Prevalence of vaccine-derived polioviruses in the environment

Hiromu Yoshida,[1] Hitoshi Horie,[2] Kumiko Matsuura,[3] Takashi Kitamura,[3] So Hashizume[2] and Tatsuo Miyamura[1]

[1] Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan

[2] Japan Poliomyelitis Research Institute, Kumegawa 5-34-4, Higashimurayama, Tokyo 189-0003, Japan

[3] Department of Virology, Toyama Institute of Health, Nakataikoyama, Kosugi-machi, Imizu-gun, Toyama 939-0363, Japan

A survey of poliovirus in river and sewage water was conducted from October 1993 to September 1995 in Toyama Prefecture, Japan. In this study, 25 isolates differentiated as type 2 vaccine-derived polioviruses (VDPVs) were characterized using mutant analysis by PCR and restriction-enzyme cleavage (MAPREC) to estimate the ratio of 481-G revertants correlated to neurovirulence in a virus population. Of these isolates, 23 (92%) comprised between 44 and 96% 481-G revertants by MAPREC. The other two isolates had revertant percentages close to the 0·6% of the attenuated reference strain. It was presumed that these 23 isolates would be variant with potential neurovirulence by MAPREC analysis. Of the 23 isolates, three were isolated from river water. Moreover, our results by MAPREC showed that type 2 poliovirus was phenotypically more variable than type 1 (69%) or type 3 (55%), as determined in previous studies. The prevalence of virulent-type VDPVs in river and sewage water suggested that the oral poliovaccine itself had led to wide environmental pollution in nature. To terminate the cycle of virus transmission in nature, the ecology of VDPVs should be studied further. A hygiene programme, inactivated poliovirus vaccine immunization and well-maintained herd immunity may play key roles in reducing the potential risk of infection by virulent VDPVs.

Introduction

Although it is known that poliovirus exists widely in nature, in soil, sewage, wastewater, drinking water and food such as shellfish, there is very little evidence to connect it directly with an outbreak of poliomyelitis (Jaykus, 1997; Metcalf *et al.*, 1979; Goyal *et al.*, 1979). Because most cases of infection by poliovirus are not apparent, it is not until secondary, person-to-person spread leads to the onset of poliomyelitis that the infection is recognized. Therefore, it is difficult to address the risk of infection from the environment (Metcalf *et al.*, 1995).

The polio eradication program is close to the final stage of replacing wild-type poliovirus in the population with vaccine-type by mass live oral poliovaccine (OPV) immunization. After the termination of OPV in the near future, the possibility of an outbreak caused by vaccine-derived poliovirus (VDPV) must

be considered, since it has been shown in many studies that nucleotide substitution in the virus genome occurs gradually during replication in the human gut after OPV administration and the phenotype of excreted viruses changes from attenuated to virulent (Abraham *et al.*, 1993; Dunn *et al.*, 1990; Japan Live Poliovaccine Research Commission, 1967; Benyesh-Melnick *et al.*, 1967; Guillot *et al.*, 1994; Wood & Macadam, 1997). It is therefore difficult to distinguish whether vaccine-associated paralytic poliomyelitis (VAPP) cases are recipient VAPP or contact VAPP by sequencing the genome of excreted virus.

On the other hand, environmental surveillance is still epidemiologically important for the following reasons: (i) the results of virus surveillance retrospectively reflect the properties of virus circulating in the community (Divizia *et al.*, 1999; Shulman *et al.*, 2000; Tambini *et al.*, 1993; Pöyry *et al.*, 1988; van der Avoort *et al.*, 1995) and (ii) it assesses the potential risk of infection from the environment and food (Jaykus, 1997; Richards, 1999; Haas, 1983; Haas & Heller, 1988; Haas *et al.*, 1993). In Toyama, Japan, routine OPV immunization has been administered annually in May and October. We have shown in

Author for correspondence: Hiromu Yoshida.
Fax +81 42 561 4729. e-mail hyoshida@nih.go.jp

previous studies that VDPVs were isolated from sewage and river waters within approximately 3 months after OPV and then, in type 1 and 3 viruses, not only did genetic mutation occur with less than 1·4% nucleotide divergence from the vaccine strain, but neurovirulence also increased in some of the isolates, as indicated by mutant analysis by PCR and restriction-enzyme cleavage (MAPREC) and neurovirulence tests with transgenic mice (Matsuura *et al.*, 2000; Yoshida *et al.*, 2000; Horie *et al.*, 2002). This means that it would be possible to detect phenotypically changed vaccine strains circulating in the population retrospectively through environment surveillance provided by the MAPREC assessment.

Neurovirulence increased to varying degrees when the following changes took place in base positions of the viruses: in the case of type 1 viruses, positions 480 and 525 in the 5′ non-coding region changed respectively from G to A and from U to C; for type 2, position 481 changed from A to G; and for type 3, position 472 changed from U to C (Kawamura *et al.*, 1989; Pollard *et al.*, 1989; Evans *et al.*, 1985). Chumakov *et al.* (1991, 1994) developed the MAPREC method to estimate the ratio of revertants in a virus population that correlated with neurovirulence in monkey (Chumakov *et al.*, 1991, 1994; Chumakov, 1999; Taffs *et al.*, 1995; Rezapkin *et al.*, 1994, 1999). MAPREC is a very sensitive method of determining the ratio of revertants, so, in type 1 and type 2 viruses, the results of MAPREC are not always related to the monkey neurovirulence test (MNVT), but are still useful in monitoring poliovirus mutants (Chumakov, 1999; Rezapkin *et al.*, 1999). In this study, other type 2 strains were examined by MAPREC. Moreover, we report on the ecology of polioviruses in the environment, given the previous data on type 1 and type 3 viruses (Yoshida *et al.*, 2000; Horie *et al.*, 2002).

Methods

■ **Virus isolation from sewage and river water.** From October 1993 to September 1995, samples were collected twice monthly at a sewage plant and river-monitoring points. The collected water was concentrated by using the method described by Matsuura *et al.* (1984). Each concentrated specimen was inoculated into a total of 30 tube cultures of rhabdomyosarcoma (RD), primary monkey kidney (MK) or Vero cells and incubated at 36 °C (Matsuura *et al.*, 1984). Thirty-one isolates were identified as type 2 by polio pooled serum and differentiated intratypically as vaccine types (Yoshida *et al.*, 1997). The 25 available isolates were used for further study.

■ **MAPREC.** RNA purification, cDNA synthesis and PCR were performed as described by Taffs *et al.* (1995). The primers used for PCR were pS2-465 (sense primer; 5′ ^{431}GCTACATAAGAGTCCTCCGGCC-CCTGAATGCGGCT^{465} 3′) and pA2-483 (antisense primer; 5′ ^{520}CGC-GTTACGACAAGCCAGTCACTGGTTCGCGACCACGT^{483} 3′). The PCR product containing 481-G revertants was used for second-strand DNA synthesis using a ^{32}P-labelled sense primer and digested with *Bsp*1286I. The proportion of 481-G revertants was calculated by measuring the radioactivity in c.p.m. of *Bsp*1286I-digested and undigested DNA strands, using the equation 100 × digested DNA (c.p.m.)/[digested DNA (c.p.m.) + undigested DNA (c.p.m.)]. F207, which

is a Sabin original + 2nd passaged strain (SOM-2) derived from the P712, ch, 2ab strain, was used as a reference strain for the attenuated phenotype and was compared with environmental strains. As for type 1 and 3 polioviruses, previously reported MAPREC results were used, and data for attenuated reference strains F113 for type 1 and F313 for type 3 were also referred to (Yoshida *et al.*, 2000; Horie *et al.*, 2002).

Results and Discussion

Twenty-five type-2 polioviruses were isolated from sewage and river water within 3 months of routine OPV immunization (Fig. 1), showing the properties of vaccine-derived strains (Matsuura *et al.*, 2000). The substitution ratio of position 481 in the 5′ non-coding region of the virus genome was examined by using the MAPREC method, and 23 of 25 isolates (92%) comprised between 44 and 96% 481-G revertants by MAPREC (Fig. 2). Three isolates from the river contained 92–96% 481-G revertants. F207, as the attenuated reference strain, had 0·6% 481-G. Only two isolates were close to F207; the other 23 had a higher rate than that for the attenuated F207 strain. Taffs *et al.* (1995) reported that the stipulated cut-off of the ratio of 481-G revertants for passing or failing of type-2 vaccine viruses by MNVT was approximately 4%. Therefore, these 23 isolates were presumed to be variant with potential virulence. In type 1 virus, the stipulated cut-off value of 480-A + 525-C was approximately 5% and, in type 3, the cut-off of
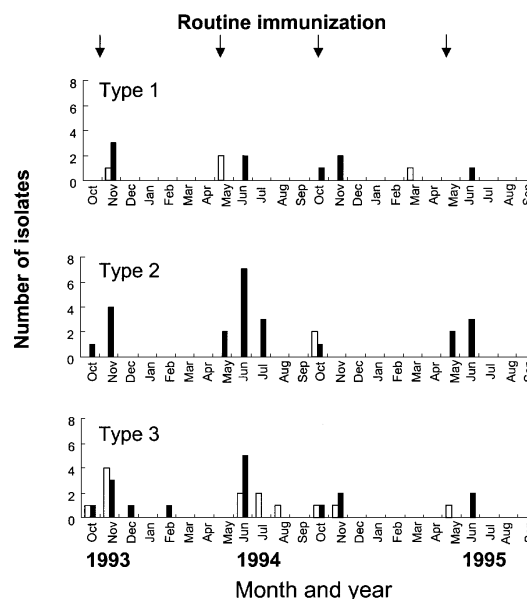

Fig. 1. Time-series of VDPVs in the environment. Routine immunization was conducted in May and October. Each serotype was isolated in the environment within approximately 3 months of OPV immunization. Variant strains (filled bars) are VDPV strains that had a value that exceeded the stipulated cut-off percentage of 480-A + 525-C for type 1, 481-G for type 2 and 472-C for type 3. Attenuated strains (open bars) are strains for which the content of 480-A + 525-C (type 1), 481-G (type 2) or 472-C (type 3) was the same as or less than for F113, F207 or F313 (attenuated reference strains). Data for type 1 and type 3 viruses were taken from previous studies (Yoshida *et al.*, 2000; Horie *et al.*, 2002).
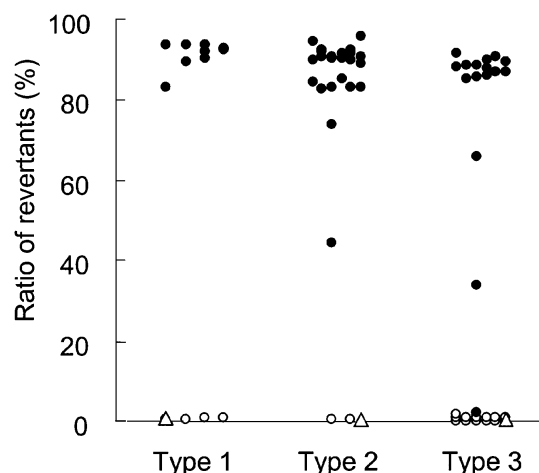
Fig. 2. Contents of 480-A + 525-C for type 1, 481-G for type 2 and 472-C for type 3 revertants in strains isolated from sewage and river water. ●, Content of 480-A + 525-C (type 1), 481-G (type 2) or 472-C (type 3) exceeded the stipulated cut-off values (approximately 5, 4 and 1%, respectively) for passing of each serotype vaccine virus by MNVT. ○, Content of 480-A + 525-C (type 1), 481-G (type 2) or 472-C (type 3) was the same as or less than F113, F207 and F313 (attenuated reference strains). △, Attenuated reference strains (F113, F207 and F313 for each serotype). Data for type 1 and type 3 viruses were reported in previous studies (Yoshida *et al.*, 2000; Horie *et al.*, 2002).

472-C was approximately 1% (Chumakov, 1999; Rezapkin *et al.*, 1994). Therefore, in type 1 examined in a previous study as shown in Fig. 2, 9 of 13 (69%) isolates contained 83–94% 480-A + 525-C by MAPREC and were presumed to be variant with potential virulence (Horie *et al.*, 2002). In type 3, 16 of 29 (55%) isolates contained 2–91% 472-C revertants (Fig. 2) (Yoshida *et al.*, 2000). Accordingly, the ratios of variant/total isolates were respectively 69, 92 and 55% in types 1, 2 and 3.

The results of our analysis of environmental strains by MAPREC showed that the ratio of revertants increased in 55–92% of isolates in each serotype, compared with the attenuated reference virus. Of the three serotypes, type 2 virus had the highest rate of mutation. As all samples from environmental sources examined were highly concentrated, the risk of infection by these isolates would be very small. However, the risk-assessment study by Haas and co-workers showed that the longer the term of exposure to water contaminated by enteroviruses, the higher the potential infection risk (Haas *et al.*, 1993; Haas & Heller, 1988; Haas, 1983). Although it is very rare in industrialized countries to have direct access to wastewater contaminated by viruses, this is not always the case in developing countries, which may have poor sewage and drinking water systems. Therefore, a risk-assessment study for virulent-type VDPVs would be epidemiologically important.

In agreement with our results, type 2 virus has been shown in other studies to have the highest rate of phenotypic mutation of the three serotypes by the T-marker test (Matyasova & Koza, 1982, 1985). Generally, type 2 poliovirus

has a higher rate of seroconversion after OPV administration than types 1 and 3, as a result of interference with the growth of types 1 and 3. Selection in the human gut might lead to high proportion of revertants in type 2.

It has been shown that isolates in the environment are genetically and epidemiologically related to those circulating in the community (Divizia *et al.*, 1999; Shulman *et al.*, 2000; Tambini *et al.*, 1993; Pöyry *et al.*, 1988; van der Avoort *et al.*, 1995; Slater *et al.*, 1990; Brancroft *et al.*, 1957). Therefore, the properties of isolates from sewage and river water would reflect those of viruses excreted from humans after OPV immunization, and, for susceptible individuals, VDPVs, which are a source of virulence, have the potential to be causative agents of poliomyelitis. As long as immunization coverage is maintained, OPV or inactivated poliovaccine will be effective in protecting against poliomyelitis caused by these VDPVs. The important point is that VDPVs in each serotype could be considered as potential causative agents of poliomyelitis, and might be spread widely in the community through contact infection, unapparent for susceptible individuals. The survey of seroconversion in the community would be useful to predict the risk of transmission of virulent-type VDPVs.

OPV immunization has contributed greatly to the poliomyelitis eradication programme. However, when the circulation of wild strains seems to have been interrupted, it is necessary to consider the possibility of the circulation of VDPVs. It has been reported that outbreaks of poliomyelitis caused by type 2 VDPV in Egypt and by type 1 in Haiti and the Dominican Republic have occurred in 2000 (Centers for Disease Control and Prevention, 2000, 2001). Environmental virus surveillance is important in considering the potential risk towards the worldwide poliomyelitis eradication programme in its final stages.

References

Abraham, R., Minor, P., Dunn, G., Modlin, J. F. & Ogra, P. L. (1993). Shedding of virulent poliovirus revertants during immunization with oral poliovirus vaccine after prior immunization with inactivated polio vaccine. *Journal of Infectious Diseases* **168**, 1105–1109.

Benyesh-Melnick, M., Melnick, J. L., Rawls, W. E., Wimberly, I., Oro, J. B., Ben-Porath, E. & Rennick, V. (1967). Studies of the immunogenicity, communicability and genetic stability of oral poliovaccine administration during the winter. *American Journal of Epidemiology* **86**, 112–136.

Brancroft, P. M., Engelhard, W. E. & Evans, C. A. (1957). Poliomyelitis in Huskerville (Lincoln) Nebraska. Studies indicating a relationship between clinically severe infection and proximate fecal pollution of water. *Journal of the American Medical Association* **164**, 836–847.

Centers for Disease Control & Prevention (2000). Outbreak of poliomyelitis – Dominican Republic and Haiti, 2000. *Morbidity and Mortality Weekly Report* **49**, 1094, 1103.

Centers for Disease Control & Prevention (2001). Circulation of a type 2 vaccine-derived poliovirus – Egypt, 1982–1993. *Morbidity and Mortality Weekly Report* **50**, 41–42, 51.

Chumakov, K. M. (1999). Molecular consistency monitoring of oral poliovirus vaccine and other live viral vaccines. *Developments in Biological Standardization* **100**, 67–74.

Chumakov, K. M., Powers, L. B., Noonan, K. E., Roninson, I. B. & Levenbook, I. S. (1991). Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. *Proceedings of the National Academy of Sciences, USA* **88**, 199–203.

Chumakov, K. M., Dragunsky, E. M., Norwood, L. P., Douthitt, M. P., Ran, Y., Taffs, R. E., Ridge, J. & Levenbook, I. S. (1994). Consistent selection of mutations in the 5′-untranslated region of oral poliovirus vaccine upon passaging in vitro. *Journal of Medical Virology* **42**, 79–85.

Divizia, M., Palombi, L., Buonomo, E., Donia, D., Ruscio, V., Equestre, M., Leno, L., Pana, A. & Degener, A. M. (1999). Genomic characterization of human and environmental polioviruses isolated in Albania. *Applied and Environmental Microbiology* **65**, 3534–3539.

Dunn, G., Begg, N. T., Cammack, N. & Minor, P. D. (1990). Virus excretion and mutation by infants following primary vaccination with live oral poliovaccine from two sources. *Journal of Medical Virology* **32**, 92–95.

Evans, D. M., Dunn, G., Minor, P. D., Schild, G. C., Cann, A. J., Stanway, G., Almond, J. W., Currey, K. & Maizel, J. V., Jr (1985). Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. *Nature* **314**, 548–550.

Goyal, S. M., Gerba, C. P. & Melnick, J. L. (1979). Human enteroviruses in oysters and their overlying waters. *Applied and Environmental Microbiology* **37**, 572–581.

Guillot, S., Otelea, D., Delpeyroux, F. & Crainic, R. (1994). Point mutations involved in the attenuation/neurovirulence alternation in type 1 and 2 oral polio vaccine strains detected by site-specific polymerase chain reaction. *Vaccine* **12**, 503–507.

Haas, C. N. (1983). Estimation of risk due to low doses of micro-organisms: a comparison of alternative methodologies. *American Journal of Epidemiology* **118**, 573–582.

Haas, C. N. & Heller, B. (1988). Test of the validity of the Poisson assumption for analysis of most-probable-number results. *Applied and Environmental Microbiology* **54**, 2996–3002.

Haas, C. N., Rose, J. B., Gerba, C. & Regli, S. (1993). Risk assessment of virus in drinking water. *Risk Analysis* **13**, 545–552.

Horie, H., Yoshida, H., Matsuura, K., Miyazawa, M., Ota, Y., Nakayama, T., Doi, Y. & Hashizume, S. (2002). Neurovirulence of type 1 polioviruses isolated from sewage in Japan. *Applied and Environmental Microbiology* **68**, 138–142.

Japan Live Poliovaccine Research Commission (1967). Evaluation of Sabin live poliovirus vaccine in Japan. IV. Marker tests on poliovirus strains recovered from vaccinees and their contacts. Marker Test Subcommittee. *Japanese Journal of Medical Science and Biology* **20**, 167–173.

Jaykus, L. A. (1997). Epidemiology and detection as options for control of viral and parasitic foodborne disease. *Emerging Infectious Diseases* **3**, 529–539.

Kawamura, N., Kohara, M., Abe, S., Komatsu, T., Tago, K., Arita, M. & Nomoto, A. (1989). Determinants in the 5′ noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. *Journal of Virology* **63**, 1302–1309.

Matsuura, K., Hasegawa, S., Nakayama, T., Morita, O. & Uetake, H. (1984). Viral pollution of the rivers in Toyama City. *Microbiology and Immunology* **28**, 575–588.

Matsuura, K., Ishikura, M., Yoshida, H., Nakayama, T., Hasegawa, S., Ando, S., Horie, H., Miyamura, T. & Kitamura, T. (2000). Assessment of poliovirus eradication in Japan: genomic analysis of polioviruses isolated from river water and sewage in Toyama Prefecture. *Applied and Environmental Microbiology* **66**, 5087–5091.

Matyasova, I. & Koza, J. (1982). Properties of poliovirus strains isolated over the period 1969–1978 from the main municipal sewerage system of the city of Prague. *Journal of Hygiene Epidemiology Microbiology and Immunology* **26**, 162–175.

Matyasova, I. & Koza, J. (1985). Properties of poliovirus strains isolated from waste waters of nurseries in Prague in 1969–1982. *Journal of Hygiene Epidemiology Microbiology and Immunology* **29**, 387–398.

Metcalf, T. G., Mullin, B., Eckerson, D., Moulton, E. & Larkin, E. P. (1979). Bioaccumulation and depuration of enteroviruses by the soft-shelled clam, *Mya arenaria*. *Applied and Environmental Microbiology* **38**, 275–282.

Metcalf, T. G., Melnick, J. L. & Estes, M. K. (1995). Environmental virology: from detection of virus in sewage and water by isolation to identification by molecular biology – a trip of over 50 years. *Annual Review of Microbiology* **49**, 461–487.

Pollard, S. R., Dunn, G., Cammack, N., Minor, P. D. & Almond, J. W. (1989). Nucleotide sequence of a neurovirulent variant of the type 2 oral poliovirus vaccine. *Journal of Virology* **63**, 4949–4951.

Pöyry, T., Stenvik, M. & Hovi, T. (1988). Viruses in sewage waters during and after a poliomyelitis outbreak and subsequent nationwide oral poliovirus vaccination campaign in Finland. *Applied and Environmental Microbiology* **54**, 371–374.

Rezapkin, G. V., Chumakov, K. M., Lu, Z., Ran, Y., Dragunsky, E. M. & Levenbook, I. S. (1994). Microevolution of Sabin 1 strain in vitro and genetic stability of oral poliovirus vaccine. *Virology* **202**, 370–378.

Rezapkin, G. V., Fan, L., Asher, D. M., Fibi, M. R., Dragunsky, E. M. & Chumakov, K. M. (1999). Mutations in Sabin 2 strain of poliovirus and stability of attenuation phenotype. *Virology* **258**, 152–160.

Richards, G. P. (1999). Limitations of molecular biological techniques for assessing the virological safety of foods. *Journal of Food Protection* **62**, 691–697.

Shulman, L. M., Handsher, R., Yang, C. F., Yang, S. J., Manor, J., Vonsover, A., Grossman, Z., Pallansch, M., Mendelson, E. & Kew, O. M. (2000). Resolution of the pathways of poliovirus type 1 transmission during an outbreak. *Journal of Clinical Microbiology* **38**, 945–952.

Slater, P. E., Orenstein, W. A., Morag, A., Avni, A., Handsher, R., Green, M. S., Costin, C., Yarrow, A., Rishpon, S., Havkin, O., Ben-Zvi, T., Kew, O. M., Rey, M., Epstein, I., Swartz, T. A. & Melnick, J. L. (1990). Poliomyelitis outbreak in Israel in 1988: a report with two commentaries. *Lancet* **335**, 1192–1195.

Taffs, R. E., Chumakov, K. M., Rezapkin, G. V., Lu, Z., Douthitt, M., Dragunsky, E. M. & Levenbook, I. S. (1995). Genetic stability and mutant selection in Sabin 2 strain of oral poliovirus vaccine grown under different cell culture conditions. *Virology* **209**, 366–373.

Tambini, G., Andrus, J. K., Marques, E., Boshell, J., Pallansch, M., de Quadros, C. A. & Kew, O. (1993). Direct detection of wild poliovirus circulation by stool surveys of healthy children and analysis of community wastewater. *Journal of Infectious Diseases* **168**, 1510–1514.

van der Avoort, H. G., Reimerink, J. H., Ras, A., Mulders, M. N. & van Loon, A. M. (1995). Isolation of epidemic poliovirus from sewage during the 1992–3 type 3 outbreak in The Netherlands. *Epidemiology and Infection* **114**, 481–491.

Wood, D. J. & Macadam, A. J. (1997). Laboratory tests for live attenuated poliovirus vaccines. *Biologicals* **25**, 3–15.

Yoshida, H., Li, J., Yoneyama, T., Yoshii, K., Shimizu, H., Nguyen, T. H., Toda, K., Nguyen, T. L., Phan, V. T., Miyamura, T. & Hagiwara, A. (1997). Two major strains of type 1 wild poliovirus circulating in Indochina. *Journal of Infectious Diseases* **175**, 1233–1237.

Yoshida, H., Horie, H., Matsuura, K. & Miyamura, T. (2000). Characterization of vaccine-derived polioviruses isolated from sewage and river water in Japan. *Lancet* **356**, 1461–1463.