*	Appearance Potency (Virus concentration) Sterility General safety test Residual moisture Identity	Potency (Virus concentration) Thermal stability	Appearance Potency (Virus concentration) Thermal stability Sterility General safety test Identity pH *<Comment #14>*
Q6. Test items; split influenza	Haemagglutinin content (Potency) Endotoxin *<Comment #15>*	Haemagglutinin content (Potency) Sterility General safety test Identity *<Comment #16>*	Appearance Haemagglutinin content (Potency) Purity (On the first 5 lots of monovalent bulks) Endotoxin	Haemagglutinin content (Potency) Protein content General safety test Test for leukopenic toxicity Test for Freedom from ether	Appearance Haemagglutinin content (Potency) Endotoxin Sterility General safety test Test for Freedom from ether Identity *<Comment #17>*	Appearance Haemagglutinin content (Potency, SRID) Purity Protein content Endotoxin Sterility General safety test Identity	Appearance Haemagglutinin content (Potency) Protein content Endotoxin	Appearance Haemagglutinin content (Potency) Endotoxin Sterility General safety test *<Comment #18>*
Q7. Test items; acellular pertussis	Potency (Mouse immunogenicity test) *<Comment #19>*	Potency (Modified intracerebral challenge assay) Residual activity of pertussis toxin (eg. HIST) Sterility Identity	Appearance Potency (Mouse immunogenicity test) (On every new final bulk) Residual activity of pertussis toxin (eg. HIST) (On every new final bulk) Endotoxin (On every new final bulk) Identity	Potency (Modified intracerebral challenge assay) Residual activity of pertussis toxin (eg. HIST) Accelerated reversion test Endotoxin General safety test Content of residual detoxifying agent	Appearance Potency (Mouse immunogenicity test) Potency (Modified intracerebral challenge assay) Residual activity of pertussis toxin (eg. HIST) Accelerated reversion test Endotoxin Sterility General safety test Adjuvant content Preservative content Content of residual detoxifying agent pH Identity *<Comment #20>*	Appearance Potency (Modified intracerebral challenge assay) Residual activity of pertussis toxin (eg. HIST) Accelerated reversion test Protein content Endotoxin (SanofiAventis product preformed progen test) Sterility General safety test Adjuvant content Preservative content Content of residual detoxifying agent (detoxification test for 42 days) pH Identity *<Comment #21>*	Appearance Potency (Mouse immunogenicity test)	Appearance Potency (Modified intracerebral challenge assay) Endotoxin Sterility General safety test Adjuvant content Preservative content pH Identity *<Comment #22>*

表　ロットリリース制度に関するアンケート結果（つづき）

	Canada	China	EU	Japan	Korea	Taiwan	A	B
Q8. Test items; human papillomavirus (r-DNA)		<*Comment #23*>	Appearance Adjuvant content (MPL contents, if applicable) Potency (in vitro relative potency assay) Potency (in vivo assay) Identity of each type L1 purity (for intermediate stages) Degree of intact L1 monomer (for intermediate stages) <*Comment #24*>	General safety test Adjuvant content (MPL) Potency (in vitro relative potency assay)	Appearance Sterility Endotoxin General safety test Protein content Degree of absorption of each type Potency (in vitro relative potency assay) pH Extractable volume Identity of each type	Appearance Sterility Endotoxin Adjuvant content Degree of absorption of each type Potency (in vitro relative potency assay) pH Identity of each type	Appearance Potency (in vitro relative potency assay)	Appearance <*Comment #25*>
Q9. For a new product, at what stage do you usually start a collaboration/discussion with a manufacturer regarding development and/or transfer of testing methodology and capability?	Late pre-application stage, e.g. clinical trial stage Immediately before application Post-application stage Post-approval stage <*Comment #26*>	Early pre-application stage, e.g. non-clinical trial stage	Late pre-application stage, e.g. clinical trial stage Immediately before application <*Comment #27*>	Post-application stage	Early pre-application stage, e.g. non-clinical trial stage Late pre-application stage, e.g. clinical trial stage Immediately before application Post-application stage Post-approval stage <*Comment #28*>	Immediately before application Post-application stage	Post-application stage Post-approval stage <*Comment #29*>	Early pre-application stage, e.g. non-clinical trial stage Late pre-application stage, e.g. clinical trial stage Immediately before application Post-application stage Post-approval stage
Q10. What specifications/standards are used to judge the test results of independent testing?	Marketing authorization dossier Official compendium International guidelines <*Comment #30*>	Marketing authorization dossier Official compendium	Marketing authorization dossier Official compendium	Official compendium <Comment #31>	Marketing authorization dossier Official compendium	Official compendium	Marketing authorization dossier Official compendium <*Comment #32*>	Marketing authorization dossier Official compendium International guidelines

表　ロットリリース制度に関するアンケート結果（つづき）

	Canada	China	EU	Japan	Korea	Taiwan	A	B
Q11. Do you have any routine system to disclose the data obtained by lot release testing to the relevant manufacturer?	Yes *<Comment #33>*	No	No *<Comment #34>*	No	No	No	No	Yes *<Comment #35>*
Q12. Do you have any legal or formal system to disclose the data obtained by lot release testing to a third party if detailed data are requested?	Yes *<Comment #36>*	No	No *<Comment #37>*	No	No	No	Yes *<Comment #38>*	No
Q13. Have you stored samples for future reference?	Yes	Yes	Yes	No <Comment #39>	Yes	No	Yes *<Comment #40>*	No
Q14. Useful Websites	http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/brgtherap/gui_sponsors-dir_promoteurs_lot_program-eng.pdf	http://www.nicpbp.org.cn/directory/web/WS02/CL0108/ (Chinese Version)	www.edqm.eu	http://www.nih.go.jp/niid/en/mrbp-e.html *<Comment #41>* http://www.jpma.or.jp/english/parj/1013.html *<Comment #42>*	http://drug.mfds.go.kr (no English version)	http://www.fda.gov.tw/TC/download.aspx?cid=114 *<Comment #43>* http://www.fda.gov.tw/TC/site.aspx?sid=2008 *<Comment #44>*		

表　ロットリリース制度に関するアンケート結果（つづき）

Comments:
#1. Plasma derivatives (Ig and fractionated), Insulins, Hormones (proteins greater than 100 peptides), some (but not all) recombinant therapeutic proteins, but only for a limited number of batches (generally first 3 – 5).
#2. BGTD Lot Release Program is a risk/based approach. The release of specific lots may be based on: a) protocol review and testing, b) protocol review only c) receiving notification only Activities conducted are rationalized based on the available evidence (product history, use, evidence for consistent manufacture & testing) and documented under our Quality system. Activities are reviewed on an ongoing basis, and the level of product oversight changed as appropriate based on review of consistency of product quality or in response to emerging issues. More information in Health Canada Lot Release Guideline http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/brgtherap/gui_sponsors-dir_promoteurs_lot_program-eng.pdf.
#3. Vaccine for lot release application must be provide documents like list for reviews, including Packing list, Certificate of Analysis, National Authority release certificate, Approval License and Instruction of direction copy, Standard operating procedures for the control of animal sources of raw materials (SOP) and the Certificate guarantee source of raw materials materials are BSE free, Release protocol for Raw material, Mono bulk , Final bulk, and Final contain product, raw data of some testing items(i.e. potency,safety test,pyrogen etc). Letter for announce in process control of manufacture, sealing label, box appearance, instruction of direction same as License application and guarantee no any change.
#4. label review, appearance test, and perform trend analysis.
#5. BGTD releases FluLaval lots for the Southern Hemisphere. This product is not marketed in Canada but is subject to the same regulatory oversight as domestic product.
#6. Lot release will be done for lots which are produced domestically but are for export only, if it is required by NRA of users.
#7. Lot release will be done for lots which are produced domestically but are for export only, if it is required by the manugacturer.
#8. Some preparations are released by summary protocol review only.
#9. Same final container lot will be document reviewed only, but if the shipping cold chain temperature is excursion, it will be need check efficacy and safety test again. In domestic product, same final bulk will be filling to different lots (like filling to 10 lots from same final bulk) , just pick up 2 or 3 lots to performance efficacy test, other lots will not need to testing this test.
#10. Risk-based assessment, based on target population, number of doses, and protocol information.
#11. At least 10% of vaccine lots produced every year especially potency and stability tests; 100% of bulk monovalent polio for second reading of NVT; and appearance test for all types of vaccines.
#12. The Content residual of bovine serum albumin and Content residual of antibiotic is also tested for partial lots.
#13. Korea has only trivalent vaccine (MMR). Other test item, uniformity of dosage units test(mass variation test) is done.
#14. For sterility and general safety tests are performed at least 3 lots per year.
#15. Endotoxin testing has been discontinued for seasonal vaccines at this time due to interference in the LAL assay. We are developing an alternative test based on monocyte activation to be used for pandemic lot release and for TF vaccines.
#16. The Content of ovalbumin is also tested for partial lots.
#17. Additionally, thimerosal content (if applicable), pH, extractable volume test are done.
#18. For endotoxin, sterility and general safety tests are performed at least 3 lots per year.
#19. Vaccines are tested for potency according to pre-determined criteria included in the product test plan.
#20. Korea has only trivalent vaccine (DTaP, TdaP for adult). Additionally, thimerosal content (if applicable), extractable volume test are done.
#21. Taiwan doesn't have single pertussis vaccine, here performed test item including all of kind acellular base combined vaccine like DTaP, DTaP-IPV, DTaP-IPV-Hib, DTaP-IPV-Hib-HB
#22. For endotoxin, sterility, adjuvant content, preservative content and general safety tests are performed at least 3 lots per year.
#23. There is no licensed HPV vaccine available in China now.
#24. For potency, if an in vitro assay is used to determine the antigen content, it must be done on the final lot. If an in vivo assay is used, it should be done on the final bulk or on a lot of finished product derived it.
#25. Human papillomavirus (rDNA) distributed in Indonesia are imported vaccines and have certificate of release from origin country.
#26. The discussions occur at different stages depending on the product. Many times discussions on product testing and specifications take place at an early stage (during clinical trials) or during the review of the New Drug Submission (NDS). It is important to note that not all products are tested in clinical trials in Canada, but manufacturers can request pre-submission meeting with BGTD. In addition, discussions regarding product testing including new assays and qualification of new reference standards are part of the review of post-approval changes (see Health Canada Guideline http://www.hc-sc.gc.ca/dhp-mps/alt_formats/pdf/prodpharma/applic-demande/guide-ld/postnoc_change_apresac/noc_pn_quality_ac_sa_qualite-eng.pdf)

表　ロットリリース制度に関するアンケート結果（つづき）

Comments:
#27. Early pre-application stage; exchange this early is possible but less common. Late pre-application stage; most often it is in the late clinical trial stages ie: once it is clear there will be an intention to submit for licensing.
#28. Case by case.
#29. Depends on the nature of the product and the nature of the test.
#30. The specifications are product-specific and are approved as part of the Marketing Authorization. The Marketing Authorization review and the lot release activities are integrated in BGTD. Product specifications may be based on Pharmacopoeia (e.g. European and US), WHO recommendations or the test results which could be product-specific. Please note that Canada does not have a National Pharmacopoeia.
#31. Provisions of monograph may refer the relevant specifications in the Marketing Authorization.
#32. Pharmacopoeial Standards have priority; specifications as approved in dossier for products where there is no monograph at the time of registration
#33. BGTD has direct communication with all relevant manufacturers to discuss any issues regarding testing methodologies, product quality issues, new assays etc. Some manufacturers request regular meetings (e.g. once or twice a year) to discuss Lot Release activities with BGTD, even if no issues are identified.
#34. On the request of relevant manufacturer, the testing data may be disclosed.
#35. By using trend analysis; comparability study between NCL and manufacturer.
#36. BGTD has Memorandum of Understanding agreements with several regulatory agencies. Under these agreements the disclosure of confidential information including testing results is permitted. It is important to note that for some of these agreements (e.g. European Official Medicines Control Laboratory network) manufacturers have agreed to allow BGTD to discuss this information. In addition, under special situations BGTD can request the permission of manufacturers to exchange confidential information with a third party.
#37. On the request of third parties to the releasing OMCL data may be shared, this will depend on who the third party is (eg confidential exchange with another NCL or authority will be treated differently than a request from the general public) and on the freedom of information policies in the different member states.
#38. Section 61 of the Therapeutic Goods Act for disclosure of any information to another NRA.
#39. Samples are not necessarily stored according to the institutional regulations. They may be stored in some individual divisions.
#40. Usually only until expiry.
#41. This site offers the Minimum Requirements for Biological Products (English version)
#42. This site offers the English information about pharmaceutical administration and regulations in Japan.
#43. This site offers the lot release information (Chinese version). Its information will be update monthly , all of NQC pass product (including vaccine, blood product, Antitoxin). It will be provide brand name, lot number, Packing form, expiry date, sealing dosage , release date, and so on.
#44. This site offers the application information about pharmaceutical administration and regulations in Taiwan.

III. 研究成果の刊行に関する一覧表

Ⅲ. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mutsuyo Takayama-Ito, Kazuo Nakamichi, Hitomi Kinoshita, Satsuki Kakiuchi, Ichiro Kurane, Masayuki Saijo, Chang-Kweng Lim	A sensitive in vitro assay for the detection of residual viable rabies virus in inactivated rabies vaccines	Biologicals	42	42 - 47	2014
Takizawa K, Nakashima T, Mizukami T,Kuramitsu M, Endoh D, Kawauchi S, Sasaki K, Momose H, Kiba Y, Mizutani T, Furuta RA, Yamaguchi K, Hamaguchi I.	Degenerate polymerase chain reaction strategy with DNA microarray for detection of multiple and various subtypes of virus during blood screening.	Transfusion	53	2545 - 2555	2013
Odaka C, Kato H, Otsubo H, Takamoto S, Okada Y, Taneichi M, Okuma K, Sagawa K, Hoshi Y, Tasaki T, Fujii Y, Yonemura Y, Iwao N, Tanaka A, Okazaki H, Momose SY, Kitazawa J, Mori H, Matsushita A, Nomura H, Yasoshima H, Ohkusa Y, Yamaguchi K, Hamaguchi I.	Online reporting system for transfusion-related adverse events to enhance recipient haemovigilance in Japan: a pilot study.	Transfus Apher Sci.	48	95 - 102	2013

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Krayukhina E, Uchiyama S, Nojima K, Okada Y, Hamaguchi I, Fukui K.	Aggregation analysis of pharmaceutical human immunoglobulin preparations using size-exclusion chromatography and analytical ultracentrifugatio n sedimentation velocity.	J Biosci Bioeng.	115	104 - 110	2013
Okajima K, Iseki K, Koyano S, Kato A, Azuma H.	Virological Analysis of a Regional Mumps Outbreak in the Northern Island of Japan–Mumps Virus Genotyping and Clinical Description.	Jpn. J. Infect. Dis	66(6)	561 - 563	2013
Abe M, Tahara M, Sakai K, Yamaguchi H, Kanou K, Shirato K, Kawase M, Noda M, Kimura H, Matsuyama S, Fukuhara H, Mizuta K, Maenaka K, Ami Y, Esumi M, Kato A, Takeda M.	TMPRSS2 is an activating protease for respiratory parainfluenza viruses.	J Virol.	87	11930 - 11935	2013
Nagata S, Maedera T, Nagata N, Kidokoro M, Takeuchi K, Kuranaga M, Takeda M and Kato A.	Comparison of the live attenuated mumps vaccine (Miyahara strain) with its preattenuated parental strain.	J Vaccines Immun. 1		13 - 21	2013
Wood D, Elmgren L, Li S, Wilson C, Ball R, Wang J, Cichutek K, Pfleiderer M, Kato A, Cavaleri M, Southern J, Jivapaisarnpong T, Minor P, Griffiths E, and Sohn Y.	A Global Regulatory Science Agenda for Vaccines.	Vaccine	31	163 - 175	2013
多屋　馨子	副反応報告と救済制度	公衆衛生	Vol.78 No.2	86 - 92	2014

IV. 研究成果の刊行物・別刷

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

A sensitive *in vitro* assay for the detection of residual viable rabies virus in inactivated rabies vaccines

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ABSTRACT

Rabies is a viral disease transmitted through bites from rabid animals and can be prevented by vaccines. Clinically used rabies vaccines are prepared from inactivated rabies viruses grown in cell cultures or embryonated eggs. In Japan and across the world, tests that confirm complete inactivation, such as the *in vivo* suckling mouse assay, in which suckling mice are intracerebrally inoculated with vaccine products, are required for quality control. In this study, we developed a novel cell-based immunofluorescence assay that does not require mice for testing rabies vaccine inactivation for human use. The sensitivity of this cell-based *in vitro* assay was 5.7 times that of the *in vivo* suckling mouse assay, with a detection limit of one focus forming units per ml of test sample. This newly developed *in vitro* assay may replace the established *in vivo* suckling mouse assay for confirming viral vaccine inactivation.

1. Introduction

Rabies is a zoonotic viral disease of the central nervous system caused by rabies viruses that can infect almost every mammalian species. Upon presentation of symptoms of rabies infection, the mortality rate is 100%. It is estimated that approximately 55,000 patients die of rabies each year [1], and the World Health Organization (WHO) estimates that more than 15 million people worldwide receive annual post-exposure vaccinations to prevent disease progression.

Rabies is a viral disease that can be prevented using vaccines that in humans are used for pre- and post-exposure prophylaxis. Currently, rabies vaccines for human use are produced by cultivating rabies viruses in cell cultures or embryonated eggs and then inactivating them using phenol, β-propiolactone, N-tributylphosphate, or formalin. Inactivation is a crucial step in the manufacture of rabies vaccines, and two cases of inactivation failures have been reported. In Brazil, 18 people died of rabies caused by incomplete rabies virus inactivation [2]. In 2004, the Food and Drug Administration (FDA) identified the presence of non-inactivated rabies virus in a single product lot using an inactivation test before product release; subsequently, manufacturers recalled vaccine lots produced during the same period [3].

Inactivation tests that confirm the absence of residual infectious rabies virus are conducted in purified bulk samples and/or final products. In several countries, National Regulatory Authorities (NRAs) and manufacturers use the *in vivo* suckling mouse assay to test rabies vaccine inactivation [4–9]. In this procedure, 10 or more suckling or adult mice are intracerebrally inoculated with vaccine products and the appearance of rabies-associated symptoms is subsequently monitored for 21 days. If any mice die or show symptoms between days 4 and 21, brain samples are collected and tested for residual infectious rabies virus by detecting rabies virus antigens in the samples or by injecting brain samples into five additional mice. The Japanese Pharmacopoeia (JP) [7] and minimum requirements for biological products (MRBP) [8] require testing of purified bulk samples and final products using the *in vivo* suckling mouse assay. According to the Japanese MRBP [8], at least 30 suckling mice must be intracerebrally injected with 20 μl vaccine product and observed for 21 days.

It has been suggested that a cell-based *in vitro* assay could be used instead of the suckling mouse assay to avoid the use of animals and to facilitate data replication [10,11]. Both WHO and the European Pharmacopeia (EP) permit *in vitro* alternatives for testing rabies vaccine inactivation [4,12]. Moreover, the report and recommendation of the European Centre for Validation of Alternative Methods (ECVAM) workshop 48 state that "the test for residual live virus should be conducted on the bulk sample using cell cultures, and tests in mice and rabbits should be conducted as

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finished product tests" [13]. Only one report published in German by Blum et al. [14] demonstrates the use of an immunofluorescence assay with the same detection limits as those of the mouse test. Therefore, in this study, we developed a novel sensitive *in vitro* assay for assessment of rabies vaccine inactivation.

2. Material and methods

2.1. Vaccine

The freeze-dried inactivated rabies vaccine used in this study was produced in Japan for human use, and was purchased from Kaketsuken (Kumamoto, Japan). According to the manufacturer's package insert, one reconstituted vaccine dose (1.0 ml) contains more than 10^7 LD_{50} inactivated high egg passage (HEP)-Flury rabies virus strain and less than 0.2-mg gelatin as stabilizer. The potency of one dose is at least 2.5 IU of rabies antigen. The virus was propagated in primary chick embryo cell cultures and was subsequently inactivated by incubating with 0.02 volumes of beta-propiolactone at 37 °C for 60 min. The inactivated virus was concentrated by ultrafiltration, purified by ultracentrifugation, and then dispensed into individual vials and lyophilized [15]. Vaccine was resuspended in 1.0 ml of provided distilled water according to the package insert.

2.2. Virus

We used the HEP-Flury rabies virus vaccine strain originating from Japanese vaccine seed stock [16,17]. This strain is lethal to suckling mice, but not to adult mice when administered by intracerebral inoculation [18]. The working virus stock was prepared from infected Neuro-2a cells and stored at −80 °C until use. Viral titers were determined by titration in Neuro-2a cells as previously described [19]. The virus titer of the working stock was 1.0×10^8 focus forming units (ffu)/ml.

2.3. Mice

One-day-old specific pathogen-free outbred ddY mice were purchased from Nippon SLC Inc. (Shizuoka, Japan). Eight suckling mice were placed in a cage with their untreated mother. All animal procedures were conducted in accordance with the guidelines of the National Institute of Infectious Diseases (NIID) in Japan. Symptoms in mice were observed every day, and mice were euthanized upon becoming moribund.

2.4. Cell cultures

Neuro-2a cells were kindly provided by Dr. Satoshi Inoue (National Institute of Infectious Diseases, Tokyo, Japan), and Baby Hamster Kidney (BHK-21) cells were kindly provided by Dr. Kinjiro Morimoto (Yasuda Women's University, Hiroshima, Japan). HEK-293 cells were purchased from RIKEN BioResource Center (Ibaraki, Japan). Neuro-2a and BHK-21 cells were grown in Dulbecco's modified Eagle's medium (D5796, D-MEM, Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA), 100-U/ml penicillin, and 100-μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA). HEK-293 cells were grown in Eagle's minimum essential medium (M4655, E-MEM, Sigma–Aldrich, St. Louis, MO, USA) containing 10% FBS, 0.1-mM nonessential amino acids (Life Technologies, Carlsbad, CA, USA), 100-U/ml penicillin, and 100-μg/ml streptomycin. For virus inoculation, cells were cultured in medium containing 2% FBS or 0.2% bovine serum albumin (BSA; A2153, Sigma–Aldrich, St. Louis, MO, USA). Neuro-2a and BHK-21 cells were generally passaged twice per week at split ratios of 1:6 and 1:10, respectively. HEK-293 cells were passaged once per week with a split ratio of 1:8. Neuro-2a cells were seeded into 24- or 96-well culture plates (Techno Plastic Products AG, Trasadingen, Switzerland) at a density of 6×10^4 cells/cm^2 one day before inoculation. BHK-21 and HEK-293 cells were seeded into 24-well plates at a density of 1×10^5 cells/cm^2. All cultures were incubated in a humidified incubator at 37 °C with 5.0% CO_2, unless stated otherwise.

2.5. Direct immunofluorescent assays (DIFA)

In general, cells were cultured in 24- or 96-well culture plates for one day and then inoculated with diluted rabies virus. After 48–72 h, the culture medium was removed and allowed to dry in a safety cabinet for 10 min, and was then fixed in cold acetone for 20 min. After fixation, cells in culture plates were washed once with 0.01-M phosphate-buffered saline [PBS(−), Wako Pure Chemical Industries, Osaka, Japan] and stained with fluorescein isothiocyanate (FITC)-labeled anti-rabies mAb (Fujirebio Inc, Tokyo, Japan) for 40 min at 37 °C in a humidity chamber. Subsequently, cells in culture plates were washed 3 times in PBS(−) and culture plates were examined using a fluorescence microscope with an excitation filter BP460-490, dichroic mirror DM500, and barrier filter BA520IF (Olympus, Tokyo, Japan). Antigen-positive cells were identified as those containing small fluorescent cytoplasmic granules. All wells were screened in lines from top to bottom at 40× magnification, and rabies-specific fluorescent granules were confirmed at high magnification (200×). As positive and negative controls for DIFA, we used the HEP-Flury-infected or mock-infected Neuro-2a cells prepared on 12-well microscope slides (Iwaki, Tokyo, Japan).

2.6. In vivo suckling mouse assay

The *in vivo* suckling mouse assay was performed according to the Japanese MRBP [8]. Mice aged 4 days or younger were intracerebrally inoculated with 20-μl vaccine product. After inoculation, mice were monitored for 21 days for clinical symptoms and death. Mice deaths occurring within 4 days of inoculation were regarded as rabies unrelated death and were excluded from further analysis. Mice showing signs of rabies such as paralysis and convulsions at day 21 were included in analyses of rabies-related deaths.

3. Results

3.1. Selection of the most sensitive cell line

Sensitivities of Neuro-2a, BHK-21, and HEK-293 cells to rabies virus infection were determined. Neuro-2a and BHK-21 cells are commonly used in rabies studies, and HEK-293 cells are reportedly as sensitive as Neuro-2a cells in rapid rabies virus isolation experiments [20]. Confluent monolayers of BHK-21, Neuro-2a, and HEK-293 cells were cultured in 24-well plates and inoculated with 10-fold serial dilutions of the HEP-Flury strain in D-MEM containing 2% FBS (DMEM2). After 48 h incubation at 37 °C, cells were fixed and stained with FITC-labeled anti-rabies mAb as described above. Culture plates were observed under a fluorescent microscope at 200× magnification. The number of rabies antigen-positive foci did not differ between cell types, but foci in Neuro-2a cells were larger than those in BHK-21 and HEK-293 cells (Fig. 1). Thus, Neuro-2a cells were selected for further analyses.

3.2. Determination of optimal culture conditions

Neuro-2a cell culture conditions may influence virus-detection efficiencies. Accordingly, we analyzed the effects of incubation temperatures, culture medium supplements, and times of

Fig. 1. Immunofluorescent signals of rabies virus antigens in cells infected with the HEP-Flury strain. Neuro-2a (left panel), BHK-21 (middle panel), and HEK-293 (right panel) were inoculated with the HEP-Flury rabies virus strain at a multiplicity of infection of 0.5 ffu. Original magnification, 200×.

incubation. Neuro-2a cells in 96-well plates were inoculated with 10-times serially diluted virus (range 0–1000 ffu/well; 3 or 5 wells for each dose). Subsequently, cells were incubated with 100-μl diluted virus suspensions and were assayed using DIFA as described above. Data from three independent experiments are shown in Fig. 2. Although detection rates at 35 °C were slightly higher than those at 37 °C (Fig. 2A), this difference was not statistically significant ($p = 0.315$). Because the HEP-Flury strain is propagated in cells at 35 °C in vaccine production process, it is likely that low incubation temperatures slightly increased viral proliferation. Culture medium supplements (Fig. 2B) and time of incubation (Fig. 2C) did not affect residual virus detection rates. Consequently, in subsequent experiments, cells were cultured in DMEM2 at 35 °C for 72 h.

3.3. Subculture of culture fluids increased the detectability of residual virus

Because additives and large quantities of inactivated virus particles in original inoculums may affect the infectivity of residual active viruses by inhibiting viral replication or occupying viral receptors, viral proliferation might be enhanced by subculturing culture fluids. Assay designs are shown in Fig. 3. In these experiments, 2-ml reconstituted vaccine product was spiked with 20 ffu of the HEP-Flury strain, and 10 ml of DMEM2 was then added. Neuro-2a cell medium was removed from 96-well plates. Mixtures of DMEM2 and vaccine containing virus were dispensed onto the plates and cells were incubated at 35 °C for 72 h (original culture). Then, 50-μl aliquots of culture fluid were transferred onto Neuro-2a

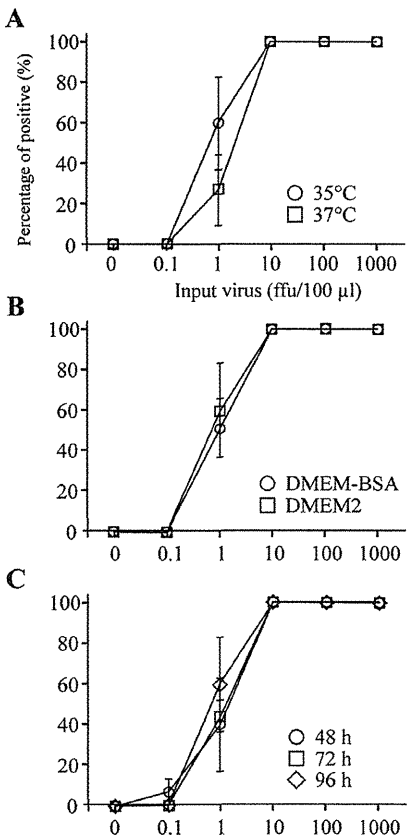

Fig. 2. Analysis of optimal culture conditions. Neuro-2a cells were cultured in 96-well plates and were inoculated with serial 10-fold dilutions of the HEP-Flury strain. Cells were incubated (A) for 72 h in DMEM2 at 35 °C or 37 °C, (B) for 72 h in DMEM containing 0.2% BSA or 2% FBS at 35 °C, or (C) for 48, 72, or 96 h in DMEM2 at 35 °C. After incubation, cells were fixed and viral antigen-positive cells were identified using DIFA.

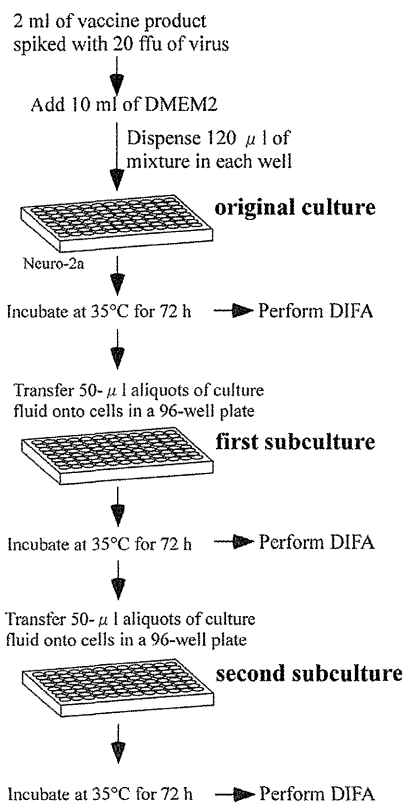

Fig. 3. Schematic of assay design for subculture of the culture fluid. Neuro-2a cells were cultured in 96-well plates and were inoculated with 2 ml of vaccine product spiked with 4 ffu of the HEP-Flury strain. After 72-h incubation, culture fluids were transferred onto newly prepared Neuro-2a cells and were incubated for a further 72 h. Culture fluids were then transferred again onto fresh Neuro-2a cells. After incubation, cells were fixed, and antigen-positive cells were identified using DIFA.