standardization activities, and can also be used to evaluate the appropriateness of the reference materials used and/or the need for new ones.

Reference Reagents are developed to improve standardization of assays. They are becoming increasingly important in the context of new vaccines, such as multicomponent vaccines. In many cases, the Reference Reagents are established and prepared by the manufacturer as they are often product specific. These Reference Reagents should be calibrated in International Units, against an International Standard where one exists.

5.2.6 Standards

The intention of the WHO International Standards is to serve as a basis for calibration of secondary standards (e.g. regional and national standards) (*14*). Generally, the International Standards are not used directly in the assays as a working standard. The regional or national standard is calibrated against the International Standard, to make a common working standard available to NCLs and manufacturers.

The regional or national standards should be established by a collaborative study, which should include the manufacturers. Practical aspects of secondary standard preparation need to be considered at regional level, and a suitable concept for development, establishment, distribution and use of regional reference preparations should be put in place.

5.2.7 Practical considerations

The number of samples of the final lot or upstream components requested by NCLs should be appropriate for the testing required, and the sampling procedures should ensure the representativeness of the lot in question. A system should be in place for recording, tracking and appropriate storage of all samples upon receipt from the manufacturer.

It may be necessary to obtain product-specific reference materials or reagents from the manufacturer. The amount requested should be relevant to the amount of testing to be performed and should not place undue stress on the supply of the materials, as stocks of these are often limited.

The time required for testing is an important issue, as it can greatly influence the supply chain and can have a significant impact when products have short shelf-lives. This can be of particular concern when in vivo tests, which can take several weeks to complete, are involved. Under certain circumstances, the NRA/NCL may agree to receive samples from manufacturers before they have completed their own test procedures, so that testing by the NCL is done in parallel. In such cases, the lot cannot be released by the NCL until all the test results from the manufacturer have been received (including the completed and signed final summary protocol with their test results). The NCL should evaluate

the risk and benefit of parallel testing, taking into account the frequency of rejection of lots by either the manufacturer or the NCL.

When animals are used for testing, the NCL should be aware of the potential variability of the source, housing and handling of animals. It is desirable to apply the "3R" principles (reduction, replacement, refinement) to minimize the use of animals, for ethical reasons. Validated in vitro alternatives should be favoured wherever possible. However, the type of testing should be driven by the scientific need for valid relevant data. Moreover, in the spirit of minimizing animal testing worldwide, agreements should be sought with the NCL of the exporting country or with other NCLs, in a mutual recognition or collaborative agreement, in order to utilize the results of animal testing already performed by another NCL.

5.2.8 Release specifications

NRA/NCL lot release should pertain only to products that have a valid marketing authorization in which specifications have been approved by the competent NRA of the country using the vaccine.

Since these specifications are used to judge the test results, it is important to have a mechanism in place to allow the testing NCL to be aware of the latest version of the approved licence specifications. Ideally, the responsible NCL staff should be involved in assessing the test methods, validity criteria and product specifications in the decision-making process for marketing authorization.

5.2.9 Evaluation of NCL results

The NCL test results should be assessed against the specifications approved in the marketing authorization dossier. It is understood that the variability expected in the results for a given test method for a given product should already be taken into account in the specifications. To be in compliance with the marketing authorization, the test results should fall within the defined acceptance criteria, which are based on the validated methodology used by the NCL, and the specifications approved in the marketing authorization (*15*).

The NCL should clearly define its retest policy and determine how, if applicable, the combination of results is carried out and how these results are evaluated. The acceptance criteria should also be predefined and laid down in relevant SOPs.

The NCL should have a predefined standard procedure for dealing with results that do not comply with the specifications. This should include confirmation that the results reflect the actual quality of the lot tested and are not due to analytical error by the NCL, or to the influence of variables unrelated to the product.

The manufacturer should be notified when an OOS result is confirmed and exchanges should ensue to try to identify the cause of the discrepancy.

A test report, including the results and outcome of all of the testing, should be prepared for final evaluation of the lot and the decision-making process.

A feedback mechanism from the NCL to the NRA and/or the GMP inspectorate is highly advisable, in order to coordinate and optimize regulatory actions (e.g. urging licence variation or refinement of product specification based on trend analysis).

6. Data monitoring

All critical quantitative data from quality-control results, and especially potency, from the manufacturer or other sources, should be used for trend analysis as an essential part of lot release. Statistical analysis should be conducted once sufficient data have been accumulated. The alert or warning limits and action limits of consistency trends should be defined on statistical grounds. In general, when data are distributed normally, ±2 and ±3 standard deviations of the mean are set for the alert or warning limits and action limits respectively. The variability and precision of the test should be considered when defining the limits. Care should be taken in interpreting such limits when they are based on small datasets. Trend analysis of key parameters may be requested from manufacturers or from the responsible NRA/NCL. More complex specific trend analysis statistical methods can be used when sufficient data and expertise are available, particularly when data are not normally distributed. In addition, a set of data from a certain period (e.g. 6 months or 1 year) should be analysed statistically, compared to data of the previous period, in order to detect any significant differences or shift in trends.

An SOP should be developed to describe this tracking and trending of manufacturers' and, where available, the NCL's results. This procedure will describe parameters to be tracked and trended, the frequency of periodic reviews, criteria for judgement, and actions to be taken in the case of outlier results, etc.

6.1 Trend analysis including data from the NCL

In cases where independent testing of lots is performed at the NCL, all data from these tests, including performance of reference standards and controls, should also be trended and analysed. It should be kept in mind that not all countries test all consecutive lots from a manufacturer. In such cases, the trends should be interpreted with caution and additional information from the manufacturer may be required, either directly or through contact with the relevant national inspectorate.

6.2 Comparison of results of the manufacturer with those of the NCL

Results from the NCL should be compared with those of the manufacturer. Any systematic differences should be documented. Any differences in trends should

be investigated and resolved, in collaboration with the manufacturer. Testing by the NCL may, however, occur months after the manufacturer's release, so this should be taken into consideration when the NCL makes the comparison.

7. Evaluation of the lot and the decision-making process

7.1 Establishment of decision-making procedures

The authority responsible for issuing a release certificate may differ between countries. Therefore, it is critical that the roles and responsibilities of both the NRA and the NCL are clearly defined, particularly when they are separate entities. When all elements are available for final evaluation, a formal decision-making process should be in place to decide whether the lot can be released. An SOP should be in place to describe clearly the process and required elements for the final decision. Good coordination and communication are needed, especially when different bodies are involved in this process.

In order to provide continuity and to develop expertise on each product, it is desirable that product specialists are assigned the responsibility for managing the relevant information for particular products. A general lot release process chart should be in place, outlining the lot approval process and the persons responsible for each activity.

The competent authority's approach to independent lot release should be appropriately described in the NRA/NCL process charts. Procedures should cover the options used: release upon review of summary protocol only and/or release upon review of summary protocol plus independent testing by the NCL. They should also define how and by whom the final decision is taken on the basis of the formal written conclusions of the defined options used. SOPs or documents are necessary to cover the essential elements presented below.

1. An SOP for summary protocol review should describe acceptance criteria for the completeness of the summary protocol, and all reviewing steps up to and including the final conclusion on the summary protocol (e.g. need for manufacturer's correction, review of corrected pages, investigation, conclusion).

   The NRA/NCL should produce a formal written conclusion regarding the summary protocol review. A summary decision form should be filled out to ensure compliance with approved specifications and should be signed by the responsible staff.

2. An SOP should describe the acceptance criteria for NCL test results and record all the individual test results in certificate(s) of analysis.

For the lot release following independent testing by the NRA/NCL, a formal written conclusion form containing the outcome of test results should be developed. A summary decision form should be used to capture the test results and ensure compliance with approved specifications, and should be signed by the responsible staff.

A retest policy should be developed in accordance with general quality assurance principles, in order to define the policy for retesting and handling of OOS results. In addition, an SOP should be in place to give guidance on retest policy according to product-specific recommendations (e.g. combination of results, calculation method). In the event of non-compliance, a full traceability investigation should be conducted on test reports and the manufacturer should be contacted for further investigation. As part of the quality assurance, in the event of derogation, an SOP should outline the decision-making process, including documentation and written criteria to support the decision made.

3. An SOP should be available that describes the acceptance criteria for release of vaccines in exceptional cases, which deviate from the normal procedure. Examples include release for an emergency/crisis situation, urgent need due to a critical supply shortage, when information is pending regarding correction of the summary protocol, or in the event of discrepancies between the test results of the NCL and the manufacturer. The procedure should be developed on the basis of a risk–benefit analysis that takes into account all available information. This should be applied only by the staff officially responsible for signing the release certificate. Documentation supporting compliance with approved specifications (summary protocol review and test reports, if applicable) should be included.

All steps in the decision-making process should be documented.

7.2 Recognition of, and confidence in, lot release by other NRAs/NCLs

In cases where a lot has already been released by another NRA/NCL, it may be possible to accept that lot for release on the basis of the existing release certificate. Processes for doing this may range from a list of countries that are acceptable to the importing country, through to mutual recognition agreements. Examples are described below.

Establishment of mutual recognition agreements is a legal approach. Many NRAs/NCLs use such agreements to: enhance international regulatory cooperation in order to maintain high standards of product safety and quality; reduce the regulatory burden for NRAs/NCLs and manufacturers; and improve the free flow of goods and increase the accessibility of medicinal products

globally. Reciprocal mutual recognition of release certificates involves a number of legal aspects that should be addressed; however, the key to successful mutual recognition is the building of mutual confidence between the interested parties. This requires strong collaboration and communication between the different NRAs/NCLs and a good level of transparency.

Examples of agreements range from accepting the test results provided by another NCL, thus avoiding repeat testing and facilitating harmonization without compromising the safety and quality of the product, to full mutual recognition of the lot release certificate. The test results provided by another NCL can thus be used in addition to the protocol review by the local NRA/NCL, when they lot release the product.

Situations may exist where a two-way recognition of certificates or test results is not possible, owing to technical or other limitations. However, even in cases where reciprocity is not attainable, an NRA/NCL may still wish to recognize a release certificate from another NRA/NCL. This should be possible, provided the releasing NRA/NCL has clearly established procedures that are transparent and relevant to the NRA/NCL wishing to recognize the certificate or test results.

These types of approaches provide the advantage of limiting repeated evaluation and testing, and serve to streamline the release procedure.

It is important to note that the product manufacturers should be involved in the establishment of an agreement to share product information, since there are issues of confidentiality that need to be addressed.

When these types of arrangements are foreseen, specific SOPs should be developed to establish clearly what information is necessary and how it should be received and processed before final release on to the local market is accepted.

7.3 Release certificate issued by the NRA/NCL of a producing/releasing country for United Nations procurement

The responsible NRAs/NCLs are required to issue a certificate of release for vaccines that are distributed through United Nations agencies (*16*). Vaccines distributed through United Nations agencies are prequalified by WHO, to ensure that the products comply with the quality and safety standards established by the Organization. This release certificate is issued on the basis of, as a minimum, a review of the lot summary protocol for the relevant lot.

The responsible NRA/NCL plays a key role in ensuring that products meet the specifications outlined in the marketing authorization and WHO recommendations. This is achieved by maintaining regulatory oversight, assessing and approving changes to manufacturing processes – including testing and specifications, compliance with GMP – and PMS of AEFI. The release certificate issued by the responsible NRA/NCL should be forwarded by the United Nations agencies to the NRA/NCL of the receiving country, and the summary protocol will be provided upon request.

The receiving country may wish to review the summary protocol to develop its competency and have an overview of the vaccine quality.

In some countries, testing is undertaken on the product received by a competent laboratory, in order to strengthen the NCLs' capacity and obtain information on the quality of the product at the receiving site. If a deficient result is detected, the responsible NRA/NCL should be consulted.

8. Lot release certificate

A release certificate for each vaccine lot should be issued by the NRA/NCL and sent to the manufacturer, confirming that the particular lot meets the approved specifications and related provisions. The release certificate is the official document authorizing the manufacturer to release the lot on to the market. The certificate may include the following information:

- name and address of the manufacturer;
- site(s) of manufacturing;
- trade name and/or common name of product;
- marketing authorization number;
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary);
- type of container;
- number of doses per container;
- number of containers/lot size;
- date of start of period of validity (e.g. manufacturing date) and/or expiry date;
- storage condition;
- signature and function of the authorized person and the agent authorized to issue the certificate;
- the date of issue of the certificate;
- the certificate number.

Other details – such as dosage form, strength of the product, registration code (NRA/NCL code for lot release) – may also be included in the certificate, according to the requirements of different countries.

The conclusion should be included clearly in the certificate, stating, for example: "The lot mentioned above complies with the relevant specification in

the marketing authorization and provisions for the release of biological products and has been approved for release". The statement should also give an indication of the basis for the release decision (e.g. evaluation of the summary protocol, independent laboratory testing, specific procedures laid down in defined document, as appropriate).

For lots that fail to comply with the provisions, a different form should be issued, ideally with a different colour from the approval certificate, which clearly states that the lot is non-compliant.

It is advisable that the language on the lot release certificate is the national language, with an English translation of the information.

Authors and acknowledgements

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The second draft of the Guideline was prepared by the drafting group at a meeting held in Cairo in March 2008. The third draft of the guideline was prepared by the group, taking into account comments on the second draft from national vaccine regulatory authorities and the vaccine industry.

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The sixth draft was prepared by Dr D. Lei, World Health Organization, Geneva, Switzerland, and the drafting group, on the basis of comments from the Expert Committee on Biological Standardization and the participants in the Committee meeting of 2009 and comments from the public consultation.

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Appendix 1

A model procedure to document the decision-making process in lot release

This Appendix is intended to assist NCLs in documentation of the information and the process used in the evaluation of specific issues in vaccine lot release. Examples include:

- release of vaccine lots in emergency situations such as a vaccine shortage due to a disease outbreak, natural disaster, manufacturing problems (e.g. OOS) or other unforeseen circumstances;
- periodic evaluation of the frequency of independent testing (to consider modification, suspension or continuation of the current strategy);
- periodic evaluation of tests performed for lot release of a particular product (to consider deletion, inclusion or modification of given tests).

Since each situation is specific, it is expected that modifications to the structure and content of this template may be required in order for it to be applicable to different issues.

1. Issue

Define the problem/issue to be analysed.

2. Purpose/objective

Outline the purpose and/or objectives of this analysis (for instance, to evaluate the consistency of production of a vaccine) and explore whether changes to the frequency of independent testing or elimination of a specific test are justified on the basis of the consistency of production.

3. Background

Give a brief history of the problem/issue and identify critical information.

4. Issue analysis

List all key components of the issue to be analysed, taking into account relevant information from the NCL/NRA and manufacturers. Justify the results/conclusions with regulatory and scientific data, including published and unpublished information.

5. Options analysis

- List all the options considered to address the issue/problem, including the status quo.
- List and discuss the positive and negative aspects of each option.
- Outline the proposed solution or accepted alternative and why it was selected.
- If relevant, discuss the benefits and costs of the proposed solution compared to the benefits and costs of the other solutions.

6. Considerations

Identify any additional relevant information. For instance, discuss with other NCLs that are responsible for releasing this vaccine in other countries, in order to share information regarding production and quality control of this vaccine.

7. Recommendations

Indicate what the recommendation is and who is responsible for its approval.

8. Implementation and evaluation plan

Show how the proposed changes will be implemented in terms of timing, organizational and personnel changes and resource allocation.

Indicate when and how the proposed changes will be evaluated and against what benchmarks.

9. References and attachments

Include any references, reports and relevant information used in the risk analysis, such as GMP inspection report, regulatory post-marketing unit report, quality-control product report from the NCL, and/or a summary of decisions regarding variations submitted for regulatory approval.

I approve the recommendation proposed in this analysis,

_____

Dr [insert name]
Director of National Control Laboratory

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

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Seroepidemiological study of hepatitis B virus markers in Japan

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ABSTRACT

Background: In Japan, since 1986, selective vaccination has been implemented as a hepatitis B prevention strategy. The target of vaccination is the infant born to a hepatitis B surface antigen (HBsAg)-positive mother. The current Japanese hepatitis B prevention strategy focuses on reducing the number of HBV carriers but overlooks the risk to susceptible populations. We conducted a nationwide HBV seroepidemiological study to explore the next hepatitis B control strategy.
Methods: We used sera derived from healthy individuals collected nationwide from 2005 through 2011 to investigate the HBsAg seroprevalence among children aged 4–9 years and 10–15 years (3000 samples) and hepatitis B core antibody (HBcAb) seroprevalence among people 10–39 years of age (600 samples).
Findings: Among sera from 3000 children, 5 (0.17%) specimens were HBsAg-positive. There was no significant difference in HBsAg prevalence between age groups. Among 600 samples, 15 (2.5%) were HBcAb-positive. Out of 15 samples, 4 were from teenagers. Both HBsAg- and HBcAb-positive sera were found mainly in the Southern area of Japan.
Conclusion: The prevalence of HBsAg among children was 0.17% in the present study. This is higher than the prevalence reported in previous studies performed in the local area or in blood donors. The prevalence of HBcAb is also higher than we estimated. One of the reasons for this discrepancy from previous studies may be due to the small sample size and the impact of HBV high-endemic areas included in the present nationwide study. Nevertheless, our findings revealed that the opportunities for acquiring HBV infection in the susceptible population were more frequent than we thought, especially in some localities. Hepatitis B vaccination should be introduced into the routine child immunization program for susceptible populations, and the selective vaccination program should be continued for high-risk children.

1. Introduction

Hepatitis B is caused by the hepatitis B virus (HBV) transmitted through blood and body fluids [1–3]. Infection can occur by exposure to contaminated blood or body fluids such as through blood transfusion, sexual contact, close contact with HBV-infected family or community members, or mother-to-infant transmission during birth. The outcomes of HBV infection include asymptomatic infection, or acute or chronic hepatitis B. Adults with chronic hepatitis B have a 15–25% risk of dying prematurely from HBV-related cirrhosis and hepatocellular carcinoma (HCC) [1]. It is estimated that more than 2 billion people worldwide have HBV infection. Of them, approximately 360 million people are chronically infected, and 600,000–780,000 HBV-related deaths occur annually [1,2]. HBV infection, especially chronic hepatitis B, is a global health concern. The development of chronic hepatitis B is related to the age at which a person acquires HBV infection. Approximately 80–90% of people infected perinatally and 30–50% of people infected before 6 years of age develop chronic infection [2]. After that, the risk of acquiring chronic infection is less than 5%. Most people who develop chronic infection during early childhood become asymptomatic chronic carriers, who carry HBV in their blood and body fluids for many years and can spread the infection to others. Thus, prevention of HBV infection during early childhood is a very important issue for hepatitis B prevention.

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Universal vaccination beginning at birth, as well as other successful hepatitis B vaccination strategies, have resulted in a dramatic reduction in HBV transmission in many countries. As of 2013, 182 out of 194 World Health Organization (WHO) member states had incorporated hepatitis B vaccination as an integral part of their national infant immunization programs [4]. An estimated 81% of the 2013 birth cohort received 3 doses of hepatitis B vaccine. In Japan, universal vaccination has not been conducted. Instead, since 1986, a selective vaccination program "the mother-to-infant HBV transmission prevention program" has been implemented as a hepatitis B prevention strategy [5]. The target of vaccination is the infant born to a hepatitis B surface antigen (HBsAg)-positive mother. Vaccination protocol has been improving over time. Hepatitis B immunoglobulin (HBIG) is administered at birth only and the hepatitis B vaccine is administered at birth, and at age one month and 6 months since 2013. Coupled with HBV screening of blood products, this selective vaccination strategy has decreased the number of hepatitis B patients. HBsAg seroprevalence among first-time blood donors declined from 0.161% among people born between 1982 and 1985 to 0.055% among people born between 1986 and 1989 [6]. The latest report from Iwate prefecture showed a low HBsAg prevalence, 0.017%, among children [7]. The current Japanese hepatitis B prevention strategy focuses on reducing the number of HBV carriers but overlooks the risk to susceptible populations. We conducted a nationwide-scale HBV seroepidemiological study to evaluate the effect of the current hepatitis B prevention strategy and to explore the next hepatitis B prevention strategy. We studied HBsAg seroprevalence among healthy children and hepatitis B core antibody (HBcAb) seroprevalence among healthy people 10–39 years of age. HBsAg and HBcAb are the representative markers of HBV infection and anamnesis, respectively. The findings of the present study are expected to describe the current status of HBV infection in Japan.

2. Materials and methods

2.1. Study design and subjects

This was a nationwide cross-sectional study consisting of HBsAg sero-surveillance among healthy children aged 4–15 years and HBcAb sero-surveillance among healthy individuals aged 10–39 years. All 3600 serum samples, 3000 samples for HBsAg test and 600 samples for HBcAb test, were obtained from the National Serum Reference Bank, National Institute of Infectious Diseases, Japan. A multistage random sampling technique was employed for subject selection in this study. The sample supply was restricted because of the limited stored number of samples.

2.2. The National Serum Reference Bank, National Institute of Infectious Diseases, Japan

In order to assess the domestic immune status against vaccine-preventable diseases, consistent seroepidemiological surveillance has been conducted in Japan since 1962 under the program of the National Epidemiological Surveillance of Vaccine Preventable Diseases (NESVPD) [8]. This population-based surveillance program is implemented by the Ministry of Health, Labour and Welfare, Japan, and the prefectural governments. Sera obtained from representative healthy donors are collected every year from a wide range of age groups from the representative prefectures and submitted to NESVPD. All serum samples are collected voluntarily, and each donor provides written informed consent. The samples are obtained mainly during regular health check-ups including those conducted among school children. Leftover samples of patients from hospitals are not included. The remaining sera are stored at −80 °C in the National Serum Reference Bank, National Institute of Infectious Diseases, Japan. Every stored serum sample and its epidemiological data are anonymized using an ID number. In this study, we retrieved data on age, sex, sampling locality (prefecture) and sampling year.

Fig. 1. Sample distribution for hepatitis B surface antigen (HBsAg) and/or hepatitis B core antibody (HBcAb) surveillance. Numbers in map show the prefectures: the Northern area (1, Miyagi, 2, Yamagata, 3, Fukushima), the Eastern area (4, Niigata, 5, Ibaraki, 6, Tochigi), the Central area (7, Yamanashi, 8, Nagano, 9, Aichi), the Western area (10, Fukui, 11, Kyoto, 12, Yamaguchi), and the Southern area (13, Fukuoka, 14, Saga, 15, Miyazaki, 16, Kagoshima).

2.3. HBsAg sero-surveillance

A total of 3000 serum samples were subjected to the HBsAg test (Table 1). Samples were obtained from healthy children aged 4–15 years in 15 prefectures from 2005 through 2011 (Fig. 1). The numbers of boys, girls, and unknown were 1583, 1414 and 3, respectively. The prefectures were classified into five areas, the Northern, Eastern, Central, Western and Southern areas, based on geographical and conventional classifications. Approximately 350 samples were prepared in each cohort for ages 4–8 years (Table 1). Additionally, approximately 165 samples were prepared in each cohort from ages 9 to 15 years (Table 1). Analysis was performed separately for the 4–8 age group and the 9–15 age group. The required sample size for this study was calculated by the following formula:

$$n = \frac{(Z^2 pq)}{D^2}$$

where n is the sample size; Z is the confidence interval (alpha = 5%, 1.96); D is the margin of error (0.5%); $q = 1 - p$; and p = expected prevalence in target population.

In this case, p was predicted with at most 1% according to previous studies [9,10], therefore, we set p as 1%.

$$n = \frac{(1.96)^2 \times 0.01 \times 0.99}{(0.005)^2} = 1522$$

The number of samples in the 4–8 age group was 1837, which was enough for the minimum recommended sample size. However, the number of samples in the 9–15 age group was 1163, which was less than the number of the minimum sample size if the prevalence was 1%.

HBs Ag positivity was determined by the commercial enzyme linked immunosorbent assay (ELISA) kit Enzygnost® HBsAg (Siemens Healthcare Diagnostics Products GmbH, Marburg,

Table 1
Samples for hepatitis B surface antigen surveillance.

Age	Northern area			Eastern area		Central area		
	Miyagi	Yamagata	Fukushima	Ibaraki	Niigata	Yamanashi	Nagano	Aichi
4	31	50	43	30	48	6	25	0
5	22	47	45	41	45	3	39	0
6	21	38	35	28	38	22	38	0
7	21	33	33	32	32	40	23	5
8	17	32	24	23	39	33	51	4
9	13	19	14	14	18	13	15	2
10	14	17	16	29	17	11	15	2
11	16	19	14	17	20	16	14	0
12	12	15	9	19	16	11	11	0
13	10	15	11	21	13	7	12	0
14	10	14	15	17	12	13	18	0
15	8	9	17	16	16	17	18	0
Total	195	308	276	287	314	192	279	13

Age	Western area			Southern area				Total
	Fukui	Kyoto	Yamaguchi	Fukuoka	Saga	Miyazaki	Kagoshima	
4	10	16	46	54	20	3	0	382
5	23	22	32	59	20	3	0	401
6	13	15	37	57	19	2	0	363
7	14	21	18	64	14	3	0	353
8	10	12	22	53	13	4	1	338
9	7	6	15	16	9	2	0	163
10	11	15	1	14	1	2	1	166
11	9	18	2	18	0	4	0	167
12	4	15	16	11	23	2	3	167
13	5	10	23	12	23	2	3	167
14	4	12	31	17	0	2	1	166
15	2	13	32	9	5	2	3	167
Total	112	175	275	384	147	31	12	3000

Germany). Enzygnost HBsAg was used according to the manufacturer's instructions except for sample dilution. In the modified procedure, we used 100 μL of twice diluted serum instead of 100 μL of undiluted serum that the manufacturer specified. We used phosphate-buffered saline containing 0.2% bovine serum albumin as a diluent. The linearity in the lower titer range of the modified procedure was validated until 25 mIU/mL (Fig. 2). We prepared a positive control that contained 50 mIU/mL of HBsAg adjusted from the national HBsAg sensitivity panel [11]. We placed 100 μL of twice diluted serum sample or positive control on the ELISA plate. To eliminate the false positive, the sample for which the optical density (OD) value was two times higher than that of the positive control was considered as HBsAg positive. HBsAg-positive sera were subjected to genotyping by the "Easy-to-use phylogenetic analysis system" previously mentioned [12].

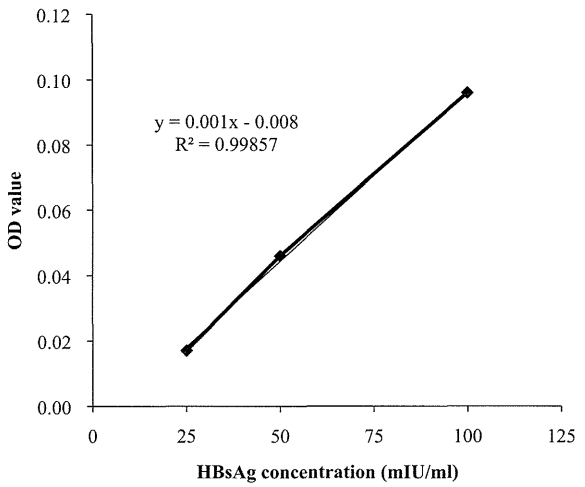

Fig. 2. Validation of a modified enzyme-linked immunosorbent assay (ELISA). Reference samples were adjusted to 25, 50 and 100 mIU/mL of hepatitis B surface antigen (HBsAg) concentration. Each sample was diluted twice, then applied for ELISA according to the manufacturer's instructions. Assay linearity within 25–100 mIU/mL was confirmed.

2.4. HBcAb sero-surveillance

A total of 600 serum samples were subjected to the HBcAb test (Table 2). The samples were obtained from healthy individuals aged 10–39 years from 13 prefectures from 2010 to 2011 (Fig. 1). The numbers of males and females were 260 and 340, respectively. We prepared 200 samples in each age group: teens, twenties and thirties. The recommended sample size for this surveillance was calculated by the following formula;

$$n = \frac{(Z^2 pq)}{D^2}$$

where n is the sample size; Z is the confidence interval (alpha = 5%, 1.96); D is the margin of error (1.25%); $q = 1 - p$; and p = expected prevalence in target population. We set p as 2.5% based on our preliminary study.

$$n = \frac{[(1.96)^2 \times 0.025 \times 0.975]}{(0.0125)^2} = 600$$

HBc Ab positivity was determined by the commercial ELISA kit, Enzygnost Anti-HBc monoclonal (Siemens Healthcare Diagnostics Products). Enzygnost Anti-HBc monoclonal was used according to the manufacturer's instructions.

Table 2
Samples for hepatitis B core antibody surveillance.

Age	Northern area			Eastern area			Central area	
	Miyagi	Yamagata	Fukushima	Ibaraki	Tochigi	Niigata	Yamanashi	Nagano
10–19	20	19	18	20	0	18	10	10
20–29	19	17	8	16	20	19	5	8
30–39	14	21	9	16	20	21	5	8
Total	53	57	35	52	40	58	20	26

Age	Western area		Southern area			Total
	Fukui	Yamaguchi	Fukuoka	Saga	Miyazaki	
10–19	17	22	18	18	10	200
20–29	12	32	20	16	8	200
30–39	10	32	18	18	8	200
Total	39	86	56	52	26	600

2.5. Statistical analysis

The chi-square independence test was performed by using Statcel ver. 2 (OMS Publishing Inc., Saitama, Japan). A P value of <0.05 was considered statistically significant.

2.6. Ethical considerations

This study design, including ethical issues, was approved by the National Serum Reference Bank, the National Institute of Infectious Diseases, Japan.

3. Results

3.1. HBsAg sero-surveillance

Three thousand serum samples from children aged 4–15 years were obtained between 2005 and 2011 from 15 prefectures nationwide. Among the samples, we found 5 (0.17%) HBsAg-positive sera (Table 3) from 3 prefectures, Saga, Fukuoka and Miyagi. All but one of the samples were from the Southern area. We confirmed that the five HBsAg-positive samples were derived from independent individuals from their data on age, sex, and sampling year. Of the 5 positive samples, three were from boys; there were no significant differences in HBsAg-positive status between boys and girls (P=0.75). All samples were HBcAb-positive except for one sample from Miyagi. The positive samples consisted of one from a 5-year-old child, two samples from 6-year-old children, and two samples from 11-year-old children. Two samples were HBV-genotype B, and three samples were HBV-genotype C. Sequencing the HBV S region n/t 245-712 from two children from Saga yielded a closely related sequence (GenBank number: LC049563 and LC049564). Sequencing the HBV S region n/t 245-712 from two children from Fukuoka yielded a common sequence (GenBank number: LC049561 and LC049562). The remaining positive serum sample from Miyagi was unable to be subjected to sequencing because of its low PCR yield. The HBsAg prevalence values among children aged 4–8 years and aged 9–15 years were 0.16% (3/1837, 95% confidence interval: 0–0.35%) and 0.17% (2/1163, 95% confidence interval: 0–0.41%), respectively. There was no significant difference of HBsAg seroprevalence between the two age groups (P=0.95).

Table 3
Hepatitis B surface antigen-positive samples.

No.	Age	Sex	Prefecture	Year of sampling	Genotype	HBcAb
1	5	F	Saga	2010	B[a]	+
2	6	M	Saga	2010	B[a]	+
3	6	F	Miyagi	2008	C	–
4	11	M	Fukuoka	2006	C[b]	+
5	11	M	Fukuoka	2010	C[b]	+

[a] Nos. 1 and 2 had closely related sequence.
[b] Nos. 4 and 5 had common sequence.

3.2. HBcAb sero-surveillance

Six hundred serum samples from healthy individuals aged 10–39 years were obtained between 2010 and 2011 from 13 prefectures. Of them, we found 15 (2.5%, 95% confidence interval 1.25–3.75%) HBcAb-positive sera (Table 4) from 9 prefectures. As with HBsAg prevalence, many HBcAb-positive sera were also found in the Southern area, especially in Fukuoka (4 positives) and Saga (4 positives). The positive samples consisted of four samples from teenagers, one sample from a person in their twenties, and ten samples from persons in their thirties. There was no significant difference in HBcAb-positive status by sex (P=0.43). HBcAb prevalence values among people who were born before and after 1986, the year that the selective vaccination program was launched, were 3.4% (10 out of 291) and 1.6% (5 out of 309), respectively. After the introduction of the selective vaccination, HBcAb prevalence decreased. However, there was no significant difference between them (P=0.15).

4. Discussion

In Japan, the selective vaccination policy "mother-to-infant HBV infection prevention program" was started in 1986. In the present study, we conducted a nationwide-scale HBV seroepidemiological

Table 4
Hepatitis B core antibody-positive samples.

No.	Prefecture	Age	Sex
1	Miyagi	33	M
2	Ibaraki	38	F
3	Niigata	**10**	M
4	Fukui	38	M
5	Nagano	32	F
6	Yamaguchi	31	F
7	Fukuoka	**11**	M
8	Fukuoka	30	F
9	Fukuoka	31	F
10	Fukuoka	32	M
11	Saga	**16**	F
12	Saga	23	F
13	Saga	35	M
14	Saga	37	M
15	Miyazaki	**16**	M

Age in boldface indicates HBcAb-positive teenagers.