8.3. Research on regulatory processes

Many regulators are restricted by law from allowing third-party access to information or samples that they may hold relating to specific products. Access to raw data or to samples for academics to design and test new approaches, or verify existing ones, may therefore need agreements to be established with the manufacturers who provided the information or samples in the first place. In the past, manufacturers have shared samples with regulators and academics, and the results of any research study, when published, are usually blinded so as not to identify any one manufacturer's product. The use of samples submitted for lot release for research purposes likewise needs to be agreed by manufacturers.

Concerns also relate to the release of confidential data to National Immunization Technical Advisory Groups (NITAGs) for the purpose of re-evaluating the benefits/risks of vaccines. While there may be a need to expedite the path of new vaccines through licensure to recommendations for use in national immunization programs, the roles and responsibilities of regulators and advisory groups on immunization are different and need to be clearly delineated. Nevertheless, ways of improving interaction between the two parties, respecting clear roles and responsibilities and within the bounds of confidentiality, should be further explored. For example, a more efficient exchange of information between the two types of organizations might benefit the public at large by facilitating, where appropriate, more timely access to vaccines.

8.4. Limited pool of regulatory science expertise for vaccines

Regulation of biological medicines, including vaccines, is a specialized task. It requires evaluators skilled and experienced in the manufacture and control of biologicals, the target diseases, animal modelling, and assessment of clinical evidence in the target population—often infants. The developers and manufacturers of vaccines are often the primary employers of persons with this range of expertise. Countries with established vaccine manufacturers will generally have a greater pool of experts than other countries where little, or no, expertise in vaccine manufacture or control may exist.

Currently, vaccine-manufacturing facilities are based in a small number (n 40–45) of countries. These companies export and supply all other countries. Well-resourced regulatory authorities can recruit from a pool of experts without any major conflicts of interest. A subset of these authorities has dedicated the resources to maintaining a research effort required for regulatory science so that only a small number of effective regulatory science institutions for vaccines ($n < 10$) exists throughout the world.

However, with appropriate coordination, the regulatory authorities in less-well-resourced countries, even with limited infrastructure, can contribute to global regulatory science activities in a number of ways. These include specific joint collaborative research projects; surveillance data reports; or collection of clinical or product samples that may facilitate research by established institutions. These activities should be linked into existing training programs and other capacity-building activities in participating countries, such as the Global Vaccine Safety Initiative [81].

9. Proposed cross-cutting strategies and actions to support a global regulatory science agenda

Specific gaps that can be addressed by a global regulatory science agenda have been described in detail in preceding sections. In addition, a number of cross-cutting strategies and actions are identified below.

9.1. Sample repositories

Prior to adoption in regulatory settings, new assays and reference preparations developed in research laboratories must be validated for regulatory use, ideally using a diverse range of vaccine samples as differences in formulation and manufacturing processes can markedly affect test results. Access to a range of vaccine lots that have passed or failed existing tests is also critical for evaluating new testing methods. For example, OPV lots that had passed or failed the monkey neurovirulence test were essential during the development and international collaborative evaluation of the MAPREC and transgenic mouse tests (see above). Moreover, discrepancy in testing results between regulators can be resolved through sharing of samples, assays, and/or standards. Such samples are useful to evaluate new methods, particularly if they are on the borderline between 'pass' and 'fail' in existing tests. The establishment of one or more international sample repositories is therefore one cross-cutting strategy to support a global regulatory science agenda. AIDS Reagents repositories such as the NIH AIDS Research and Reference Reagent Program and NIBSC Centre for AIDS Reagents illustrate how this might be done. It could be envisaged that WHO Collaborating Centres would act to facilitate sample storage and exchanges in the wider international community.

9.2. International, regional, and national reference preparations

Biological products, including vaccines, are quantitated in terms of the biological activity they contain. For a vaccine, this is the amount of immunogen, defined as the amount of material required to generate an acceptable immune response in recipients. For many vaccines, the immune response generated in animal models is used to quantitate immunogenicity. Currently, in vitro methods (e.g., ELISA) which measure antigen—rather than immunogen—content are of interest as alternates. As the assays involve biological activity measured in a biological assay, and measurement of physical mass does not necessarily correlate with outcome, it is often scientifically inappropriate to express results from such assays in grams or SI units. Similar considerations apply to the measurement of antibody responses in clinical trials or serum surveys.

It is important to be able to compare results between studies, between sites, and over time. Comparison is made possible by the inclusion of a reference material in the relevant assays, and expressing the results obtained with the unknown relative to results with the reference. This principle has been applied since the early days of biologicals and has proved extremely powerful. International reference materials are established by WHO. The process involves preparation of the material in a stable form, usually lyophilised in a large number of ampoules containing the same amount of material to within tight limits. This material is then assayed by a number of interested and competent laboratories using assays they consider suitable. Other materials are assayed at the same time and the results analyzed to establish whether expression of results relative to the reference material reduces the variation between and within laboratories. The extent to which variation is reduced by expressing the results in terms of the common candidate reference material can be very striking.

While WHO reference materials are made in batches of several thousands, the supply is clearly limited, so the purpose of the WHO primary standard is to calibrate secondary regional or national reference materials for use in routine assays. In some countries, the National Control Laboratory prepares standards calibrated against the primary standard. However, preparation of national standards is costly and expertise to do so is limited in many countries. Preparation of regional secondary reference materials is preferred. The regional activities of the European Directorate for the Quality of

Medicines (EDQM) and the South East Asian Regional Office of WHO are excellent examples.

9.3. Active safety surveillance in selected low- and middle-income countries when new vaccines are introduced

Many countries have limited capacity and experience in implementing epidemiological vaccine safety studies. In the past, the availability of comprehensive vaccine safety assessment systems in the US and EU has also served the global need to evaluate new vaccines because most new vaccines were manufactured and introduced in the US and Europe prior to use elsewhere. However, new vaccines (e.g., meningitis A, malaria) are now being introduced or soon will be introduced either exclusively in the developing world or concurrently with their release in Europe and the US. Also, many vaccines that are procured globally are manufactured outside of the EU or US. Therefore, developing global and regional capacity to evaluate vaccine safety is highly desirable both to assure the safety of the world's vaccine supply, and also to prevent perceived vaccine safety concerns from undermining successful vaccination programs. Serious medical events of unknown origin, which can occur in temporal association with vaccination, can be mistakenly attributed to vaccines, thus derailing vaccination programs that would otherwise be very beneficial for the population [82].

Unfortunately, most low- and middle-income countries do not have the resources or technical capacity to implement timely and accurate traditional epidemiological studies of vaccine safety. Lack of training and absence of accurate population denominators are among the contributing reasons. In addition, most countries do not have the population size needed for the evaluation of very rare AEs. This highlights the need for taking a collaborative international approach, led by WHO, to the epidemiological investigation of serious and rare vaccine safety concerns.

To demonstrate that it is feasible to establish a collaborative, WHO-supported consortium of vaccine safety researchers and their respective organizations from developed and middle-income countries, a proof-of-concept study that investigated the risk of Guillain-Barré Syndrome (GBS) following H1N1 pandemic influenza vaccination was initiated. Medical hospitalization databases or registries were utilized to assess the risk of a medical outcome (GBS) following vaccination. Before choosing a study methodology, the consortium first analyzed what the basic requirements would be for a collaborative approach of this kind (intended to be inclusive of low- and middle-income countries). It was concluded that the ideal methodology would need to be simple so it could be implemented easily, and standardized for all sites. It was also decided that the investigation needed to be timely and use only resources already available in the local public health system, and avoid the need for population denominators (as they are either unavailable or grossly inaccurate in most low- and middle-income countries). Therefore, a cohort study design, which usually requires significant organization and resources in addition to accurate denominator population, would not have been suitable. Although a case control study design could be affordable, bias would be a significant problem (and difficult to resolve).

The consortium, therefore, chose for this proof-of-concept study a self-controlled case series (SCCS) methodology because of its flexibility and applicability to countries where population denominator information may not be available. The SCCS method [83,84] has been shown to be valid and efficient compared to alternative approaches (e.g., cohort and case-control designs) [85–87]. In an SCCS study, the individuals are essentially matched to themselves. Because of this implicit control of within-person characteristics, this design efficiently controls for all potentially non-time-dependent confounding characteristics, including demographics, co-morbid conditions, genetic susceptibility, and other characteristics that might not be measurable. As this collaborative approach would primarily be needed for serious (and mostly rare) AEs, it was determined that hospitalized cases should be adequate. Moreover, the requirement for an SCCS design is not to identify all cases of a disease, but to obtain an unbiased set of cases. For this purpose, the identification of all hospitalized cases of a serious disease or event during a period pre-specified by the research team would be appropriate. To allow data from the different sites to be comparable, a common study process and a standardized Brighton Collaboration case definition were used by all sites. The success of this initial proof-of-concept study is currently being analyzed. This study and approach can be expanded further in the future to include low-income countries, as well.

9.4. Coordination of regulatory science efforts

The international nature of vaccine supply and demand, plus increasingly complex vaccine supply chains, coupled with limited resources, strongly argue for international coordination of regulatory science efforts. Accordingly, a number of bi- or multi-lateral agreements between like-minded countries are being developed. In addition, and due to the global nature of access to vaccines, a globally coordinated effort is required. There is a strong history of WHO-led workshops and consultations in this area—for example, the series of workshops on pandemic influenza spearheaded by WHO/US FDA/HC in 2006, three years prior to the H1N1 outbreak in 2009. These proactive efforts enabled regulatory agencies to be better-positioned to identify the regulatory gaps, formulate strategies, and provide meaningful guidelines. Very importantly, the WHO umbrella enables developing countries to participate, for example, in the development and validation of international reference standards and lot release assays.

Another dimension is the interdisciplinary nature of the required coordination. In particular, linking the regulatory community with the academic research community around diseases, vaccines, and immunology would provide additional innovation and input. The examples of developing correlates of protection for pneumococcal conjugate vaccines [12] clearly indicate the regulatory benefits to be gained from high-quality academic research on target diseases and immunology.

9.5. Global regulatory science exchange and capacity-building

Scientists who fully understand how to apply science to address regulatory needs primarily work with or are closely affiliated with NRAs, and several NRAs employ research-reviewer scientists. Opportunities for regulatory science exchange between scientists in countries without regulatory science programs and those with advanced regulatory science programs will provide an important means toward regulatory science capacity-building.

As an example, the US FDA has a relatively new regulatory science capacity-building program developed initially within the National Center for Toxicological Research (NCTR). This International Scientist Exchange Program, or ISEP, provides an opportunity for scientists from countries with less-developed or no regulatory science programs to receive training in FDA laboratories for a period of three to six months. By actively working in a regulatory science research environment, the scientists learn and practice the core principles of regulatory science necessary to support the development of regulatory systems in their home country. While this program is still relatively new, it provides one example of an approach that could be broadened

to incorporate a network of ongoing regulatory science programs that are more advanced, whereby interested scientists from developing countries could choose the laboratory of interest based on the availability of resources and identified need. The potential benefit of this type of program is to inculcate more than just scientific training, per se, but also the philosophy of how to apply science to address regulatory needs, thus promoting an increased scientific base for advancing science-based policy and decision-making.

10. Conclusions

Inputs and ideas have been obtained from a broad range of regulators and synthesized into a proposed global regulatory science agenda for vaccines. Regulators have responded with great interest and have enthusiastically engaged in this initiative. Numerous historical and recent examples demonstrate the value and impact of applied regulatory science research on vaccine safety, efficacy, quality, and performance. Regulatory science research is clearly critical to developing relevant, robust methods to evaluate vaccine quality.

Current needs in regulatory science research include research to improve methods to measure vaccine potency to avoid or reduce use of animals or to increase the predictive value of the assay; identify appropriate methods to assess novel vaccine production methods; and develop and validate new high-resolution analytic methods for assessing vaccine quality and safety, such as NMR, mass spectrometry, and high-throughput sequencing. New tools should be developed to better evaluate the quality and breadth of immune responses and predict toxicity of adjuvants. Non-clinical methods should be developed to better evaluate cell-mediated immune responses to identify immune correlates of protection following the use of vaccines. Given the complex nature of endpoints for clinical studies of different type of diseases and the vaccines used to prevent them, multiple immune correlates should be considered. The development of highly predictive correlates of safety should have high priority on a regulatory research agenda. The development of innovative approaches to clinical trial design so as to speed up the evaluation of promising new vaccines, minimize the number of ineffective candidates that proceed to phase 3 studies, and promote efficient use of resources is also highly desirable.

Regulatory science should also explore new approaches to vaccine clinical trial design by taking advantage of improvements in post-marketing safety studies. Enhancing post-market surveillance of vaccine safety by using novel real-time surveillance methods as well as mining of social media is also envisaged. It would also be beneficial to develop standard benefit-risk methodology to aid regulatory decisions on vaccines, including refining risk-benefit analysis for use throughout a licensed vaccine's lifecycle.

Additional linkages are needed with science and technology communities to nurture the proposed innovations in vaccine regulation. An agreed Global Regulatory Science Agenda will enable synergies to be established where none currently exist. Further, such an agenda will facilitate the spread of regulatory expertise and the benefits of regulatory science to the less-well-resourced countries. Indicators to monitor and evaluate the progress of such activities could be targeted but not be limited to narrowing the gaps in regulatory sciences between the developed and developing countries and increased convergence of regulations on vaccines. This concept paper can be used to develop a plan of action to implement the first ever global regulatory science agenda.

Conflict of interest

All authors declare no conflict of interest.

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副反応報告と救済制度

多屋馨子

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はじめに

2013年3月30日に予防接種法の一部が改正され，2013年4月1日から施行された(法律第8号)．この改正で，予防接種後副反応報告が病院，あるいは診療所の開設者，または医師に義務づけられた．予防接種後の一定期間に，政令で定められた症状が認められた場合や，重篤な有害事象が認められた場合は，厚生労働大臣に予防接種後副反応報告書を提出する．本稿ではその制度と，別に定められている健康被害救済制度について概要を記述する．

予防接種の種類

2013年12月現在，薬事法に基づいて製造販売承認され，国内で接種可能なワクチンの種類を示す(**表1**)[1]．なお，国家備蓄されている痘そうワクチンやA(H5N1)亜型のインフルエンザワクチンはこの表には含めていない．

わが国では予防接種法に基づく定期の予防接種(以下，定期接種)と，臨時の予防接種(以下，臨時接種)，2009年に発生したインフルエンザ(H1N1)pdmのように，感染力は強いが病原性が季節性インフルエンザと同等の場合などに実施される新臨時接種の3つの制度がある．

定期接種には，国の積極的な勧奨があり受けるよう努める義務(以下，努力義務)があるA類疾病と，国の積極的な勧奨はなく努力義務もないB類疾病がある[2]．臨時接種は国の積極的な勧奨があり努力義務もあるが，新臨時接種は国の積極的な勧奨はあるが努力義務がない．以上の枠組みのいずれにも属さない予防接種(以下，任意接種)は，国の積極的な勧奨はなく努力義務もない．

予防接種後健康被害救済制度

ワクチンは，製造，検定，流通過程，接種の過程で全く過誤なく実施されたとしても副反応が発生する可能性があるため，過失に対する補償や賠償ではない健康被害救済制度が必要であり，法律に基づいた制度がある．

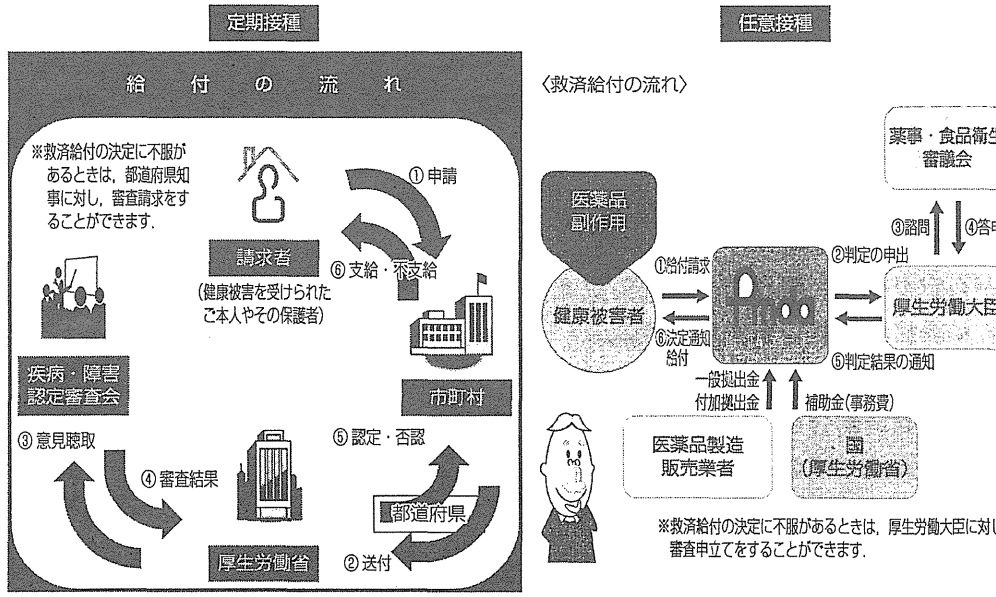
健康被害救済給付は，定期接種・臨時接種・新臨時接種については予防接種法に基づいて，任意接種は医薬品医療機器総合機構(Pharmaceuticals and Medical Devices Agency; PMDA)法に基づいて行われている．健康被害救済申請は，本人あるいは保護者が提出するが，定期接種の場合は実施主体である市区町村に，任意接種の場合は，PMDAに申請書を提

表 1　日本で接種可能な予防接種の種類(2013 年 12 月現在)：26 種類(国家備蓄のワクチンを除く)

	定期接種(年齢は政令で規定)：14 種類	任意接種：12 種類
A 類疾病	BCG	水痘
	MR(麻疹風疹混合)	流行性耳下腺炎(おたふくかぜ)
	麻疹	ロタウイルス 1 価
	風疹	ロタウイルス 5 価
	Hib(インフルエンザ菌 b 型)	黄熱
	肺炎球菌(13 価結合型)	OPV(生ポリオ)
	DPT-IPV(ジフテリア・破傷風・百日咳・不活化ポリオ混合)	B 型肝炎
	DPT(ジフテリア・破傷風・百日咳混合)	肺炎球菌(23 価多糖体)
	IPV(不活化ポリオ)	破傷風トキソイド
	日本脳炎	A 型肝炎
	DT(ジフテリア・破傷風混合)	狂犬病
	HPV(ヒトパピローマウイルス)2 価	成人用ジフテリアトキソイド
	HPV(ヒトパピローマウイルス)4 価	定期接種対象ワクチンを政令で定められた年齢以外で受ける場合
B 類疾病	インフルエンザ	

生ワクチン　不活化ワクチン・トキソイド

(筆者作成)



(厚生労働省リーフレット：「ご存じですか？予防接種後健康被害救済制度」より引用抜粋)

〔日本医師会・日本薬剤師会・医薬品医療機器総合機構(PMDA) リーフレット：「誰よりも知ってほしい．伝えてほしい．医薬品副作用被害救済制度」より引用抜粋〕

図 1　予防接種健康被害救済制度の略図

出する(**図 1**)[2,3]．

定期接種の場合は市区町村で健康被害調査委員会が開催され，その結果とともに都道府県を通して厚生労働省に申請される．厚生労働省では，疾病・障害認定審査会で審議され，当該ワクチンによって発生したことが明らかな場合や

表2　予防接種制度別予防接種後健康被害救済給付額

	給付額の比較		
	臨時接種及び A類疾病の定期接種	B類疾病の定期接種	(参考)医薬品副作用被害救済制度 生物由来製品感染等被害救済制度
医療費	健康保険等による給付の額を除いた自己負担分	A類疾病の額に準ずる	健康保険等による給付の額を除いた自己負担分
医療手当	通院3日未満(月額)　33,600円 通院3日以上(月額)　35,600円 入院8日未満(月額)　33,600円 入院8日以上(月額)　35,600円 同一月入通院(月額)　35,600円	A類疾病の額に準ずる	通院3日未満(月額)　33,600円 通院3日以上(月額)　35,600円 入院8日未満(月額)　33,600円 入院8日以上(月額)　35,600円 同一月入通院(月額)　35,600円
障害児養育年金	1級(年額)　1,520,400円 2級(年額)　1,215,600円		1級(年額)　844,800円 2級(年額)　675,600円
障害年金	1級(年額)　4,860,000円 2級(年額)　3,888,000円 3級(年額)　2,916,000円	1級(年額)　2,700,000円 2級(年額)　2,160,000円	1級(年額)　2,700,000円 2級(年額)　2,160,000円
死亡した場合の補償	死亡一時金　42,500,000円	・生計維持者でない場合 遺族一時金　7,084,800円 ・生計維持者である場合 遺族年金(年額)　2,361,600円 (10年を限度)	・生計維持者でない場合 遺族一時金　7,084,800円 ・生計維持者である場合 遺族年金(年額)　2,361,600円 (10年を限度)
葬祭料	201,000円	A類疾病の額に準ずる	201,000円
介護加算	1級(年額)　834,200円 2級(年額)　556,200円		

(注1) 単価は平成25年4月現在.
(注2) 具体的な給付額については，政令で規定.
(注3) B類疾病の定期接種に係る救済額については，医薬品副作用被害救済制度の給付額を参酌して定めることとされている
※新たな臨時接種の給付水準は，臨時接種及びA類疾病と，B類疾病の間の水準とする.
(厚生労働省健康局結核感染症課予防接種室：予防接種リサーチセンター主催予防接種従事者研修会資料より引用抜粋)

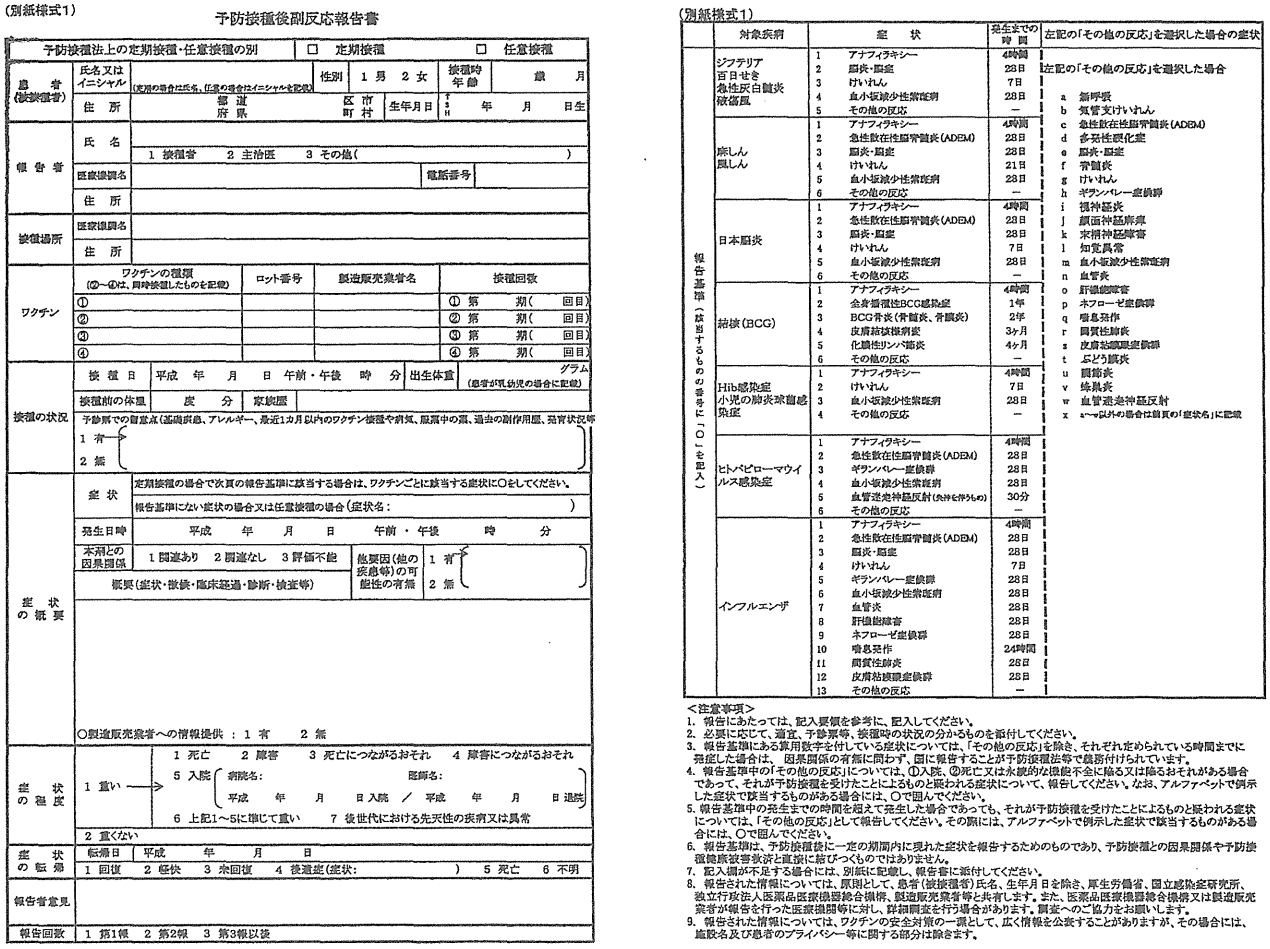
その可能性が否定できない場合で，厚生労働大臣が認定した場合に救済給付が決定される.

定期接種・臨時接種・新臨時接種については外来・入院ともに救済給付の対象となるが，任意接種については入院相当の医療が給付の対象となる．救済給付額は政令で定められているが，救済給付が認められた場合，救済される医療費・医療手当は定期接種・臨時接種・新臨時接種・任意接種とも同じであるが，障害が残った場合や死亡にいたった場合は，定期接種A類疾病・臨時接種と，新臨時接種，定期接種B類疾病，任意接種で救済給付額に違いがある(**表2**)[2].

予防接種後副反応報告制度の概要

2013年の法改正以前は，定期接種と任意接種で報告方法が異なり，制度が複雑であったが[4]，今回の法改正で，効率的に一本化されることになった.

また，今回の法改正で予防接種に関する評価検討組織が新たに設置され，厚生科学審議会予防接種・ワクチン分科会のもとに，①予防接種基本方針部会，②研究開発及び生産・流通部会，③副反応検討部会の3つの部会が設置された．予防接種後副反応は，③の「厚生科学審議会予防接種・ワクチン分科会副反応検討部会」と，「薬事・食品衛生審議会医薬品等安



（別紙様式1）

予防接種後副反応報告書

予防接種法上の定期接種・任意接種の別			□ 定期接種		□ 任意接種	
患者（被接種者）	氏名又はイニシャル（死亡の場合は氏名、任意の場合はイニシャルを記載）		性別	1 男 2 女	接種時年齢	歳 月
	住所	都道府県 区市町村		生年月日	T・S・H 年 月 日生	
報告者	氏名					
		1 接種者 2 主治医 3 その他（ ）				
	医療機関名				電話番号	
	住所					
接種場所	医療機関名					
	住所					

ワクチン	ワクチンの種類（②～④は、同時接種したものを記載）	ロット番号	製造販売業者名	接種回数
	①			① 第 期（ 回目）
	②			② 第 期（ 回目）
	③			③ 第 期（ 回目）
	④			④ 第 期（ 回目）

接種の状況				
	接種日	平成 年 月 日 午前・午後 時 分	出生体重	グラム（患者が乳幼児の場合に記載）
	接種前の体温	度 分	家族歴	
	予診票での留意点（基礎疾患、アレルギー、最近1カ月以内のワクチン接種や病気、服薬中の薬、過去の副作用歴、発育状況等）			
	1 有 →			
	2 無			

症状の概要				
	症状	定期接種の場合で次頁の報告基準に該当する場合は、ワクチンごとに該当する症状に○をしてください。		
		報告基準にない症状の場合又は任意接種の場合（症状名： ）		
	発生日時	平成 年 月 日 午前・午後 時 分		
	本剤との因果関係	1 関連あり 2 関連なし 3 評価不能	他要因（他の疾患等）の可能性の有無	1 有 → 2 無
	概要（症状・徴候・臨床経過・診断・検査等）			
	○製造販売業者への情報提供 ： 1 有 2 無			

症状の程度		
	1 重い →	1 死亡 2 障害 3 死亡につながるおそれ 4 障害につながるおそれ 5 入院（病院名： 医師名： 平成 年 月 日入院 ／ 平成 年 月 日退院） 6 上記1～5に準じて重い 7 後世代における先天性の疾病又は異常
	2 重くない	

症状の転帰		
	転帰日	平成 年 月 日
	1 回復 2 軽快 3 未回復 4 後遺症（症状： ） 5 死亡 6 不明	
報告書意見		
報告回数	1 第1報 2 第2報 3 第3報以後	

（別紙様式1）

報告基準（該当するものを1つに○を記入）

対象疾病		症状	発生までの時間	左記の「その他の反応」を選択した場合の症状
ジフテリア 百日せき 急性灰白髄炎 破傷風	1	アナフィラキシー	4時間	左記の「その他の反応」を選択した場合 a 無呼吸 b 気管支けいれん c 急性散在性脳脊髄炎（ADEM） d 多発性硬化症 e 脳炎・脳症 f 脊髄炎 g けいれん h ギランバレー症候群 i 視神経炎 j 顔面神経麻痺 k 末梢神経障害 l 知覚異常 m 血小板減少性紫斑病 n 血管炎 o 肝機能障害 p ネフローゼ症候群 q 喘息発作 r 間質性肺炎 s 皮膚粘膜眼症候群 t ぶどう膜炎 u 関節炎 v 結節炎 w 血管迷走神経反射 x a～w以外の場合は前頁の「症状名」に記載
	2	急性散在性脳脊髄炎（ADEM）	28日	
	3	脳炎・脳症	7日	
	4	けいれん	28日	
	5	その他の反応	—	
麻しん 風しん	1	アナフィラキシー	4時間	
	2	急性散在性脳脊髄炎（ADEM）	28日	
	3	脳炎・脳症	28日	
	4	けいれん	21日	
	5	血小板減少性紫斑病	28日	
	6	その他の反応	—	
日本脳炎	1	アナフィラキシー	4時間	
	2	急性散在性脳脊髄炎（ADEM）	28日	
	3	脳炎・脳症	28日	
	4	けいれん	7日	
	5	血小板減少性紫斑病	28日	
	6	その他の反応	—	
結核（BCG）	1	アナフィラキシー	4時間	
	2	全身播種性BCG感染症	1年	
	3	BCG骨炎（骨髄炎、骨膜炎）	2年	
	4	皮膚結核様病変	3ヶ月	
	5	化膿性リンパ節炎	4ヶ月	
	6	その他の反応	—	
Hib感染症 小児の肺炎球菌感染症	1	アナフィラキシー	4時間	
	2	けいれん	7日	
	3	血小板減少性紫斑病	28日	
	4	その他の反応	—	
ヒトパピローマウイルス感染症	1	アナフィラキシー	4時間	
	2	急性散在性脳脊髄炎（ADEM）	28日	
	3	ギランバレー症候群	28日	
	4	血小板減少性紫斑病	28日	
	5	血管迷走神経反射（失神を伴うもの）	30分	
	6	その他の反応	—	
インフルエンザ	1	アナフィラキシー	4時間	
	2	急性散在性脳脊髄炎（ADEM）	28日	
	3	脳炎・脳症	28日	
	4	けいれん	7日	
	5	ギランバレー症候群	28日	
	6	血小板減少性紫斑病	28日	
	7	血管炎	28日	
	8	肝機能障害	28日	
	9	ネフローゼ症候群	28日	
	10	喘息発作	24時間	
	11	間質性肺炎	28日	
	12	皮膚粘膜眼症候群	28日	
	13	その他の反応	—	

<注意事項>
1. 報告にあたっては、記入要領を参考に、記入してください。
2. 必要に応じて、追宜、予診票等、接種時の状況の分かるものを添付してください。
3. 報告基準にある算用数字を付している症状については、「その他の反応」を除き、それぞれ定められている時間までに発症した場合は、因果関係の有無に関わず、医に報告することが予防接種法等で義務付けられています。
4. 報告基準中の「その他の反応」については、①入院、②死亡又は永続的な機能不全に陥る又は陥るおそれがある場合であって、それが予防接種を受けたことによるものと疑われる症状について、報告してください。なお、アルファベットで例示した症状で該当するものがある場合には、○で囲んでください。
5. 報告基準中の発生までの時間を超えて発生した場合であっても、それが予防接種を受けたことによるものと疑われる症状については、「その他の反応」として報告してください。その際には、アルファベットで例示した症状で該当するものがある場合には、○で囲んでください。
6. 報告基準は、予防接種後に一定の期間内に現れた症状を報告するためのものであり、予防接種との因果関係や予防接種健康被害救済と直接に結びつくものではありません。
7. 記入欄が不足する場合には、別紙に記載し、報告書に添付してください。
8. 報告された情報については、原則として、患者（被接種者）氏名、生年月日を除き、厚生労働省、国立感染症研究所、独立行政法人医薬品医療機器総合機構、製造販売業者等と共有します。また、医薬品医療機器総合機構又は製造販売業者が報告を行った医療機関等に対し、詳細調査を行う場合があります。調査へのご協力をお願いします。
9. 報告された情報については、ワクチンの安全対策の一環として、広く情報を公表することがありますが、その場合には、施設名及び患者のプライバシー等に関する部分は除きます。

図2　予防接種後副反応報告書（医師報告用）

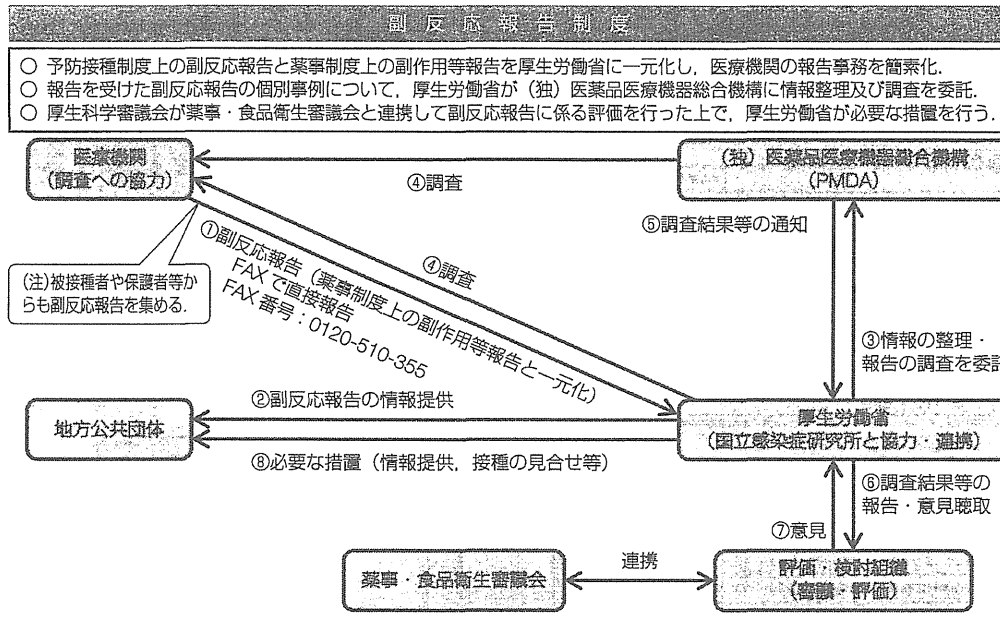
全対策部会安全対策調査会」の合同開催で検討が行われている〔座長：(奇数回)副反応検討部会部会長の桃井眞里子国際医療福祉大学副学長，(偶数回)安全対策調査会長の五十嵐隆国立成育医療研究センター総長〕．

報告基準が策定され，報告様式が定期・任意の区別なく共通になった(**図2**)．報告対象となる症状と，接種から症状出現までの期間が記載されている．その他の反応を選択した場合は，a～w の症状・疾患名から選択する．いずれにも該当しなかった場合については，x を選択したうえで，「症状の概要」の項にある「症状名」に記載をすることになった．

薬事法上の報告とも一元化され，予防接種法に基づいて上記の報告書を送付すれば，薬事法に基づく報告もかねることになった．また，法改正までは報告書の送付に対して，保護者の同意が必要であったが，それが不要となった．別に保護者からの報告も可能となり，保護者用報告書も創設された．

報告方法は，厚生労働省へのFAX(FAX番号：0120-510-355)による直接報告となり，定期接種の実施主体である市区町村へは，都道府県を通じて厚生労働省から速やかに情報が還元されることになった(**図3**)．

現状では手書きの報告であるため，「チェック機能を搭載した電子媒体報告書」が厚生労働科学研究班「予防接種後副反応サーベイランス



（厚生科学審議会予防接種・ワクチン分科会副反応検討部会資料より引用一部改変）

図3　予防接種後副反応報告制度

表3　予防接種後副反応報告数・報告頻度

（2013年4月1日～2013年7月31日報告分：報告日での集計，インフルエンザ＊については2012年10月～2013年3月31日報告分）

ワクチンの種類	接種可能のべ人数（回数）	製造販売業者からの報告		医療機関からの報告			
				全報告数			
		報告数	（報告頻度）	全報告数	（報告頻度）	うち重篤	（うち重篤の報告頻度）
Hib（ヒブ）	1,643,852	42	0.003%	76	0.005%	40(3)	0.002% (0.0002%)
7価結合型肺炎球菌	1,551,513	53	0.003%	81	0.005%	42(3)	0.003% (0.0002%)
不活化ポリオ	483,283	4	0.001%	15	0.003%	10	0.002%
DPT-IPV	1,026,312	19	0.002%	44	0.004%	19(1)	0.002% (0.0001%)
ヒトパピローマウイルス（2価）	84,903	60	0.071%	106	0.125%	56	0.066%
ヒトパピローマウイルス（4価）	165,399	23	0.014%	102	0.062%	31	0.019%
インフルエンザ＊	50,240,735	86(5)	0.00017% (0.000012%)	301	0.0006%	53(4)	0.0001% (0.000008%)
日本脳炎	1,870,888	9	0.0005%	47	0.003%	19	0.001%

※（　）内は死亡報告数とその頻度．
※医療機関からの報告の「重篤」とは，死亡，障害，それらに繋がるおそれのあるもの，入院相当以上のものが報告対象とされているが，必ずしも重篤でないものも「重篤」として報告されるケースがある．
※製造販売業者からの副反応報告は，薬事法第77条の4の2に基づき「重篤」と判断された症例について報告されたものである．
※製造販売業者からの報告には，医療機関から報告された症例と重複している症例が含まれている可能性がある．また，その後の調査等によって，報告対象でないことが確認され，報告が取り下げられた症例が含まれる可能性がある．
※製造販売業者からの報告には，複数の製造販売業者から重複して報告されている症例が含まれている可能性がある．

（副反応検討部会資料より抜粋）

表4　予防接種後副反応報告数・報告頻度

(2013年4月1日～2013年6月30日報告分：報告日での集計)

ワクチンの種類	接種可能のべ人数(回数)	製造販売業者からの報告		医療機関からの報告			
				全報告数			
		報告数	(報告頻度)	全報告数	(報告頻度)	うち重篤	(うち重篤の報告頻度)
DPT	380,045	3(0)	0.001%	28	0.007%	17(1)	0.002% (0.0003%)
DT	627,495	1(0)	0.0002%	12	0.002%	1	0.0002%
麻しん風しん混合(MR)	1,380,873	7(0)	0.001%	34	0.002%	16(2)	0.001% (0.0001%)
麻しん	27,767	2(0)	0.007%	1	0.004%	1	0.004%
風しん	45,667	0	0%	1	0.002%	0	0%
BCG	151,988	4(0)	0.003%	41	0.027%	7	0.005%
おたふくかぜ	231,900	8(0)	0.003%	8	0.003%	6	0.003%
水痘	271,951	1(0)	0.0004%	7	0.003%	4	0.001%
ロタウイルス(1価)	158,004	20(0)	0.013%	14	0.009%	10	0.006%
ロタウイルス(5価)	119,559	8(0)	0.007%	10	0.008%	5	0.004%
肺炎球菌(23価)	125,099	11(1)	0.009% (0.0008%)	6	0.005%	1	0.0008%
B型肝炎	862,856	7(0)	0.001%	12	0.001%	6(1)	0.001% (0.0001%)
ジフテリアトキソイド	92	0	0%	0	0%	0	0%
破傷風トキソイド	187,110	3(0)	0.002%	2	0.001%	0	0%
A型肝炎	32,814	1(0)	0.003%	0	0%	0	0%

※(　)内は死亡報告数とその頻度

※医療機関からの報告の「重篤」とは，死亡，障害，それらに繋がるおそれのあるもの，入院相当以上のものが報告対象とされているが，必ずしも重篤でないものも「重篤」として報告されるケースがある．

※製造販売業者からの副反応報告は，薬事法第77条の4の2に基づき「重篤」と判断された症例について報告されたものである．

※製造販売業者からの報告には，医療機関から報告された症例と重複している省令が含まれている可能性がある．また，その後の調査等によって，報告対象でないことが確認され，報告が取り下げられた症例が含まれる可能性がある．

※製造販売業者からの報告には，複数の製造販売業者から重複して報告されている症例が含まれている可能性がある．

(副反応検討部会資料より抜粋)

の効果的な運用とその行政的な活用のあり方に関する研究(研究代表者：多屋馨子)」で作成され，厚生労働省および国立感染症研究所のホームページからダウンロード可能となるよう準備を進めているところである．

届けられた報告書は，厚生労働省と国立感染症研究所とPMDAで情報の共有が行われ，PMDAで整理と調査が行われることになった．調査結果がPMDAから厚生労働省に報告された後は，評価検討組織で審議・評価が実施され，会議の議事録および配布資料は厚生労働省のホームページに公表されている[5]．

評価検討組織で公表された資料[5]から，ワクチンごとの報告数ならびに報告頻度をまとめた(**表3・表4**)．ヒトパピローマウイルスワクチンについては，「接種部位以外の体の広い範囲で持続する疼痛の副反応症例などについて十分に情報提供できない状況にあることから，接種希望者の接種機会は確保しつつ，適切な情報提供ができるまでの間は，積極的な接種勧奨を一

時的に差し控えるべき」とされ，2013年6月14日から積極的な勧奨が差し控えられている．再開の是非については，副反応について可能な限り調査を実施し，速やかに専門家の会議による分析・評価を行ったうえで，改めて判断することとされ，2013年12月25日に副反応検討部会が予定されている．

おわりに

わが国でも予防接種後副反応報告制度(Vaccine Adverse Event Reporting System; VAERS)が予防接種法に基づいて始まったことは高く評価される．先進的とされる米国のVAERSのシステム[6]，韓国のシステムなどを参考に，異常な副反応のシグナルを探知し，迅速にアラートを出すしくみも必要である．また，過去に知られていなかった新たな副反応を検出したり，既知の副反応をモニタリングしたり，特定の副反応におけるリスク因子の同定，副反応報告が増加傾向にあるワクチンロットの検出，新しいワクチンの安全性に関するアセスメントなどを行い，ワクチンの安全性に関する研究を進めることで，国民に正しい情報を提供していくことが可能となる．今後は，VAERSに加えて，米国のVSD(Vaccine Safety Datalink)やCISA(Clinical Immunization Safety Assessment)ネットワーク[6]などのしくみの構築と，必要に応じて迅速な積極的疫学調査が必要となるであろう．

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Antigen-capture ELISA for the detection of Rift Valley fever virus nucleoprotein using new monoclonal antibodies

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ABSTRACT

Monoclonal antibodies (MAbs) raised against the nucleoprotein (NP) of Rift Valley fever virus (RVFV) were developed, and an antigen-capture enzyme-linked immunosorbent assay (Ag-capture ELISA) system was developed for the detection of RVFV NP. The assay detected RVFV antigen from culture supernatants containing as little as 7.8–31.3 pfu per 100 μl. Reactivity with various truncated NPs indicated that MAb C10-54 bound only to the full-length NP, probably due to recognition of a conformational epitope, whereas MAbs G2-36 and D5-59 bound to a linear epitope ranging from amino acid residues 195–201 in the C-terminal region. Based on the alignments of the amino acid sequence of RVFV NP, the epitope regions of MAbs G2-36 and D5-59 were completely conserved among all RVFV strains. These results suggest that the MAbs are applicable to the Ag-capture ELISA for the diagnosis of RVFV infections.

1. Introduction

Rift Valley fever (RVF), a mosquito-borne zoonotic disease that affects domestic animals and humans, is caused by infection by the RVF virus (RVFV). The disease is found in sub-Saharan areas in Africa, as well as in Egypt, the Comoros Islands, Madagascar, and the Arabian Peninsula (Shimshony and Barzilai, 1983; Shoemaker et al., 2002; Sissoko et al., 2009). Infection of RVFV causes abortions or resorption of the fetus in pregnant domestic ruminants, with newborn mortality approaching 100%, and thus can cause catastrophic economic losses. Transmission of RVFV to humans, either through contact with bodily fluids of infected animals or mosquito bites, may result in mild to moderate influenza-like symptoms and severe retinitis, encephalitis and hemorrhagic fever (Alrajhi et al., 2004; Gerdes, 2004; Shimshony, 1999). During an RVF outbreak, confirmed cases are defined as suspected or probable cases by laboratory confirmation of the presence of anti-RVFV IgM by enzyme-linked immunosorbent assay (ELISA), RVFV antigen by antigen-captured (Ag-captured) ELISA, or viral RNA by RT-PCR in serum or blood samples (Al-Hazmi et al., 2003; Bird et al., 2008; Madani et al., 2003; MMWR, 2007).

RVFV belongs to the *Phlebovirus* genus of the *Buniyaviridae* family. Like other members of the family, RVFV possesses a single-stranded tripartite RNA genome composed of three segments, namely, S, M, and L. The S segment codes for nucleocapsid protein (NP) in negative sense, and non-structural NSs protein in positive sense, using an ambisense strategy. The M segment codes for a precursor of glycoproteins Gn and Gc and two non-structural proteins of 78 kDa and 14 kDa. The L segment codes for an L protein. The nucleotide sequence of the NP gene is highly conserved among various RVFV isolates (Bird et al., 2007b). Serum antibodies against NP are detected readily early after infection and in the convalescent phase, providing a basis for the diagnosis of RVF (Fafetine et al., 2007; Jansen van Vuren et al., 2007). An Ag-capture ELISA for detecting viral NP has been applied commonly to detect RVFV, as well as various viruses, since it is the most abundant viral antigen (Al-Hazmi et al., 2003; Bird et al., 2008; Jansen van Vuren and Paweska, 2009; Ji et al., 2011; Saijo et al., 2005, 2006, 2007; Madani et al., 2003; Nakauchi et al., 2009; Velumani et al., 2008). Monoclonal antibodies (MAbs) are used often as a capture antibody for Ag-capture ELISA since they have a high specificity for antigens, and identification of the epitopes of MAbs is of crucial importance for the assessment of cross-reactivity of the assay (Saijo et al., 2005, 2006, 2007; Nakauchi et al., 2009). In this study, MAbs were raised against recombinant RVFV NP (RVFV rNP). Epitope mapping showed that these MAbs recognized highly conserved epitopes on RVFV NP, suggesting their potential application for the detection of all RVFV isolates. By using these MAbs as capture antibodies, an Ag-capture ELISA for detecting an RVFV antigen was developed.

2. Materials and methods

2.1. Recombinant baculoviruses

Ac-His-RVFV-NP baculoviruses, expressing RVFV rNP, were generated using the same strategy as previously described (Saijo et al.,

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2002). Briefly, an entire cDNA clone of NP from RVFV-MP12 was used to construct a transfer vector. RVFV NP cDNA was amplified by PCR. The amplified DNA was digested with BamHI and subcloned into the BamHI site of pQE30 vector DNA (QIAGEN GmbH, Hilden, Germany) to construct pQE30-RVFV NP. An RVFV NP DNA fragment with a six-histidine (His) tag was isolated from the pQE30-RVFV NP plasmid by digestion with EcoRI and SalI. Each extremity was then blunted with T4 DNA polymerase and subcloned into the blunt-ended BamHI site of pAcYM1 (Saijo et al., 2002) to generate pAcYM1-His RVFV rNP.

Tn5 insect cells were transfected with mixtures of linearized BacPAK6 DNA (Clontech, Mountain View, CA) and the recombinant transfer vector pAcYM1-His RVFV rNP according to the manufacturer's instructions and the procedures described by Kitts and Possee (1993). The resulting recombinant baculovirus, which expresses a His-tagged recombinant NP of RVFV (His-RVFV rNP), was designated as Ac-His-RVFV NP.

2.2. Expression and purification of rNPs

Ac-His-RVFV NP-infected Tn5 cells were incubated at 26 °C for 72 h. Then, the cells were washed twice with cold phosphate-buffered saline (PBS) solution and lysed in cold PBS solution containing 1% Nonidet P-40 (NP-40). The cell lysate was centrifuged at 13,000 × *g* at 4 °C for 10 min. The supernatant fraction was collected as a source of His-RVFV rNP for purification. The His-RVFV rNP was purified by Ni^{2+} column chromatography (QIAGEN GmbH), as previously described (Saijo et al., 2002). Sabia virus (SABV) rNP as a control was expressed and purified, as described previously (Nakauchi et al., 2009).

2.3. Establishment of MAbs

BALB/c mice were immunized subcutaneously three times with the purified His-RVFV rNP. Spleen cells were obtained 3 days after the last immunization and fused with P3/Ag568 cells using polyethylene glycol (Invitrogen). The culture supernatants of the hybridoma cells were screened by ELISA with purified His-RVFV rNP as an Ag in the presence of 2 M urea in order to exclude MAbs with a low-avidity. MAbs, designated as D5-59, C10-54, and G2-36, were purified from the culture supernatants of the respective hybridoma clones using protein-G column chromatography, as described previously (Nakauchi et al., 2009).

2.4. Polyclonal antibodies

Polyclonal antibodies were raised in rabbits by immunization with the purified His-RVFV rNP expressed in the baculovirus system, as described previously (Saijo et al., 2002). Protocols for animal experiments were approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases, Tokyo, Japan.

2.5. Indirect immunofluorescence assay

Vero E6 cells were infected with RVFV-MP12 (MOI = 0.1). After 18 h, the cells were fixed with a mixture of methanol and acetone [1:1 (v/v)]. Binding of the RVFV infected cells was performed by immunofluorescence assay (IFA), as described previously (Saijo et al., 2005).

2.6. Ag-capture ELISA

The Ag-capture ELISA was performed essentially as described elsewhere (Saijo et al., 2005, 2006, 2007; Nakauchi et al., 2009). Purified MAb D5-59, C10-54, or G2-36 was coated on 96-microwell immunoplates (Falcon; Becton Dickinson Labware) at 100 ng/well in 100 μl of PBS at 4 °C overnight, followed by blocking with PBS containing 0.05% Tween-20 and 5% skim milk (PBST-M) for 1 h at room temperature. After the plates were washed three times with PBS containing 0.05% Tween-20 (PBST), 100 μl samples containing serially diluted His-RVFV rNP or culture supernatants of Vero cells, either infected with RVFV MP12 or uninfected, were added, and the plates were incubated for 1 h at 37 °C (viruses were inactivated by treatment with 1% NP40 followed by UV irradiation for 15 min). The plates were then washed with PBST, and 100 μl of rabbit anti-serum raised against His-RVFV rNP diluted 1:500 with PBST-M was added to each well. After 1 h of incubation at 37 °C, the plates were washed with PBST, and horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) was added. The plates were further incubated for 1 h at room temperature. After another extensive washing with PBST, 100 μl of ABTS substrate solution (Roche Diagnostics) was added and the OD405 was measured with a reference wavelength of 490 nm after 30 min of incubation at room temperature. As a negative control, the ODs of wells inoculated with control Ag (SABV rNP or culture supernatants of mock-infected Vero cells) were measured. Means and standard deviations were calculated from the OD405 values of 12 negative control wells, and the cutoff value for the assay was defined as the mean plus three standard deviations.

2.7. Expression of truncated rNPs of RVFV

In order to determine the epitope region reacted with the MAbs, a series of truncated RVFV rNPs were expressed as fusion proteins with glutathione S-transferase (GST), as previously described (Nakauchi et al., 2009). Briefly, the cDNA corresponding to each of the truncated NP fragments was amplified by PCR with specifically designed primer sets. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). The GST-tagged full-length RVFV NP or truncated forms of RVFV NP were expressed in *Escherichia coli* (*E. coli*) BL21.

2.8. Western blotting

The MAbs were tested for reactivity to the GST-tagged RVFV NP fragments expressed in *E. coli* by Western blotting, as described previously (Saijo et al., 2005). Goat anti-GST antibody (GE Healthcare, Piscataway, NJ) was used for detection of GST-tagged proteins in the assay.

2.9. Plaque assay

VeroE6 cells prepared in 12-well plates were inoculated with 50 μl of 10-fold serially diluted virus samples and incubated at 37 °C for 1 h. Then the cells were cultured with 1.0 ml per well of DMEM containing 1% FCS and 1% methyl cellulose (Sigma) for 5 days. Cells were fixed with 10% formalin, irradiated under a UV lamp, and stained with crystal violet. Plaques produced by RVFV were counted under light microscopy. Titration was done in duplicate and infectivity was displayed by plaque-forming units (pfu).

3. Results

3.1. Generation of MAbs

In order to obtain MAbs against RVFV NP, BALB/c mice were immunized with purified RVFV rNP. The hybridomas were cloned and their culture supernatants were tested for reactivity to RVFV rNP by IgG ELISA. Three MAbs, designated D5-59, C10-54, and G2-36, reacted with the His-RVFV rNP by IgG ELISA, even in the presence of 2.0 M urea (data not shown). The reactivity of MAbs

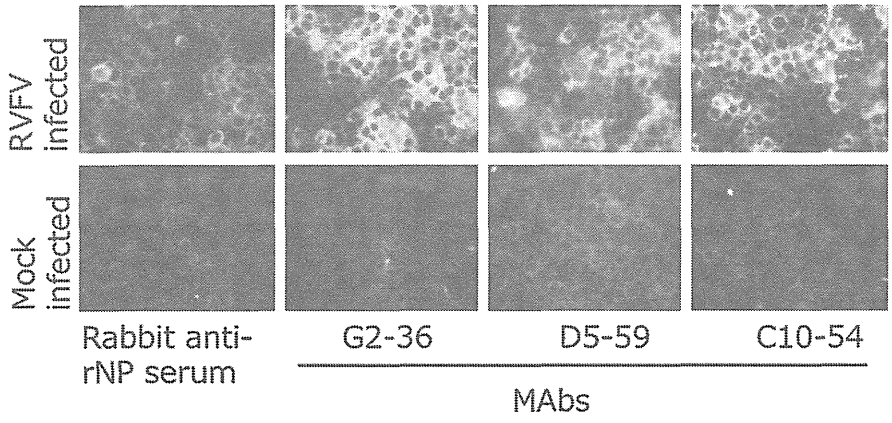

Fig. 1. Reactivity of MAbs in IFA. Vero E6 cells infected with RVFV MP12 were stained by indirect immunofluorescence with MAbs D5-59, C10-54, and G2-36. Rabbit anti-rNP serum was used as a positive control.

was examined by an indirect immunofluorescence method (Fig. 1). The RVFV-infected (MOI = 0.1), but not mock-infected, cells were stained with each of these MAbs (Fig. 1). The staining pattern was consistently similar in all MAbs tested and was characterized by a diffuse granular cytoplasmic staining, similar to that observed previously (Billecocq et al., 1996), indicating specific recognition of MAbs against RVFV NP.

3.2. Development of Ag-capture ELISA

By using these MAbs and rabbit anti-rNP serum as capture and detection antibodies, respectively, an Ag-capture ELISA for detecting RVFV NP was developed. When the His-RVFV rNP antigen was used in Ag-capture ELISA, MAbs D5-59 and G2-36 detected as little as 0.16 ng per 100 μl of rNP, whereas MAb C10-54 was more sensitive in detecting the rNP, with a detection limit of 0.08 ng per 100 μl (Fig. 2). None of these MAbs reacted with rNP of the control virus, SABV, prepared from insect cells, even at high antigen concentration (20 ng per 100 μl). RVFV antigen in the culture supernatants from Vero E6 cells infected with RVFV-MP12 was also detected in the developed Ag-capture ELISA, whereas mock-infected cells showed a negative reaction, indicating that MAbs reacted not only with recombinant NP, but also with an authentic viral NP (Fig. 3). MAbs D5-59, C10-54, and G2-36 were able to detect as little as 15.6, 7.8, and 31.3 pfu per 100 μl of RVFV, respectively.

3.3. Epitope mapping of MAbs

To determine the binding regions including epitopes of MAbs, truncated NPs were expressed in *E. coli* and analyzed for MAbs reactivity by Western blot analysis. At first, five forms of truncated rNP, as well as full-length rNP, were examined for MAbs reactivity (Fig. 4A). MAbs G2-26 and D5-59 reacted with the full-length (NP1-245) and C-terminus region (NP177-245 and NP76-245) of rNP, whereas MAb C10-54 reacted only with the full-length rNP. These results indicated that the binding region for MAbs G2-36 and D5-59 mapped within the C-terminus one-third, corresponding to amino acid residues 177–245, and that full-length RVFV NP was required for MAb C10-54 to react. MAb C10-54 could recognize a conformational epitope since it reacts weakly by Western blot against full-length rNP, probably due to epitope renaturation during or after the transfer of the protein to a membrane as reported by Zhou et al. (2007).

To narrow the region recognized by MAbs G2-36 and D5-59, additional truncated rNPs from amino acid residues 177–245 were generated and tested for reactivity by Western blot analysis. Both MAbs reacted with NP177-201 and NP195-235, but not with NP177-200 and NP196-235, indicating that minimum region for these MAbs ranged from amino acid residues 195–201 (Fig. 3B). The results also suggested essential amino acid residues 195 and 201 for MAb binding. Although one truncated NP (NP177-200) was reacted with MAbs G2-36 and D5-59, the intensities of this protein bands were significantly lower than those of the NPs containing amino acid residues 195–201. The result might be attributed to the lack of critical amino acid residue 201 on NP177-200.

To ascertain whether MAbs G2-36 and D5-59 react broadly with various RVFV isolates, the amino acid sequence of the minimum epitope region ($_{195}$TFTQPMN$_{201}$) was aligned with corresponding amino acid sequences of all known RVFV isolates, as well as those of other Phleboviruses, deposited in the GenBank database (Fig. 4). The amino acid sequence ($_{195}$TFTQPMN$_{201}$) of the epitope was conserved completely among all RVFV isolates, but was not identical to those of other Phleboviruses. These results demonstrated that MAbs G2-36 and D5-59 recognized a highly conserved linear epitope in the RVFV NP.

4. Discussion

In diagnosing many virus infections, PCR assays have excellent analytical sensitivity, but the established techniques are limited by the need for expensive equipment and technical expertise. Since the sensitivity of Ag-capture ELISA is potentially comparable to that of RT-PCR (Ji et al., 2011; Saijo et al., 2006, 2007; Velumani et al., 2008), Ag-capture ELISA represents a sophisticated approach for the diagnosis of virus infections. As a prelude to the development of such a sensitive diagnostic test for RVFV infection, a recombinant RVFV NP from insect cells infected with recombinant baculovirus was purified and novel MAbs against them were developed. MAbs D5-59, G2-36, and C10-54 reacted with His-RVFV rNP and authentic viral antigen (NP) in RVFV MP12-infected cells. Furthermore, control assays with unrelated virus (SABV) rNP and mock-infected cells revealed that MAbs were specific to RVFV NP.

Although it is difficult to compare simply the detection limits among the various assay procedures, the Ag-capture ELISA developed in this study seems to have excellent sensitivity. The detection limit of the newly developed Ag-capture ELISA (0.08–0.16 ng of rNP per 100 μl, Fig. 2) was very similar to that of a previous report, in which an *E. coli* system for the expression of RVFV rNP was used, hyperimmune sheep anti-RVFV rNP serum was used as the capture antibody, and the detection limit of the assay was 0.11 ng per 100 μl of rNP (Jansen van Vuren and Paweska, 2009).

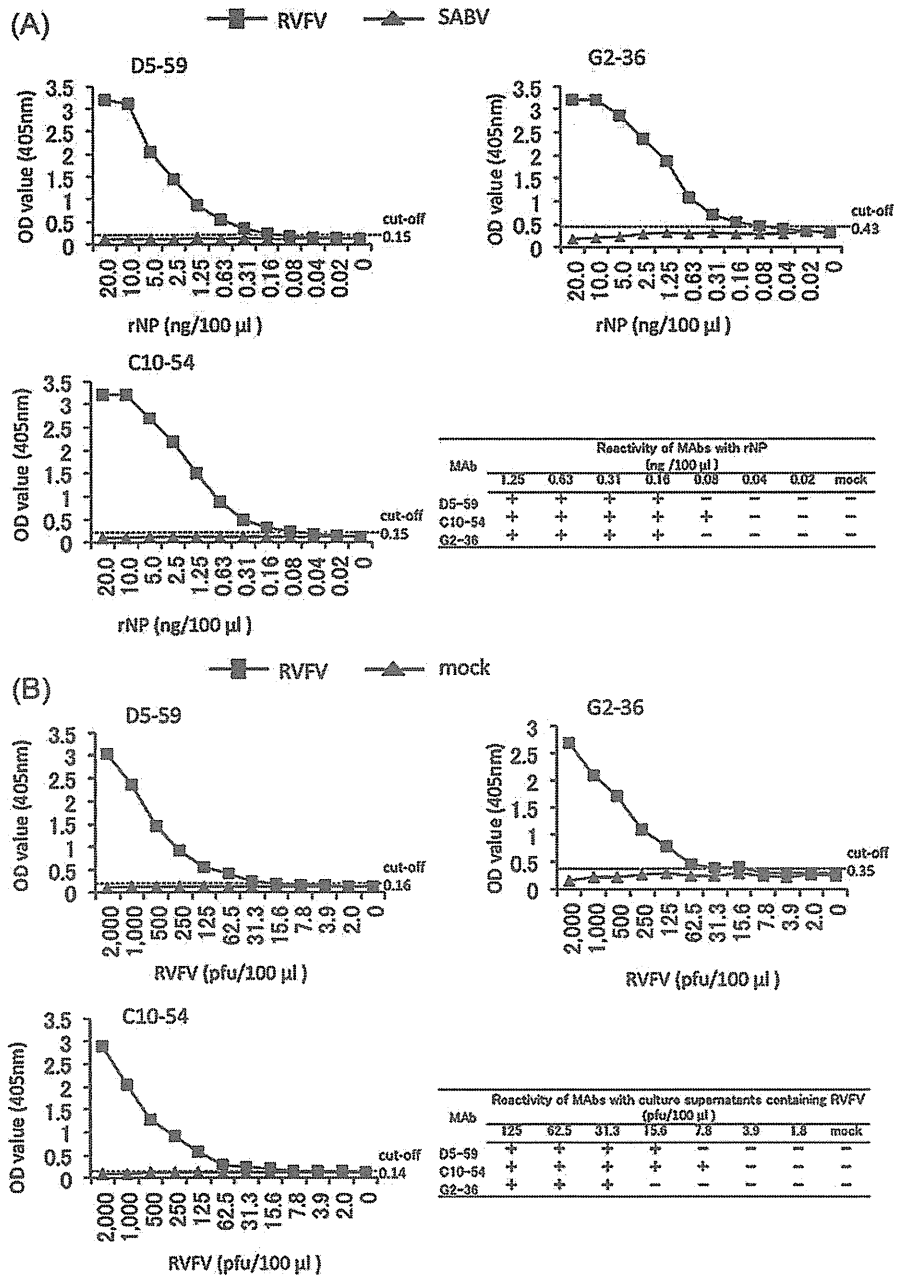

Fig. 2. Ag-capture ELISA for the detection of His-RVFV rNP (A) and authentic RVFV NP (B). MAbs D5-59, C10-54, and G2-36 were used as capture antibodies. Rabbit anti-rNP serum was used as the detecting antibody. Dashed lines indicate the cut-off for each antibody. Detection limits for each MAb are summarized in the table.

Using different amounts of authentic RVFV antigen obtained from the culture supernatants of cells infected with RVFV, as little as 7.8–31.3 pfu per 100 µl of RVFV was detected by ELISA. A real-time RT-PCR assay (Bird et al., 2007a) for detection of the RVFV genome from the same culture supernatant samples detected as little as 3.0 pfu per 100 µl (data not shown), indicating that the detection limit of the Ag-capture ELISA was slightly less than that of the real-time RT-PCR assay. However, the detection limit of this Ag-capture ELISA was approximately 10 times higher than that reported for detecting authentic RVFV antigen (Jansen van Vuren and Paweska, 2009). Thus, the newly developed Ag-capture ELISA might be useful in the diagnosis of RVFV infection.

MAbs directed against RVFV NP and their application for detecting RVFV have been reported, showing broad reactivity to RVFV strains (Martin-Folgar et al., 2010; Saluzzo et al., 1989). Since MAbs directed against RVFV NP might allow for the detecting RVFV antigen in the serological diagnosis, identification of the epitopes of MAbs is of crucial importance for the assessment of specificity of the assay system.

The Ag-capture ELISA using MAb C10-54 recognizing a conformational epitope on RVFV NP proved more sensitive than assays using MAbs D5-59 and G2-36 recognizing the linear epitope, indicating that, similar to the results shown by another diagnostic study (Velumani et al., 2008), conformation-specific MAb might

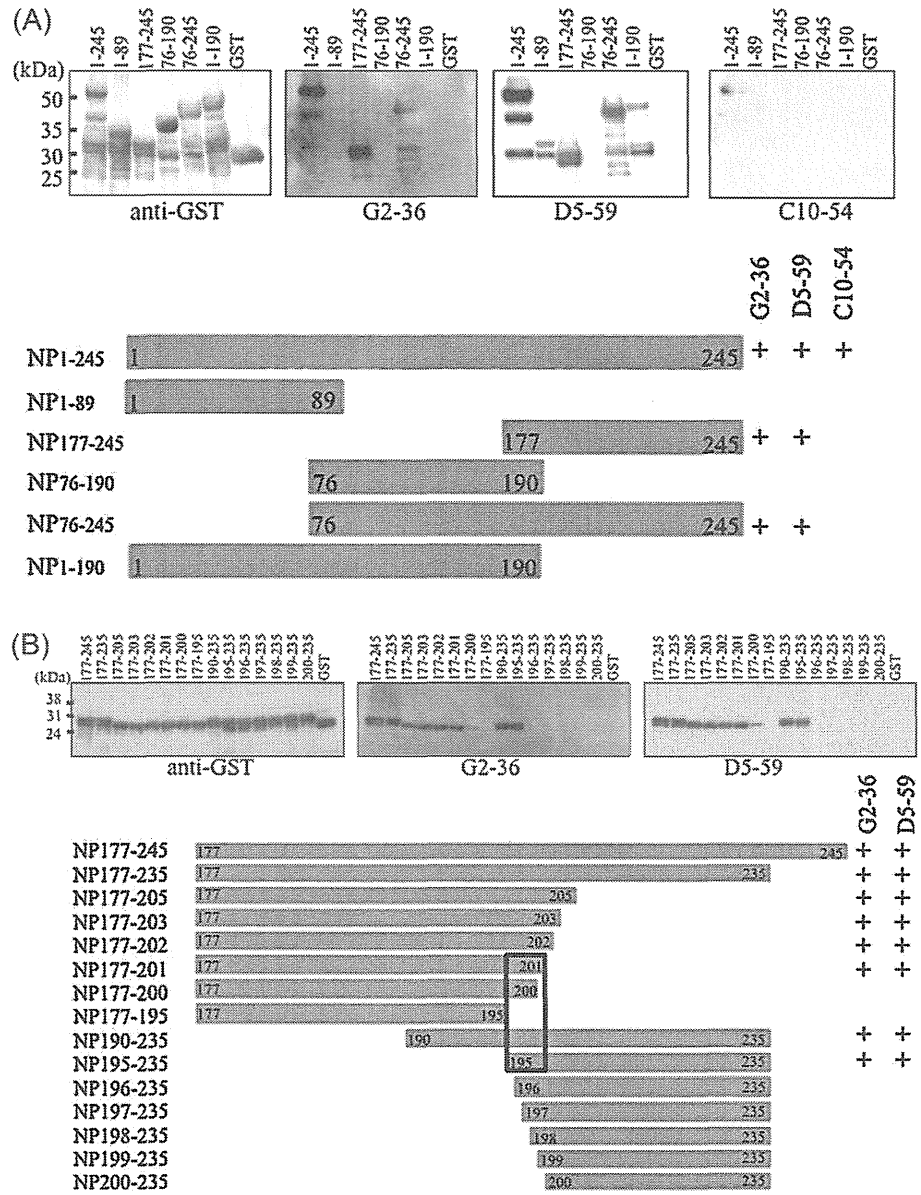

Fig. 3. Schematic representation of GST-tagged truncated RVFV rNP, and the reactivity of MAbs in Western blotting. (A) Reactivity of MAbs D5-59, C10-54, and G2-36 and anti-GST antibody to the full-length and truncated rNPs. Anti-GST antibody was used to confirm the expression of the GST-tagged rNP in *E. coli*. MAb C10-54 reacted only to full-length rNP, and G2-36 and D5-59 reacted to the C-terminal one-third region, whereas anti-GST reacted to all the rNP proteins. Intensities of faint bands observed for NP1-89 and NP1-190 by MAb D5-59 were more than 4-fold weaker than those of NP177-245, NP76-245 and full-length NP1-245 (data not shown), thus we considered that both NP1-89 and NP1-190 were negative for binding to MAb D5-59. (B) The epitope recognized by G2-36 and D5-59 was analyzed in more detail with various rNPs subdivided from the C-terminal one-third region. The numbers shown for each schematic rNP indicate the amino acid positions of the RVFV NP in each truncated NP. The epitope region ranging from amino acid residues 195–201 is shown in the box.

perform substantially better than linear epitope-specific antibodies. It is suggested that MAb C10-54 could be suitable for detecting a small amount of NP antigens, although a more detailed study is needed to determine whether this MAb is cross-reactive to all RVFV isolates. It is noteworthy that the amino acid sequence of the region including the epitope recognized by MAbs D5-59 and G2-36 was conserved among all the RVFV isolates so far deposited in the GenBank database, and that there was a significant diversity in the amino acid sequences between RVFV and other Phleboviruses. Thus, the Ag-capture ELISA using these MAbs is thought to be specific for the detection of RVFV among Phleboviruses. Thus, these MAbs may be specifically useful for detecting RVFV and for the further development of highly specific dipstick/lateral flow devices that provide sensitive, easy handling, and less time consuming assay for diagnosis of RVF.

In conclusion, an RVFV NP-detection ELISA using novel MAbs was developed, and the epitopes of these MAbs on RVFV NP were identified. Although further validation of this ELISA system is

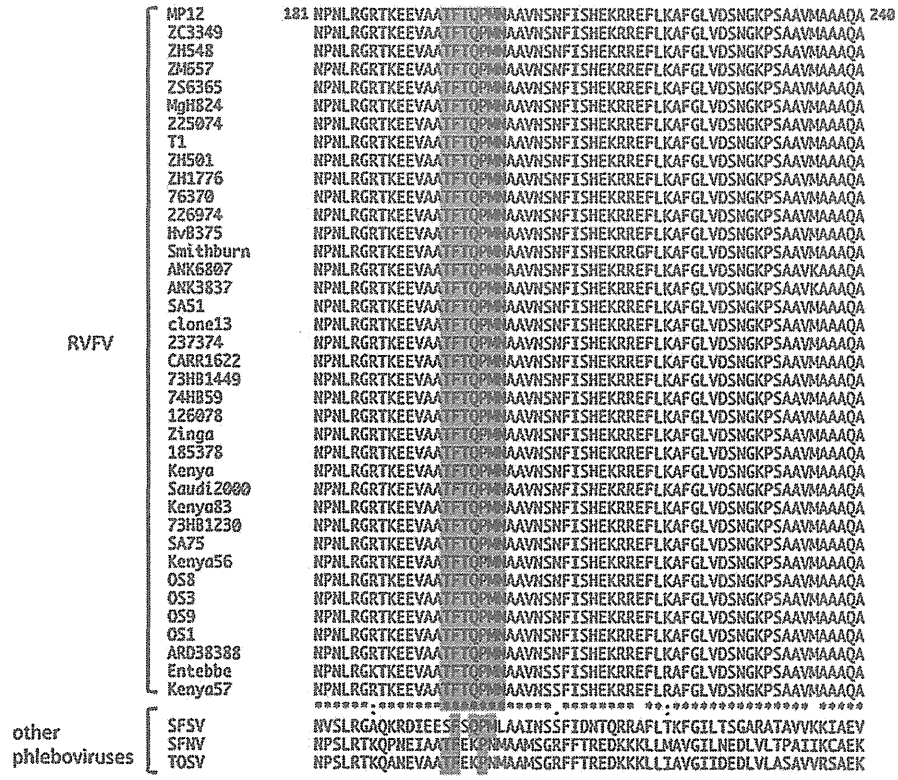

Fig. 4. Amino acid sequence alignment of NPs of RVFV isolates and other Phleboviruses. The minimum region recognized by MAbs G2-36 and D5-59 is shown as a gray box. The alignment includes only a limited number of RVFV isolates (38 strains). However, we confirmed that the amino acid sequence of $_{195}$TFTQPMN$_{201}$ was completely conserved among all RVFV isolates (98 strains) available on the GenBank database (data not shown). The amino acid sequences of the corresponding regions of the NP of other Phleboviruses were not identical.

required with a large number of clinical specimens, it may offer an effective tool for diagnosis of RVFV infection in humans and animals.

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Genotype Distribution of Human Papillomaviruses in Japanese Women with Abnormal Cervical Cytology

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Abstract: We report the prevalence and genotype distribution of human papillomaviruses (HPVs) among Japanese women with abnormal cervical cytology using the PGMY-CHUV assay, one of PGMY-PCR-based lineblot assays that was validated and shown to be suitable for the detection of multiple HPV types in a specimen with minimum bias. Total DNA was extracted from cervical exfoliated cells collected from 326 outpatients with abnormal Pap smears. Overall, 307 specimens (94%) were HPV-positive, 30% of which contained multiple genotypes. The prevalence of HPV DNA was 83% (49/59 samples) in atypical squamous cells of undetermined significance (ASC-US); 91% (20/22 samples) in atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H); 97% (130/134 samples) in low-grade squamous intraepithelial lesion (LSIL); and 99% (85/86 samples) in high-grade squamous intraepithelial lesion (HSIL). Three most frequent HPV types detected in HSIL were HPV16 (36%), HPV52 (24%), and HPV58 (14%). Our results suggest that multiple HPV infections are more prevalent in Japanese women than previously reported, and confirm that HPV52 and 58 are more dominant in their cervical precancerous lesions when compared to those reported in Western countries.

Keywords: Human papillomavirus, HPV genotyping, cervical cancer, Pap smear, abnormal cytology, HPV vaccine.

INTRODUCTION

Human papillomaviruses (HPVs) are the causative agents of cervical cancer, cervical intraepithelial neoplasia, and other anogenital cancers [1]. Among more than 100 HPV types so far identified, nearly 40 types infecting the anogenital mucosa are classified as either low- or high-risk types on the basis of their oncogenic potential [2]. A previous large-scale epidemiological study identified 15 HPV types, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, which are closely linked to the development of cervical cancer [3], with approximately 70% of cervical cancer cases worldwide attributed to HPV16 and 18 [4].

Two prophylactic HPV vaccines, one quadrivalent vaccine directed against HPV6, 11, 16, and 18 [Gardasil® (Merck and Co., Whitehouse Station, NJ, USA)], and one bivalent vaccine against HPV16 and 18 [Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium)], are now being introduced worldwide. Both vaccines exhibit a high efficacy for preventing cervical precancerous lesions caused by vaccine-targeted HPV types in clinical trials for HPV-uninfected women [5,6]. In Japan, the bivalent vaccine was first approved for use in 2009, followed by the quadrivalent vaccine in 2011, both being introduced with the expectation of reducing cervical cancer cases. However, previous epidemiological studies performed in Japanese women have shown variation in the proportion of HPV16 and 18 in cervical cancer, ranging from 50 to 70% [7-9], which makes it difficult to predict the real impact of the HPV vaccination on cervical cancer prevention in the Japanese population.

Because currently available HPV vaccines are thought to be effective only for the vaccine-targeted types, precise determination of the HPV genotype distribution in cervical cancer and its precancerous lesions is needed for assessment of the vaccine efficacy and planning of future vaccination strategies. In 2006, the World Health Organization (WHO) established the HPV laboratory network (LabNet) with the aim of harmonizing and standardizing laboratory testing procedures to promote consistent laboratory evaluation of HPV-related disease burden and monitoring of the performance of HPV vaccines. Through a series of collaborative studies for standardizing HPV genotyping assays, the LabNet has recently described a PGMY-lineblot assay (PGMY-CHUV [10]) as a reliable, low-cost HPV genotyping method [11,12]. The PGMY-CHUV assay consists of PCR with biotinylated generic PGMY09/11

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