Invited Review

Longitudinal Epidemiology of Viral Infectious Diseases Combining Virus Isolation, Antigenic Analysis, and Phylogenetic Analysis as Well as Seroepidemiology in Yamagata, Japan, between 1999 and 2018

Katsumi Mizuta*, Waka Tanaka, Kenichi Komabayashi, Shizuka Tanaka, Junji Seto, Yoko Aoki, and Tatsuya Ikeda

Department of Microbiology, Yamagata Prefectural Institute of Public Health, Yamagata 990-0031, Japan

CONTENTS:
1. Introduction
2. The principles of YPIPH: Why do we continue epidemiological studies based on virus isolation in Yamagata, Japan?
3. Microplate method in YPIPH
   3-1. Routine virus isolation
   3-2. Molecular screening of the virus genome directly from clinical specimens
   3-3. Two-step virus isolation
   3-4. Observation of cytopathic effects, screening using hemagglutination and hemadsorption test, and virus identification
4. System for stocking clinical specimens, clinical viral isolates, cDNAs, and serum specimens
5. Initial works between 2003 and 2005
6. Epidemiological and collaborative studies on several specific viruses
   6-1. Longitudinal epidemiology of EV-A71 and CV-A16
   6-2. Proposal of a new disease concept, “PeV-A3-associated myalgia/myositis”
   6-3. Human metapneumovirus
   6-4. Parainfluenza viruses
   6-5. Human coronaviruses
   6-6. Measles and Rubella viruses
   6-7. Enterovirus D68
   6-8. Saffold viruses
   6-9. Other viruses
7. Discussion

SUMMARY: We introduced a microplate method for virus isolation in the Department of Microbiology, Yamagata Prefectural Institute of Public Health (YPIPH) in 1999 in Yamagata, Japan. We have since carried out longitudinal epidemiological studies on viral infectious diseases, particularly respiratory viruses, combining traditional technologies such as virus isolation and serological techniques and newly developed molecular methods. Here, we provide an overview of our activities at YPIPH between 1999 and 2018. During the study period, we observed emerging and re-merging diseases such as those caused by echovirus type 13, enterovirus D68, parechovirus-A3 (PeV-A3), and Saffold virus. With regard to PeV-A3, we proposed a new disease concept, “PeV-A3-associated myalgia/myositis.” We also revealed the longitudinal epidemiologies of several viruses such as enterovirus A71 and coxsackievirus A16. To perform longitudinal epidemiological studies at any time in Yamagata, we established a system for stocking clinical specimens, viral isolates, complementary DNAs, and serum specimens. We have also pursued collaboration works with virology laboratories across Japan. We hope our experiences, findings, and research materials will further contribute to the development of countermeasures against viral infectious diseases and improvement in public health strategies in Yamagata, Japan, Asia, and around the world.

1. Introduction

Acute respiratory infections (ARIs) are the most common afflictions in humans, and most of them are caused by viruses. Children contract ARIs 5 to 7 times on average each year, and adults about 2 to 3 times each year (1,2). Although the pathogens most frequently associated with ARIs are the rhinoviruses, there are more than 200 respiratory viruses, falling mainly within 6 families: orthomyxoviruses, paramyxoviruses, picornaviruses, coronaviruses, adenoviruses, and herpesviruses (1,2). These viruses produce a variety of clinical manifestations such as the common cold, pharyngitis, croup, bronchitis, bronchiolitis, and bronchopneumonia (1,2).

Although a specific diagnosis of ARI can be made following virus isolation from nasopharyngeal specimens and using antigen detection techniques for some of the viruses, such as respiratory syncytial virus (RSV), adenoviruses, and influenza viruses, nucleic acid techniques such as PCR or reverse transcription PCR (RT-PCR) are also available for the determination of known viruses and are now preferable to the traditional methods such as virus isolation (3).
In the 1980s, nucleic acid techniques were not generally available, and virus isolation was still in common use in virology laboratories. In order to isolate the viruses from patients with ARIs, several cell cultures had to be used to cover the wide range of cell specificities of many respiratory viruses (4). As an alternative to this cumbersome and expensive virus isolation method, Numazaki et al. developed a microplate method, which was simpler than the conventional tube method (4).

The original HHVM 96-well microplate method, which included 4 cell lines—human embryonic lung fibroblast (HEF), human laryngeal carcinoma (HEp-2), African green monkey kidney (Vero), and Madin Darby Canine Kidney (MDCK) cells—could be used to isolate a wide variety of viruses such as influenza virus A (Flu A) and B; RSV; parainfluenza virus 1–3 (Para 1–3); mumps; adenovirus 1–6 (Ad 1–6); rhinovirus; poliovirus 1–3; coxsackievirus A16 (CV-A16); CV-B1–5; echovirus 3 (Echo 3), 6, 7, 9, 11, 21, and 22; herpes simplex virus (HSV); and cytomegalovirus (CMV) (4). The microplate method enabled the isolation of many respiratory viruses simultaneously in 1 plate while reducing labor and material requirements, allowing the processing of a large number of specimens, advancing the epidemiology of viral ARIs in the community throughout the year, and facilitating the stocking of clinical viral isolates (4).

We introduced the microplate method in the Department of Microbiology, Yamagata Prefectural Institute of Public Health (YPIPH) in 1999 in Yamagata, Japan, and have since carried out longitudinal epidemiological studies on viral infectious diseases, combining traditional technologies and newly developed molecular methods. Here, we provide an overview of our activities at YPIPH between 1999 and 2018.

2. The principles of YPIPH: why do we continue epidemiological studies based on virus isolation in Yamagata, Japan?

First, we recognized that YPIPH should function as a public health laboratory, performing its functions by providing analytic biological testing and testing-related services that protect human populations from public health issues, including infectious diseases (5). In terms of viral infectious diseases, the discovery of new diseases, pathogens, diagnostic methods, antiviral drugs, vaccines, and therapies fundamentally contribute to the development of strategies for the control of viral infectious diseases. In addition, basic research, such as epidemiological studies, development of animal models, and identification of viral receptors, is also important (Fig. 1). Among these many factors, the principles of YPIPH were designed to allow us to contribute to the control of viral infectious diseases mainly through our epidemiological studies based on virus isolation. Viral diseases new to humans may emerge in the future. Thus, longitudinal epidemiological studies performed over several decades and even centuries are needed to control viral infectious diseases. As scientific advances have been providing us with new technologies and methodologies to detect and analyze viral pathogens, we have combined the traditional and modern ones.

We are fortunate for the large contributions made by the general public and healthcare workers across Yamagata in collecting clinical specimens as well as valuable information. Furthermore, we have strong support from and collaboration with the Department of Infectious Diseases, Yamagata University Faculty of Medicine, which is at the forefront of research on Flu C (6–17).

![Fig. 1. Concepts of Yamagata Prefectural Institute of Public Health, Department of Microbiology. We contribute to the control of viral infectious diseases mainly through our own epidemiological studies based on virus isolation as well as necessary collaborative works.](image-url)
We believe that clinical viral isolates held in a public health laboratory ideally form a valuable set of research materials for the community. In addition, we are willing to share the library of viruses, cDNAs, and clinical specimens in Yamagata with collaborators under the certain circumstances to pursue our research activities and have already carried out several collaborative studies as described below.

3. Microplate method in YPIPH

3-1. Routine virus isolation

Although the original HHVM plate used 4 cell lines horizontally in one 96-well microplate (4), we later used the plate vertically (Fig. 2A) and prepared it with 6 cell lines in 1999 by adding 2 more cell lines, the rhabdomyosarcoma (RD-18S) and green monkey kidney (GMK) cell lines, which are sensitive to CV-A viruses other than CV-A16, and to enterovirus A71 (EV-A71) and CV-A16, respectively, and named it the HHVMRG plate (18). In 2003, when a severe acute respiratory syndrome (SARS) coronavirus-related outbreak occurred in China, the Vero E6 cell line was provided by the National Institute of Infectious Diseases (NIID), Japan, to all PIPHS, including YPIPH, to experiment with virus isolation from suspected SARS cases. Although SARS coronavirus was not isolated, we found that the Vero E6 cell line was useful in isolating human metapneumovirus (HMPV), and we replaced Vero with Vero E6 in 2003 (HHVe6MRG plate) (Fig. 2A) (19). We previously presented an outline of this system and the results of virus isolation between 2004 and 2005 (20). Since then, we have carried out our epidemiological studies based on virus isolation using this system in Yamagata, Japan (20). Although we introduced a human malignant melanoma cell line (HMV-II) in 2008 in order to isolate parainfluenza viruses apart from the HHVe6MRG plate, we replaced the HMVII cell line with a rhesus monkey kidney cell line (LLC-MK2) in 2011 (21,22).

The system has thus been modified gradually over time. Here, we briefly describe the system, which includes 7 cell lines in one 96-well microplate (18). In 2003, when a severe acute respiratory syndrome (SARS) coronavirus-related outbreak occurred in China, the Vero E6 cell line was provided by the National Institute of Infectious Diseases (NIID), Japan, to all PIPHS, including YPIPH, to experiment with virus isolation from suspected SARS cases. Although SARS coronavirus was not isolated, we found that the Vero E6 cell line was useful in isolating human metapneumovirus (HMPV), and we replaced Vero with Vero E6 in 2003 (HHVe6MRG plate) (Fig. 2A) (19). We previously presented an outline of this system and the results of virus isolation between 2004 and 2005 (20). Since then, we have carried out our epidemiological studies based on virus isolation using this system in Yamagata, Japan (20). Although we introduced a human malignant melanoma cell line (HMV-II) in 2008 in order to isolate parainfluenza viruses apart from the HHVe6MRG plate, we replaced the HMVII cell line with a rhesus monkey kidney cell line (LLC-MK2) in 2011 (21,22).

The system has thus been modified gradually over time. Here, we briefly describe the system, which includes 7 cell lines, that was in use as of November 2018.

We prepare the 96-well microplates for routine virus isolation as well as 24-well plates for passaging purposes on Monday and Tuesday. The compositions of the growth medium and maintenance medium (MM) are shown in Table 1. When the monolayer of each cell line is ready for specimen inoculation on Thursday, the plates are washed with phosphate-buffered saline without calcium or magnesium, and 100 μL of MM is added to each well.

| Cell lines used | Growth medium | Maintenance medium |
|-----------------|---------------|-------------------|
| HHVe6MRG plate  |               |                   |
| HEP-2           | MEM+2% FBS+1.7% glucose | MEM+2% FBS |
| Vero E6         | MEM+10% FBS  | MEM+crystallized trypsin (1 μg/mL)\(^{\text{viv}}\)+vitamin solution (4%) |
| MDCK            | MEM+8% FBS   | MEM+crystallized trypsin (2 μg/mL) |
| RD-18S          | MEM+8% FBS   | MEM+2% FBS        |
| GMK             | MEM+10% FBS  | MEM+2% FBS        |
| LLC-MK2-N       | MEM+10% FBS  | MEM+crystallized trypsin (10 μg/mL) |
| RD-A            | MEM+10% FBS  | MEM+5% FBS        |
| RD-18S-N        | MEM+10% FBS  | MEM+2% FBS        |

\(^{\text{viv}}\): We use minimum essential medium (MEM) with trypsin and that without trypsin for isolation of parainfluenza virus and PeV, respectively.

\(^{\text{viv}}\): Eagle’s MEM including antibiotics (penicillin 100 units/mL and streptomycin 100 μg/mL).

\(^{\text{viv}}\): Percentage and trypsin density indicate final concentration.

\(^{\text{viv}}\): fetal bovine serum.
Nasopharyngeal specimens are placed in tubes containing 3 mL of transport medium consisting of minimum essential medium (MEM) with 0.5% gelatin, 100 units of penicillin, and 100 μg of streptomycin per mL and transported to our laboratory at 4°C, by Thursday each week. After centrifugation of the specimens at 2,000 rpm (approx. 650 × g) for 15 min, 75 μL of the supernatant is inoculated directly onto 2 wells of each cell line in the HHVe6MRG plate and LLC-MK2 plate (Fig. 2A).

The inoculated plates are centrifuged at 2,000 rpm (approx. 450 × g) for 10 min, incubated at 33°C in a 5% CO2 incubator, and assessed for a cytopathic effect (CPE) for 14 days, except for the Vero E6 cell lines, which are observed for approximately one month without medium change in order to isolate HMPV (19) (Fig. 3A).

3-2. Molecular screening of the virus genome directly from clinical specimens

We also extract viral RNA directly from the specimens using a High Pure Viral RNA kit (Roche Diagnostics Manheim, Germany) according to the manufacturer’s instructions and then transcribe it into complementary DNA (cDNA) using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan) to amplify the viral genome through RT-PCR (Fig. 2A).

The remainder of each specimen is then stored at −80°C (Fig. 3D-a).

Fig. 3. (Color online) Observation of CPEs, screening by HA and HAd, virus identification and stocking. (A) We perform microscopic observation of CPEs 2–3 times per week up to the 4th week. (B) We perform the HA test for the MDCK cell line in order to check the growth of influenza and parainfluenza viruses, and the HAd test for the LLC-MK2-N cell line in order to track the growth of Para 1 and 3, which do not usually show CPEs, on Days 6–7 and 13–14, respectively (Fig. 4). (C) We record these results as well as related information on the recording sheets. (D) For cases CPE and/or HA and/or HAd positive or suspected, the viral fluids are passaged on the identical cell lines to get a high dose of the virus, and sometimes on different cell lines in order to check cell sensitivity, which helps us to estimate the virus through presumptive identification. We then further identify the virus using NT, HI, (RT-) PCR and sequencing methods and stock the viral isolates (b), as well as original specimens (a), cDNAs from the specimens (c), and cDNAs from viral isolates (d), at a −80°C in a freezer for further research. We preserve the consecutively numbered isolates by year of collection together with necessary information such as cell passage history and stock date (e).
3-3. Two-step virus isolation

From our experience, we found that several viruses grow slowly and/or poorly in our routine virus isolation system using microplates as well as other cell lines. Therefore, we introduced a 2-step virus isolation system (Fig. 2B), which is usually carried out, separate from our routine virus isolation method (Fig. 2A); First, we screen the virus genome directly from the nasopharyngeal specimens using genome amplification methods such as RT-PCR and real-time RT-PCR, and then, we inoculate only the genome-positive specimens onto the appropriate cell lines and perform 3–5 passages. For example, after screening, we inoculate the genome-positive specimens onto the LLC-MK2-Niigata (LLC-MK2-N) cell line for the isolation of parainfluenza viruses (PeVs) (23,24), the RD-18S-Niigata (RD-18S-N) cell line for the isolation of Saffold viruses (SAFVs) (25,26), the RD-A cell line for the isolation of EV-D68 and CV-A6, and the HeLa-ACE2-TMPRSS2 cell line for the isolation of human coronavirus 229E (HCoV-229E) (Table 2) (27). The LLC-MK2-N and RD-18S-N cell lines have better sensitivities for PeVs and SAFVs, respectively, than the LLC-MK2 and RD-18S cell lines that we have used in our laboratory. RD-A cell lines are more sensitive to recent EV-D68 and CV-A6 strains than RD-18S cell lines. We confirmed that the HeLa-ACE2-TMPRSS2 cell line has a higher sensitivity to HCoV-229E than RD-18S cell lines (27).

We regularly use the LLC-MK2-N plate for the isolation of parainfluenza viruses using MM including 229E than RD-18S cell lines. We confirmed that the HeLa-ACE2-TMPRSS2 cell line has a higher sensitivity to HCoV-229E than RD-18S cell lines (27).

We start the microscopic observation of CPEs on the 4th day (Day 4) after specimen inoculation and perform observations 2–3 times per week (Figs. 3A and 4). Based on our experience, we can estimate the type of virus before final identification through presumptive identification based on morphological changes and cell sensitivity patterns, as shown in Table 2.

Traditionally, red blood cells (RBCs) from guinea pigs and chickens were often used for hemagglutination (HA) and hemadsorption (HAd) tests to detect the growth of influenza and parainfluenza viruses, and HA inhibition (HI) and HAd inhibition (HAd-I) tests were used to identify these viruses. However, as molecular methods have been widely introduced, these technologies are currently not always used at PIPhs in Japan, except that HA and HI tests are sometimes used to detect and identify influenza viruses. In our laboratory, we still use these RBCs to detect and identify Flu A, B, and C as well as parainfluenza viruses in parallel with molecular methods. We carry out a 60-min HA test using 1% guinea pig RBCs to detect Flu A and B as well as parainfluenza viruses, whereas we perform a 45-min HA test using 0.5% chicken RBCs to detect Flu B and C on Days 6 and 13 (Figs. 3B and Fig. 4). We also perform the HAd test to detect the growth of Para 1 and 3, which

### Table 2. Typical cell sensitivity patterns\(^1\) of each virus in the microplate method and other principal cell lines in Yamagata, Japan

| Virus          | HHVe6MRG microplate\(^1\) | LLC-MK2-N          | RD-A  | RD-18S-N | Other sensitive cell line |
|----------------|---------------------------|--------------------|-------|----------|--------------------------|
|                | HEF                      | HEP-2              | Vero E6 | MDCK     | RD-18S                   | GMK                      |
| Flu            | A/B/C                    | ×                  | ×      | ×        | ×                        | ×                        | -                        | -                        | -                        |
| Para           | 1–4                      | ×                  | ×      | x        | ×                        | ×                        | -                        | -                        | -                        | HMV-II                   |
| HMPV           |                           | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| RS             |                           | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| Mumps          |                           | ×                  | ×      | x        | ×                        | -                        | -                        | -                        | -                        | -                        |
| Measles        | Vaccine strain           | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | Vero/hSLAM               |
| Wild strain    |                           | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | Vero/hSLAM               |
| CV-A2,4,10     |                           | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| CV-A16/EV-A71 |                           | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| CV-A9          |                           | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| Entero         | CV-A6/EV-D68             | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| CV-B1-5        | ×                        | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| Echo           | ×                        | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| Polio          | ×                        | ×                  | ×      | ×        | ×                        | -                        | -                        | -                        | -                        | -                        |
| PeVA           |                           | ×                  | ×      | ×        | ×                        | ×                        | -                        | -                        | -                        | -                        |
| 1              |                          | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | DO\(^2\)                | -                        |
| 3              |                          | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | DO                      | -                        |
| Rhinovirus     | A/B                      | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | -                        | -                        |
| SAFV           | 2/3                      | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | -                        | -                        |
| HCoV           | 229E                     | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | -                        | Hela-ACE2-TMPRSS2        |
| Ad             | 1–7                      | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | -                        | -                        |
| Rubella        | Vaccine strain           | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | -                        | -                        |
| HSV            |                           | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | -                        | -                        |
| CMV            |                           | ×                  | ×      | ×        | ×                        | ×                        | ×                        | ×                        | -                        | -                        |

\(^1\) Good growth; \(^2\) Poorer growth than ×; × , no growth; - , no evaluation.
\(^3\) Cell sensitivity patterns are changeable depending on the viral strains; \(^4\) Routine virus isolation; \(^5\) DO: difficult to observe cytopathic effect.
rarely show CPEs, using guinea pig RBCs on Days 7 and 14 (Figs. 3B and Fig. 4). We record these results, including those from virus isolation, HA and HAd tests, the neutralization (NT) test, RT-PCR, and sequencing (Fig. 4), as well as related information such as the clinical diagnosis and results of rapid tests, and share these data among laboratory members (Fig. 3C).

When CPE and/or HA, and/or HAd are positive or suspected, the viral fluid is passaged into fresh cells, and stepwise viral identification is performed using the NT test, the HI test, and molecular methods (Figs. 3D, Fig. 4). For several picornaviruses such as EV-A71, we have prepared polyclonal antibodies for the NT test using guinea pigs and rabbits in collaboration with Yamagata University (28,29).

Finally, we report the results to hospitals and clinics (Fig. 4).

4. System for stocking clinical specimens, clinical viral isolates, cDNAs, and serum specimens

Our basic approach is to isolate viruses from the clinical specimens and to stock clinical viral isolates as important research materials in order to advance their epidemiology. Thus, we have stored clinical viral isolates in our –80°C freezers since 1999 (Fig. 3D-b) and original clinical specimens since 2008 (Fig. 3D-a), except that poliovirus type 1, 2, and 3 isolates and their original specimens were destroyed in accordance with the global initiative (30). No poliovirus was isolated in Yamagata after 2012, when the oral poliovirus vaccine was stopped in Japan. Furthermore, we routinely extract RNA/DNA from clinical specimens and prepare cDNAs using universal (random) primers (Figs. 2, 4) in order to detect viral genomes such as those of enterovirus and PeV (Fig. 4), most of which are RNA viruses (Table 2), and store these cDNAs in –80°C freezers (Fig. 3D-c). When we carry out molecular epidemiological studies using viral isolates, we prepare cDNAs from these isolates and stock them (Fig. 3D-d). We also take part in the Japanese National Epidemiological Surveillance of Vaccine-Preventable Diseases every year and store the serum specimens at –20°C when informed consent is available from the participants or their guardians to carry out seroepidemiological studies. Preserving these materials provides us with further opportunities to advance the epidemiology of viral infectious diseases in Yamagata at any time. For example, we have succeeded in carrying out longitudinal epidemiological studies using stocked specimens, viruses, and cDNAs for EV-A71 and EV-D68 as described below.

5. Initial works between 2003 and 2005

We started our work to advance the epidemiology of viral ARIs among children, mainly using traditional technologies such as virus isolation and serological techniques. It took several years to accumulate the data necessary to present our initial papers on the situation in Yamagata. We published our first paper related to our microplate method focusing on enterovirus isolation in 2003 (18). We then published 3 papers related to Echo13, Ad7, and Flu B using virus isolation and serological techniques such as the NT test and HI test (31–33). These papers on Echo13 and Ad7 were our first studies related to emerging and re-emerging viral diseases, which later became an important theme in our research. Since then, a combination of virus isolation, genetic detection, and seroepidemiological study has regularly been used in our subsequent epidemiological
studies.

We introduced an RT-PCR method to amplify the viral genomes and detect viruses directly from clinical specimens and virus isolates around 2000. Using RT-PCR, we published our first paper related to influenza A/H1N2 in 2003 (34). In 2004, we observed an outbreak of measles virus infections at a junior high school caused by a genotype D9, which had not previously been identified in Japan (35). In that study, we introduced a genotyping method to monitor the transmission of the measles virus, using a combination of RT-PCR and sequence analysis. Genotyping thereafter became for us an important tool for advancing the epidemiology of viral infectious diseases. These approaches also became essential required methods for the elimination of measles in the revised version of the Prevention of Specific Infectious Diseases: Measles in Japan in 2013 (35,36).

Subsequently, we have carried out epidemiological studies on several viruses simultaneously using a variety of viruses isolated by microplate methods. On the other hand, we additionally sought to detect and isolate other viruses that had not been isolated by microplate methods one by one as described below.

6. Epidemiological and collaborative studies on several specific viruses

6-1. Longitudinal epidemiology of EV-A71 and CV-A16

Although hand-foot-and-mouth disease (HFMD) among children was a self-limited disease, EV-A71 has caused severe complications, mainly in the Asia-Pacific region, since around 1997 (37). We had many clinical EV-A71 isolates in Yamagata and decided to carry out longitudinal molecular epidemiology and antigenic analyses, with the aim of contributing to the control of this disease.

We finally analyzed 223 EV-A71 strains isolated between 1990 and 2013 (28,38,39). First, we suggested that distinct subgenogroups, including a novel B5, which might have been imported from neighboring countries, has had a major epidemiological impact on the local community in Yamagata (38). During the study period, we found that 6 subgenogroups appeared one after another and sometimes reappeared at intervals (28,39). Furthermore, we suggested that there was a discrepancy between the major subgenogroups circulating in the Asia-Pacific region and those in Europe (39). Serological analyses of residents in Yamagata in 2004 and sera from guinea pigs immunized with 2 distinct subgenogroup strains showed cross-antigenicity among 7 different subgenogroups, including the prototype strain (28). We proposed the idea that severe illnesses due to EV-A71 infections could be prevented through the development of a vaccine, as has been shown with the measles vaccine strategy, in which the measles vaccine strain has not been changed even though a number of subgenogroups have been reported (28).

A Singaporean research group asked us to send representative EV-A71 Yamagata isolates for each subgenogroup to evaluate a synthetic peptide vaccine produced from the subgenogroup B4 strain, and they succeeded in confirming its efficiency against the different subgenogroup strains using Yamagata isolates (40). We also pursued several basic research programs with NIID and the Tokyo Metropolitan Institute of Medical Science (29,41–43). For example, we found that the VP1 amino acid residue 145 of EV-A71 is a key residue for its receptor attachment and resistance to NT antibodies in an animal experiment (29).

CV-A16 is another causative agent of HFMD. We analyzed 220 CV-A16 strains isolated between 1988 and 2011 and categorized them into 3 genogroups; the first genogroup includes only the prototype strain, the second includes strains that had disappeared by the end of the 20th century, and the third comprised those that have been circulating since then in Yamagata (44).

6-2. Proposal of a new disease concept, “PeV-A3-associated myalgia/myositis”

PeV-A3 was first reported in 2004 and has been recognized as a particular causative agent of severe infectious diseases such as sepsis syndrome among infants (45,46). We reported PeV-A3-associated-myalgia/myositis among adults, characterized by myalgia and muscle weakness of the upper and lower limbs, which occurred in the summer of 2008 in Yamagata (23). In that study, we could detect PeV-A3 with the assistance of modern high-throughput sequencing, which was performed at NIID using the representative specimens (23). Since then, we repeatedly observed and reported PeV-A3-associated-myalgia/myositis cases in 2011, 2014, and 2016 (47–49). It is noteworthy that we observed cases among children as well in 2014 (47). In the outbreaks of 2014 and 2016, PeV-A3-associated-myalgia/myositis cases were reported in 9 other prefectures other than Yamagata, suggesting that this disease has recently been recognized across large areas of Japan (50). We succeeded in isolating PeV-A3 strains using the LLC-MK2-N cell line (23), which enabled us to measure NT antibodies against PeV-A3 among the patients suspected of PeV-A3-associated-myalgia/myositis. The results of NT antibody titration strongly suggested a relationship between PeV-A3 and myalgia/myositis (23). In the low PeV-A3 activity season in 2017, when we did not detect any PeV-A3 in our routine community surveillance, we found a sporadic case of PeV-A3-associated-myalgia/myositis in Yamagata and suggested the importance of maintaining a careful clinical watch to identify further cases of this disease (51). We also carried out a seroepidemiological study on PeV-A1, PeV-A3, and PeV-A6 using Yamagata isolates (52). We hope that the mechanism of the disease will be resolved, the situation regarding the prevalence of this disease abroad will be known well, and laboratory diagnostic techniques for PeV-A3 will be developed and made generally available (50,53).

6-3. Human metapneumovirus

We succeeded in isolating HMPV using the Vero E6 cell lines (19). In clinical settings, rapid test kits for the detection of viral antigens are quite useful for the clinical management of patients. We carried out a comparative study among real-time RT-PCR, the newly developed immunochromatographic kit method for HMPV, and virus isolation methods (54,55). These data contributed to the kit being approved by the government and becoming commercially available in Japan in 2014. As a part of our studies on HMPV, we
revealed that its epidemics had a seasonal pattern, which peaks between March and April in Yamagata (56). Our longitudinal epidemiological study between 2004 and 2009 revealed that genotypes A2 and B2 had been in endemic circulation as the major genotypes almost every year, whereas other genotypes had appeared less frequently (57). We also measured genotype-specific seroprevalence (58), investigated HMPV infection among family members (59), and developed a virus-based enzyme-immunosorbent assay for HMPV antibodies (60).

6-4. Parainfluenza viruses
Parainfluenza viruses appear to be the cause of approximately 5% of common cold illnesses (2). Although we can isolate parainfluenza viruses using the Vero E6 and MCDK cell lines in the HHVe6MRG plate (20), we introduced the HMV-II cell line in 2008, and the isolation frequencies of parainfluenza viruses subsequently increased (21). Based on the virus isolation data of Para 1–3 over 8–10 years, we were able to reveal several epidemiological characteristics. First, unlike Para 1, Para 3 shows clear seasonality with yearly outbreaks in the spring-summer season, and Para 2 tended to appear biannually in autumn-winter (21,56). Second, Para 3 infections are common in infants and young children (0–1 years old), whereas Para 1 and 2 infections tend to be more common in young children (3–4 years old) (56). We succeeded in isolating one Para 4 strain with the HMV-II cell line in October 2011 for the first time; thus, we added the LLC-MK2(-N) cell line, which was reported to be sensitive to the isolation of Para 4, and thereafter detected and isolated 43 Para 4 strains between October 2011 and February 2012 (22). Using Yamagata isolates, we carried out molecular epidemiological studies on Para 1 and Para 3 (61,62). In collaboration with NIID, we revealed the role of the transmembrane serine protease TMPRSS2 as an activating protease for the replication of parainfluenza viruses (63).

6-5. Human coronaviruses
HCoV are viruses that frequently associate with ARIs such as rhinoviruses (2). Although we continued performing virus isolation using respiratory specimens for over a decade, we did not isolate any HCoVs. Thus, we attempted to detect HCoV genomes retrospectively using RT-PCR directly in the clinical specimens that had been collected since 2010. Using specimens collected for 4 years since January 2010 until December 2013, all 4 HCoVs (HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1) were detected, with annual variations mainly in winter (64). Furthermore, we observed that HCoV-OC43 caused an outbreak during the 2014–2015 influenza season and again in June 2016 in Yamagata (65,66). In terms of virus isolation, we were able to reveal several epidemiological characteristics. First, unlike Para 1, Para 3 shows clear seasonality with yearly outbreaks in the spring-summer season, and Para 2 tended to appear biannually in autumn-winter (21,56). Second, Para 3 infections are common in infants and young children (0–1 years old), whereas Para 1 and 2 infections tend to be more common in young children (3–4 years old) (56). We succeeded in isolating one Para 4 strain with the HMV-II cell line in October 2011 for the first time; thus, we added the LLC-MK2(-N) cell line, which was reported to be sensitive to the isolation of Para 4, and thereafter detected and isolated 43 Para 4 strains between October 2011 and February 2012 (22). Using Yamagata isolates, we carried out molecular epidemiological studies on Para 1 and Para 3 (61,62). In collaboration with NIID, we revealed the role of the transmembrane serine protease TMPRSS2 as an activating protease for the replication of parainfluenza viruses (63).

6-6. Measles and Rubella viruses
We reported an outbreak of measles due to genotype D9 at a junior high school in 2004 (35), and we further found an imported case of genotype D9 infection from Thailand in 2009 (68). We used the Vero/hSLAM cell line for measles virus isolation (69). However, we have also isolated vaccine-derived measles viruses from children with ARI using the cell lines we routinely use, such as Vero E6, GMK, and HEp-2 cells, on the HHVe6MRG plate. It was, however, initially difficult for us to identify measles virus in specimens from patients who were not clinically suspected measles cases (69,70). Furthermore, we experienced another difficulty in identifying vaccine-derived rubella virus, which showed CPEs on the Vero E6 cells (69). These experiences suggested that laboratory technologists in public health institutes require adequate information regarding vaccination history before conducting virus isolation using cell culture techniques (69). Our experiences are reflected in Table 2.

Japan achieved measles elimination in March 2015 (36). However, in 2017, we experienced the largest measles outbreak in Yamagata to date, including 38 modified measles, which were characterized by mild illness and an atypical clinical course in a patient who had insufficient immunity, and 22 typical measles cases (71). We investigated this outbreak in Yamagata to contribute to the improvement of strategies against measles in the post-measles elimination era. Three out of 7 primary cases produced 50 transmissions, with each patient responsible for 9–25 transmissions (71). These 3 patients were aged 22–31 years with no history of vaccination, developed typical measles, and maintained contact with the public during their symptomatic period (71). These findings suggested that, in general, those aged 20–39 years with insufficient immunity against measles virus should be provided with additional doses of vaccine to prevent measles importation and endemcity (71). We also verified the effectiveness of real-time RT-PCR in that outbreak and found that throat swabs and peripheral blood mononuclear cell specimens were useful for the detection of the measles virus genome among modified measles cases and that the high viral loads suggested by real-time RT-PCR for pharyngeal specimens are important for predicting super-spreading events/primary cases as typical measles (72).

6-7. Enterovirus D68 (EV-D68)
EV-D68, which was first isolated in 1962, was only sporadically reported worldwide until 2010 (73). Since Imanuma et al. detected EV-D68 in children with severe ARIs in the Philippines in 2008–2009, EV-D68 has been regarded as an emerging pathogen associated with outbreaks of respiratory illness with varying degrees of seriousness (73,74). Although only limited cases of EV-D68 infections had been reported up to 2014, outbreaks of EV-D68 have been reported in Canada, Europe, and Asia as well as in the USA since then (75). Furthermore, EV-D68 has been associated with not only respiratory illnesses but also neurological features such as muscle weakness and flaccid paralysis in both children and adults since the outbreak in 2014 (74).

We observed picornavirus-like CPEs on RD-18S cell lines between August and October 2010 in Yamagata and identified those isolates as EV-D68 using a molecular method (76). We further tried to detect
EV-D68 genomes using stocked cDNAs, which were prepared from respiratory specimens, and succeeded in detecting EV-D68 genomes in specimens from 2005, 2006, 2007, and 2009 (76). In this study, we found that genetically distinct clusters of EV-D68, i.e., lineages 1, 2, and 3, which were later proposed as clades C, B, and A, respectively, by Tokarz et al. (77), co-circulated in Yamagata. The results of this study also suggested that difficulties associated with EV-D68 isolation lead to an underestimation of the prevalence of EV-D68 infections (76). We further detected 2 EV-D68 sporadic cases in 2013 and 46 cases in 2015 at an outpatient pediatric clinic in Yamagata and found that most cases with EV-D68 infection were managed as outpatients (78). In contrast, there has been a strong focus on the admission of most of the severe cases of EV-D68 to hospitals in other parts of the world. As a collaborative study with Tohoku University Graduate School of Medicine, we analyzed the antigenic and receptor binding properties of EV-D68 (79).

6-8. Saffold viruses (SAFVs)

In 2007, SAFV was initially recovered from a fecal specimen collected in 1981 using the cell culture method and was classified as member of the genus Cardiovirus, family Picornaviridae as a novel human cardiovirus (80). Since then, we have carried out several preliminary studies of SAFVs using RT-PCR and sequencing methods (81,82). We further reported an outbreak of SAFV type 2 (SAFV2) over a 4-month period with a peak in October 2009 and proposed that SAFV2 is one of the causative agents of ARIs (83). In that outbreak, we succeeded in detecting the SAFV2 genome but failed to isolate the virus. However, we succeeded in isolating SAFV2 using the RD-185-N cell line with a 2-step virus isolation method (Fig. 2B, Table 2) (25). Combining molecular detection and virus isolation as well as phylogenetic analysis, we carried out a longitudinal epidemiological study of SAFVs between 2008 and 2015, with the results of this study suggesting that SAFV3 is another possible causative agent of ARIs among children as well as SAFV2 and that their infections occur mainly in the autumn season in Japan (84).

6-9. Other viruses

We isolated a total of 2,587 Ad1-6 strains between 1988 and 2007 in Yamagata, and the phylogenetic tree based on hexon protein indicated that the Yamagata isolates and reference strains branched depending on serotype, with hypervariable regions stably conserved as serotype-specific regions for a long period with only minor genomic variations (85).

We isolated 249 mumps viruses between 1999 and 2013 and carried out sequence analysis of the small hydrophobic gene. These data demonstrated that the genotype G strains have been endemic as the major type over a wide area of Japan since 2001, although the genotype G strains (G-cluster 1) that emerged after 2011 differed from the earlier strains (G-cluster 2) (86).

We have also published several papers related to Flu A, Flu B, CV-A9, and rhinovirus (87-91).

7. Discussion

With the aim of advancing the epidemiology of viral infectious diseases in a community such as Yamagata, one basic benefit of the microplate method is that it can be used to process a large number of clinical specimens throughout the year. We have collected over 2,000 specimens per year from patients (mainly infants and children) in Yamagata. Our preliminary study between 2004 and 2005 indicated that our virus isolation rate was 37.8% (1,551/4,107) (20). Of course, if we only targeted viruses that are easy to isolate, such as influenza viruses, the virus isolation rate would increase. However, this is not our purpose, and rather, we aim to isolate as wide a range of viruses present in the respiratory specimens as possible. In this context, we are pleased that we are now able to isolate a wider range of viruses than we were between 2004 and 2005. We preserve the specimens in the freezer, numbering them successively by year of collection, such as 2017-1, 2017-2, and 2017-3, together with the viral isolate and necessary information such as cell passage history and stock date (Fig. 3D-b, e). We stock viruses using the internationally authentic methods such as A/YAMAGATA/10/2017(H3) for influenza viruses and MV/1/Yamagata.JPN/10.17(D8)-473 for measles virus as well as "2017-56;FluAH3" (Fig. 3D-e) and "2017-473; Measles," respectively, to identify them within the overall spectrum of viral infectious diseases in Yamagata in 2017. To improve our system, the staff at our laboratory have shared ideas and modified our virus isolation, detection, and analyzing methods as well as the system for stocking research materials little by little over the last 2 decades, as described in this manuscript.

Currently, classical and traditional technologies, such as virus isolation and serological techniques, are uncommon and have been replaced by molecular methods. However, we still believe that a combination of useful traditional and modern, more analytically powerful technologies is important and necessary to achieve our goal of developing control measures against viral infectious diseases in the community.

Although we have published a several studies related to noroviruses (92,93), our main target has been respiratory viruses. We started to perform epidemiological studies on viral ARIs among children using the microplate method in 1999 in Yamagata and thereafter published several initial works. We then combined the traditional methods with molecular methods and accumulated our data through longitudinal epidemiological studies. In these works, we observed emerging and re-emerging diseases such as those caused by Ad7, Echo13, EV-D68, PeV-A3, and SAFVs. We insisted on isolating viruses, and we succeeded in isolating several viruses such as HMPV, PeV-A3, PeV-A4, PeV-A6, Para 4, SAFV2 and 3, EV-D68, HCoV-229E, vaccine-related measles virus, and vaccine-related rubella virus for the first time in our laboratory. With regard to PeV-A3, we proposed a new disease concept, “PeVA3-associated myalgia/myositis,” using a combination of our technologies, including virus isolation, the NT test, RT-PCR, sequence analysis, and phylogenetic analysis. Genotyping was also useful for estimation of the transmission and/or distribution of viruses, particularly for measles virus and EV-A71.

Our studies are based on epidemiological studies. Of course, the good relationships maintained among YPIPH, health centers, hospitals, clinic, and Yamagata University are of the primary importance. Many specialized skills from associated disciplines are required
to develop control measures against viral infectious diseases, and we cannot perform all of them by ourselves in our laboratory (Fig. 1). Thus, our intention has been to collaborate in our research activities with various universities and research institutes. As we have many clinical viral isolates, we have provided Yamagata strains of Flu A–C, Para 1–4, HMPV, enteroviruses, RSV, measles virus, rhinovirus, rubella virus, PeV-A3, Ad 1–6, HSV, and SAFV to public health laboratories, universities, and other research laboratories across Japan and to several international laboratories since 2007 (Fig. 5). In 2007, the Infectious Diseases Control Law was revised, and the regulations for manipulating microbiological pathogens became stricter. We prepared material transfer agreement forms for providing the viruses, and we have kept them since then.

It is also important for us to share our findings in Yamagata with the public health and infectious disease control sectors through research papers and textbooks. For example, our papers were referenced in several textbooks related to EV-A71 (28,37,38), PeVs (23,47,94), HMPV (55,56,58,60,95–97), EV-D68 (76,98,99), SAFV (81–83,100), Flu C (10,11,101), and norovirus (92,93,102). Our first 2 papers related to EV-A71, in particular, were referenced in the textbook “Viral Infections of Humans, Epidemiology and Control,” suggesting that genotyping and molecular epidemiological methods, which can be used to estimate the transmission and/or distribution of viruses, are important approaches for the control of infectious diseases and that the direction of our research to date has been appropriate (28,37,38).

In conclusion, we hope our experiences, findings, and research materials will contribute to the development of countermeasures against viral infectious diseases and improve public health strategies in Yamagata, Japan, Asia, and around the world.

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Overview of Activities at Yamagata PIPH

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