Wild birds are thought to act as reservoirs of JEV in nature (and may also act as amplifiers), since many species of birds have been proven to develop high viremia titers ($1–3 \times 10^4$ per ml) for as long as 4 days [5,8,9]. Although a number of seroepidemiological studies have shown that antibodies against JEV are detected in many mammalian species [5,10–14], only pigs are considered to be important as amplifiers in the JEV transmission cycle, because of their high viremia titer and the duration of viremia [15]. In fact, JEV has been shown to be transmitted from experimentally infected birds and pigs to mosquito vectors [16]. Although humans and horses develop fatal encephalitis, these two species do not exhibit high enough levels of viremia to infect mosquito vectors. Thus, humans and horses are incidental dead-end hosts [17].

Pathogenesis

Most JEV infections in humans are asymptomatic. The ratio of symptomatic to asymptomatic infections ranges from 1:25 to 1:1000 [18,19]. Symptomatic infections in humans exhibit a variety of manifestations ranging from febrile illness to meningitis, polio-like acute flaccid paralysis and encephalitis [19]. The incubation period of JEV infection is 5–15 days. Various factors, including the mosquito species, the frequency of exposure to mosquito bites, the circulating strains of JEV [20], and host factors, such as age, gender, genetic background and existing antibodies against JEV and/or other flaviviruses [18,21–24], affect the ratio of symptomatic to asymptomatic infections. Generally, the case fatality rate ranges from 5 to 40% [17]. Approximately 20–50% of the survivors have psychoneurological sequelae [25–31]. The availability of medical facilities also affects patient outcomes, with better medical environments contributing to reduced fatality rates, which, in turn, may bring about an increased percentage of sequelae in the survivors [19]. Children and young adults are generally believed to be a high-risk group, and comparative studies have indicated that these populations exhibit more severe manifestations and clinical outcomes [26,32].

Geographical distribution & epidemiology

Japanese encephalitis virus is distributed in East, South and Southeast Asia, and its distribution continues to expand, although it is not thought to extend towards the north because of the absence of mosquito vectors in northern areas. In 1995, JEV emerged in the Torres Strait Islands, which are northern islands of Australia [33], and JEV activity has been observed annually in this region – with the exception of 1999 – based on serological surveys. JEV has also been isolated from mosquitoes in the Cape York Peninsula on the Australian mainland [34], and JEV activity has been reported in Pakistan [35]. Thus, JEV is expanding to the south and west.

Approximately 3 billion people are at risk of JEV infection [36]. A surveillance system to monitor infectious diseases, including JEV, has been established, and approximately 10,000 cases have been reported via this system [36,201]. Although many cases may remain undetected, 30,000–50,000 cases have been reported worldwide annually; along with 10,000 deaths. There are two patterns of seasonal prevalence [5,36,202]. The first pattern is observed in tropical regions, such as Malaysia, Singapore and Thailand. In these regions, transmission occurs throughout the year, although a peak of cases is observed after the start of the rainy season. The second pattern is observed in temperate/subtropical regions, such as Japan, Korea and China, where the epidemic season starts in May/June and lasts until September/October. This pattern of seasonal prevalence reflects the fact that the density and activity of mosquito vectors increases when rice cultivation starts in these regions.

Based on the nucleotide sequence of the envelope (E) gene, all JEV strains are classified into five genotypes [37]. The geographical distribution of each genotype suggested that JEV originated from the Indonesia–Malaysia region, and was then transported to other regions [38,39]. Although the mechanisms of JEV migration remained uncertain, it is now thought that JEV is transported by migrating birds and wind-blown mosquito vectors [5]. Recently, the dominant genotype changed, and a new genotype emerged in several countries. In Australia, JEV strains, belonging to genotype I, have been isolated since 2000, while all previous isolates (from 1995 to 2000) had belonged to genotype II [40]. Genotype shifts from III to I have been observed in Thailand and Vietnam since the 1980s and the late 1990s, respectively [41,42]. Similarly, JEV strains belonging to genotype I have been isolated in Japan and China since the early to mid-1990s and the 1980s, respectively [43–46]. Although the mechanisms behind these genotype shifts remained unclear, it was clear that they could occur repeatedly and independently in separate regions. Thus, it is important that antiviral drugs and vaccines are developed to be effective against all genotypes.

Antivirals

There are no specific/nonspecific antiviral reagents available against JE, although a number of researchers have attempted to find/develop such drugs [47,48]. Interferon (IFN) is one of the most well-studied antiviral drugs. IFNs are produced in response to microbial infections, and induce more than 300 IFN-stimulated genes to combat infection [49]. Several of these genes encode proteins, such as Mx1, RNaseL and protein kinase R, which possess direct antiviral activities [50]. As therapeutic drugs against other viral infections, IFN-α2, IFN-β and IFN-γ have been approved by the US FDA. Importantly, IFN-β has been used as an antiviral drug against infections with hepatitis C virus, which belongs to the flavivirus family [51], suggesting that IFN may be effective against JE as well. Thus, trials using IFN to treat JEV infection have been conducted. Treatment with IFN-α effectively inhibited JEV replication *in vitro* [52,53]. However, a randomized double-blind clinical trial in Vietnam revealed that treatment with IFN-α2a did not improve the clinical outcome for JE patients compared with a placebo control [54]. However, only one dose of IFN-α2a via one route was administered in this trial and, thus, different outcomes may be achieved by either using a higher dosage, an alternative route of administration or by combination therapy using other drugs.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic purine nucleoside analogue, first reported in 1972 [55,56], with broad-spectrum antiviral activity. Ribavirin has been tested widely against both DNA and RNA viruses *in vitro* and *in vivo* [55,56]. The mechanisms behind the antiviral effects induced by ribavirin include inhibition of inosine monophosphate dehydrogenase (IMPDH), immunomodulatory effects, inhibition of viral capping, inhibition of viral polymerase activity and induction of error catastrophes [56]. For flaviviruses, ribavirin has been shown to induce antiviral effects, predominantly by depletion of the intracellular GTP pools resulting from the inhibition of IMPDH in a model of yellow fever virus (YFV) [57]. *In vitro* studies have demonstrated that ribavirin treatment is also effective against JE [53]. However, a recent randomized-controlled trial conducted in India revealed that oral intake of ribavirin did not show any efficacy against JE [58]. Thus, further evaluations using a different dosage and/or route are required to determine the effectiveness of ribavirin treatment.

A number of other antiviral compounds have been identified and evaluated against JEV infection. Minocycline, which has been used as an antibiotic drug, has been shown to possess neuroprotective activities, and minocycline treatment has been shown to inhibit West Nile virus (WNV) replication and apoptosis in neuronal cells *in vitro* [59]. More recently, it was reported that minocycline treatment reduced blood–brain barrier damage induced by JEV infection in mice [60]. Furthermore, curcumin, aloe-emodin, 2-(2-Methyl-quinoline-4ylamino)-*N*-(2-chlorophenyl)-acetamide, bovine lactoferrin, α-glucosidase inhibitors, PI-88 and dehydroepiandrosterone have been reported as effective antiviral drugs *in vitro* [61–67]. Also, a pentoxifylline, *N*-methylisatin-β-thiosemicarbazone derivative, as well as arctigenin and rosmarinic acid, have been reported as effective antiviral drugs *in vivo* [68–71].

In recent decades, advances in molecular technology and our understanding of the RNA replication and degradation machinery have led to the emergence of novel nucleic acid-based antiviral drugs. Antisense oligonucleotides and small interfering RNAs (siRNAs) have been a major antiviral strategy against numerous RNA viruses [72]. Antisense peptide nucleic acid is able to suppress viral proliferation *in vitro* [73]. In addition, phosphorodiamidate morpholino oligomers (PMOs) have been demonstrated to be effective against a broad spectrum of flaviviruses *in vitro*, and PMO treatment provided partial protection in a WNV model [74]. siRNA has been shown to be a potent therapeutic drug *in vivo* [75,76], and modification of siRNA with a neurotropic glycoprotein of rabies virus made it possible to deliver siRNA to the brain transvascularly, resulting in effective treatment [77]. This technology demonstrated that the delivery of nucleic acid-based antiviral drugs to specific organs/cells could be achieved by selecting the appropriate modifying proteins.

To be effective *in vivo*, many of the drugs described must be administered soon after, or even before, JEV infection. Therefore, alternative administration methods/routes need to be explored, along with the possibility of antiviral combination therapies.

Vaccines

Under the current situation in the development of antivirals, protection of humans from JEV infection or disease development is an important element in the effective control of JEV. Although vector control is an effective measure for reducing virus transmission between amplifier hosts, and from amplifiers to humans [5], and the fact

that swine vaccination is also effective at reducing the efficiency of JEV transmission to humans [78], it is generally accepted that vaccination of humans themselves is the most effective measure that to reduce susceptibility to JEV infection.

The introduction of a vaccine against JE for human use in Japan in 1954, and later in other endemic countries, contributed towards a reduction in the number of cases of JEV infection. Currently, Japan, Korea, Nepal, China and Thailand are conducting nationwide routine vaccine programs, and India, Nepal, Sri Lanka, Malaysia and Vietnam have a program operating in certain areas [79,203]. In Japan, Korea and Taiwan, JE has been well controlled as a result of a nationwide vaccination program [5]. JEV infection is not only a threat to people living in endemic areas; since 1945, a total of 179 cases have been reported in travelers and US soldiers [80,81]. Therefore, vaccination has been considered an effective measure to prevent JEV-related diseases in travelers as well. Several JE vaccines have been developed and used for the inhabitants of endemic countries and for travelers from non-endemic countries (Table 1), these include: mouse brain-derived formalin-inactivated vaccine, primary hamster kidney cell (PHK)-derived formalin-inactivated vaccine, live-attenuated vaccine, and Vero cell-derived formalin-inactivated vaccine. Each of these vaccines are discussed in the following sections.

Mouse brain-derived inactivated vaccine

The mouse brain-derived formalin-inactivated vaccine was developed in 1954 in Japan using the Nakayama strain [82], and was proven to protect humans from disease [83]. The purification protocols were gradually improved, resulting in a highly purified inactivated vaccine [82], which was produced by seven Japanese manufacturers. Since 1989, the Beijing-1 strain has been used for vaccine preparation instead of the Nakayama strain for domestic use [84]. For many years, this mouse brain-derived inactivated vaccine was the only internationally approved vaccine, and the technology was transferred to several countries, including Korea, Taiwan, Thailand, India, Vietnam and Russia [5,36,85,86]. In addition, Connaught Laboratories Inc. in the USA (now known as Sanofi Pasteur) distributed this vaccine for travelers [87]. Generally, two or three doses are administered to induce durable immunity in vaccinees, and a booster immunization is required every 2–3 years [85,88]. Large-scale trials showed that a two-dose vaccination regimen provided 81–95% protective efficacy [89,90], and 97–100% seroconversion rates [91,92]. In these trials, no serious adverse events were observed. Mild (e.g., redness of injection site) and moderate (e.g., fever and headache) side effects were observed in less than 6% of the participants. Systemic reactions to the JE vaccine were thought to be caused by the presence of gelatin in the vaccine, included as a stabilizer [93,94]. However, a similar incidence rate (0.6–0.7 per million doses) was observed after removal of the gelatin components from the JE vaccine [95].

PHK cell-derived inactivated vaccine

A cell culture-derived formalin-inactivated vaccine, prepared in PHK cells using the Beijing-3 (P-3) strain, was used for many years in China [85]. This PHK cell-derived inactivated vaccine was only licensed in China, and since its development in 1968, approximately 70 million doses have been produced and administrated every year. Despite the widespread use of this vaccine in China and its relatively low cost per dose compared with other vaccines [96], this vaccine has been replaced by the live-attenuated vaccine.

Live-attenuated vaccine

A live-attenuated vaccine developed using strain SA14-14-2 has been available in China since 1988 [85]. The SA14-14-2 strain was obtained by passing the parental SA14 strain 11 times through mouse brains, followed by over 100 passages through PHK cells, and plaque purifications [97]. Sequence analysis of the SA14 strain and the SA14-14-2 strain revealed that there are 45 nucleotide differences, resulting in 15 amino acid differences, between these strains [98]. Further comparative studies using other attenuated strains derived from strain SA14 indicated that residues E-138, E-176, NS2B-63, NS3–105 and NS4B-106 are the candidate amino acid substitutions responsible for its attenuation [99]. Since 1988, more than 20 million doses of this live-attenuated vaccine, approximately 50% of the global production of JE vaccines, have been administered annually. Field trials in China showed that a one- to three-dose regimen exhibited 95–100% efficacy [100]. Only mild or moderate adverse events were observed, and in less than 5% of the participants [101]. Evaluation of the long-term immunogenicity resulting from immunization with this vaccine showed that a single dose could induce long-lasting immunity (seropositive rate of 89.9% at 4 years and 63.8% at 5 years post-immunization) [102].

Since it is an inexpensive vaccine (US$0.75/

Table 1. List of vaccines against Japanese encephalitis reviewed.

Vaccine	Manufacturer(s)	Strain	Status
Mouse brain-derived formalin-inactivated vaccine	Biken, Denka Seiken, Kaketsu-ken, Chiba, Takeda (Japan), Sanofi Pasteur (USA), Green Cross (South Korea), Central Research Institute (India), National Institute of Preventive Medicine (Taiwan), Government Pharmaceutical Organization (Thailand), National Institute of Hygiene (Vietnam)	Nakayama Beijing-1	Most manufacturers have ceased production
Primary hamster kidney cell-derived formalin-inactivated vaccine	Beijing, Shanghai, Wuhan and Changchun Institute of Biological Products (China)	Beijing-P3	Replaced by live-attenuated vaccine
Live-attenuated vaccine	Chengdu Institute of Biological Products (China)	SA14-14-2	Approved in China, Nepal, India, Sri Lanka, Korea and Thailand
Vero cell-derived formalin-inactivated vaccine	Intercell (Austria), BIKEN (Japan)	SA14-14-2 Beijing-1	Intercell vaccine was approved in Europe, Australia, USA and Canada, and the BIKEN vaccine was approved in Japan

dose in Asia) compared with the BIKEN-inactivated vaccine (US$5.00/dose in Asia), and requires only a single dose to induce protective immunity, it appears to be suitable for mass vaccination programs outside of China as well. However, there are theoretical concerns about the acquisition of a back mutation, conferring virulence, since the vaccine is a live virus [103]. In the case of the YFV 17D live-attenuated vaccine, virulent YFV was isolated from a patient who had developed YF vaccine-related encephalitis [104]. Recently, the live-attenuated JE vaccine has been approved in Nepal, India, Sri Lanka, Korea and Thailand [85]. Studies conducted in Nepal and India showed that a single dose of the vaccine exhibited high efficacy (more than 95%), similar to that observed in China [105–107], and the efficacy remained high (96.2%) 5 years after the single-dose vaccination [108].

Vero cell-derived inactivated vaccine

As an alternative to the mouse brain-derived inactivated vaccine, novel vaccines using large-scale cell-culture technology were developed. In early 2009, Vero cell-derived inactivated vaccines (IC51; Ixiaro® [Intercell AG, Austria] and freeze-dried JE Vaccine [BIKEN, Japan]), were licensed. Although these two vaccines, both prepared from JEV-infected Vero cells, are similar, the IC51 vaccine contains an adjuvant.

The IC51 vaccine has been licensed in Europe, Australia, the USA and, more recently, in Canada. IC51 is a formalin-inactivated vaccine developed using the SA14-14-2 strain of JEV [86]. In preclinical trials, IC51 showed higher immunogenicity than the BIKEN mouse brain-derived inactivated vaccine in mice [109]. In clinical trials, a two-dose immunization with IC51 exhibited a seroconversion rate of 98%, while three doses of the mouse brain-derived inactivated vaccine exhibited 95% seroconversion. Consistently, IC51 caused the development of higher titers of neutralizing antibodies than the mouse brain-derived vaccine (geometric mean titers [GMT] were 1:244 and 1:102, respectively) [110]. Mild and moderate adverse events were observed in approximately 10% of the participants, and no severe or acute allergic reactions were observed [111], which was similar to the frequency of adverse events induced by the mouse brain-derived inactivated vaccine [110]. A long-term evaluation 6 months after the first immunization showed that IC51 vaccination maintained detectable neutralizing antibodies in 95% of the population, whereas the mouse brain-derived inactivated vaccine maintained neutralizing antibodies in only 74% of the population [112]. After 12 months, IC51 vaccination conferred a seropositive rate of 83% [112]. These trials indicated that the IC51 vaccine possessed equivalent or even higher immunogenicity than the mouse brain-derived inactivated vaccine.

Since the two-dose regimen could induce durable immune responses, a single-dose regimen was worth examining. Thus, a trial using a single-dose schedule was conducted. Since 6 µg of viral protein was used in the two-dose regimen, 12 µg was used for the single-dose evaluation. Although the first immunization in the two-dose regimen showed a seroconversion rate of 39.8% with a GMT of 1:11, the single-dose regimen showed a seroconversion rate of 65.8% with a GMT of 1:23, on day 28 [113]. However, the seroconversion rate reached 97.3% after the

second immunization in the two-dose regimen with a GMT of 1:218, while it decreased to 41.2% in the single-dose regimen with a GMT of 1:11, on day 56 [113]. Regardless of the higher immunogenicity of the 12-μg dose than the 6-μg dose, comparable populations showed local and systemic adverse events between these two regimens [113]. The single-dose schedule might be useful for travelers to endemic areas who do not have enough time to receive the vaccine with the two-dose schedule. Since the IC51 vaccine was initially only approved for adults, a trial to examine the IC51 vaccine in children was conducted. In this trial, 3- and 6-μg doses of the IC51 vaccine and the mouse brain-derived inactivated vaccine were compared in a two-dose regimen. Seroconversion rates of 95.7, 95.2 and 90.9% with a GMT of 1:201, 1:218 and 1:230 were shown 56 days after the first immunization, respectively [114]. No severe adverse events were observed in any of the three groups, and no significant differences in the frequency of the adverse events were detected between the three vaccination regimens [114]. Thus, the IC51 vaccine appeared to be safe and effective for children, as well as adults.

At approximately the same time that the IC51 vaccine was approved in Europe, Australia and the USA, the Freeze-dried JE vaccine, produced by BIKEN, which is also developed in Vero cells, obtained licensure in Japan. A Japanese company, Kaketsuken, also developed a Vero cell-derived vaccine independently, which exhibited similar immunological and physicochemical properties to those shown by the mouse brain-derived vaccine [115,116]. In a Phase I clinical trial of the Kaketsuken vaccine, the Vero cell-derived vaccine showed a 96.7% seroconversion rate with a GMT of 1:155, while the mouse brain-derived vaccine showed a 92.9% seroconversion rate with a GMT of 1:123, after the first immunization [117]. Following the second and third immunizations, both vaccines showed a 100% seroconversion rate. During the observation period, only mild adverse events were noted. The adverse event rates observed after the first, second and third immunization with the Vero cell-derived vaccine were 3.3, 3.3 and 6.7%, whereas those with the mouse brain-derived vaccine were 26.7, 13.8 and 10.7%, respectively [117]. Since participants of this clinical trial were 25–30-year-old adults, another trial recruiting 6–90-month-old children was conducted. In this trial, both Vero cell-derived and mouse brain-derived vaccines exhibited 100% seroconversion rates after the third immunization, and the Vero cell-derived vaccine induced neutralizing antibodies with GMTs of 1:309 and 1:9120 after the second and third immunizations, respectively [118]. Although the Vero cell-derived vaccine induced higher neutralizing antibody titers than the mouse brain-derived vaccine, the Vero cell-derived vaccine developed mild and moderate adverse events more frequently than the mouse brain-derived vaccine [118]. This vaccine is currently under review for licensure.

The initial clinical trial of the BIKEN Vero cell-derived vaccine revealed a 100% seroconversion rate following two doses of vaccination in 6–90-month-old children [119]. However, adverse events caused by the Vero cell-derived vaccine were observed at higher rates than with the mouse brain-derived vaccine [119]. Thus, a new vaccine preparation containing a smaller amount of E protein (5, 2.5 and 1.25 μg) was examined next. In this clinical trial, the seroconversion rates following the second and third immunizations with 2.5 μg of the vaccine were 99.2% with a GMT of 1:263 and 100% with a GMT of 1:5834, respectively. Mild and moderate adverse events were observed. For instance, fever was observed in 9.8, 10.7 and 4.9% of participants following the first, second and third immunizations, respectively. However, no severe adverse events were observed [120]. Although immunization with 5 μg of the vaccine could induce 100% seroconversion, which was slightly higher than with the 2.5-μg immunized group, higher adverse event rates were observed in the 5-μg immunized group than in the 2.5-μg immunized group [121,122]. Thus, the lower dose (2.5 μg) of this vaccine was approved for children aged 6–90 months. Based on the results of the ongoing clinical trials, this Vero cell-derived vaccine may also be approved for 9–13-year-old children.

These trials conducted by Intercell, Kaketsuken and BIKEN demonstrated that Vero cell-derived vaccines are an alternative to the mouse brain-derived vaccine. The Kaketsuken and BIKEN Vero cell-derived vaccine does not contain any adjuvant, unlike the IC51 vaccine that contains aluminum hydroxide. The BIKEN Vero cell-derived vaccine requires three doses to induce high, long-lasting immune responses, in contrast to the IC51 vaccine that requires two doses. In addition, the Kaketsuken and BIKEN vaccine is used for those living in endemic countries, whereas the IC51 vaccine is primarily used for travelers who do not need long-lasting immune responses.

Debate over the necessity for continued JE vaccine use in Japan

In Japan, the number of JE cases has dramatically decreased since 1967, when the use of a well-refined vaccine was introduced nationwide. Prior to that year, more than 1000 cases had been reported annually, and that number decreased to less than ten cases since 1992 [123]. Similarly, the number of JE cases has decreased in Korea to less than ten cases each year since the introduction of an immunization program in 1971 [124]. The incidence rates in Taiwan also decreased from 2.05 per 100,000 in 1967, to 0.03 per 100,000 in 1997, as a result of the introduction of a vaccination program in 1968 [125]. The successes observed in these three countries clearly show that JE is vaccine preventable.

Although no severe adverse events have been reported in the clinical trials of the mouse brain-derived inactivated vaccine [85], post-vaccination acute disseminated encephalomyelitis (ADEM) cases and severe neurological complications have been reported at incidence rates of 0.2 per 100,000 to one per 50,000 [126]. There have been at least 23 cases of ADEM after JE immunization, including probable cases from 1994 to 2007 (~55 million doses) in Japan, according to the adverse event reporting system established in 1994 [127,128]. In July 2004, a 14-year-old girl developed ADEM at 11 days after JE vaccination. Since this was a severe case, and the number of annual ADEM cases had been increasing at that time, the Japanese government (the Ministry of Health, Labour and Welfare) decided to suspend their recommendation of the mouse brain-derived vaccine for routine immunizations in 2005 [128,129,204]. At the same time, the Japanese government stated that there was no scientific evidence to demonstrate the relationship between this case of severe ADEM and JE vaccination. However, there was also insufficient scientific evidence to rule out this possibility, and the Vero cell-derived vaccine was already in the final stages of development. Following the Japanese government's decision, manufacturers anticipated a decrease in demand, and stopped producing the mouse brain-derived vaccine in 2005. The decision also met a large number of responses from other countries. In particular, the Global Advisory Committee on Vaccine Safety (WHO) did not favor this government's decision [130].

Observation of ADEM during a certain post-vaccination period has been reported for a variety of vaccines, including those against JE, rabies and influenza [126]. The substance most likely to induce ADEM is myelin-basic protein or its related proteins derived from the brain tissue [131]. However, a correlation between ADEM and JE vaccination has not been reported. A significant correlation with ADEM had been observed in the cases of a Semple rabies vaccine and a vaccinia virus that had been used as a vaccine against smallpox virus [132]. In these cases, the patients who developed ADEM post-vaccination exhibited high levels of antimyelin-basic protein antibodies [133]. Although the incidence rates of ADEM have been reduced by using neurological tissue-free duck embryo- or human diploid cell-culture-derived rabies vaccines, post-vaccination ADEM is still being reported with low incidence rates (<1/25,000) [132]. Thus, contamination of myelin-basic protein could be one of the potential causative agents of ADEM development, but it was not the sole determinant as far as the rabies vaccine was concerned.

In the case of the JE vaccine, undetectable levels of myelin-basic protein (<2 ng/ml) were detected in the BIKEN mouse brain-derived inactivated vaccine [88]. The threshold amounts of myelin that induce myelitis in guinea pigs and mice are 75 and 400 µg, respectively [134,135]. In addition, the amount of protein derived from tissues or cells in the JEV vaccine preparation is less than 40 µg/ml, as specified by the minimum requirements for biological products, which was recently changed from less than 80 µg/ml [205]. Thus, the amount of myelin contained in the BIKEN vaccine is extremely small and, therefore, unlikely to induce myelitis in humans. Owing to advances in large-scale cell-culture technology, next-generation vaccine development has shifted towards cell-culture-derived vaccines. As expected, there were no significant differences in the induction of expression of inflammation-related genes, such as TNF-α and chemokines, in rats between the Vero cell-derived vaccine and mouse brain-derived JE vaccines [136]. These results imply that both vaccines possess similar reactivity in animals.

In addition, recent surveys revealed that the incidence of ADEM in children in Japan was unaltered by the government's decision to suspend recommendation of the mouse brain-derived vaccine. Specifically, the number of ADEM cases in children (<15 years old) was 0.33 per 100,000 in 2003–2004, before the government's decision, while the frequency in 2005–2006, after the government's decision, was 0.34 per 100,000 [206]. During this period, the vaccination rate in children drastically decreased. The vaccinated population among 3-year-old children was over

80% before 2004 (over 4,000,000 doses), while it decreased to 20% in 2005, and 4% in 2006. If the mouse brain-derived vaccine was the source of ADEM, the frequency of ADEM should have declined as the vaccination rate declined. These surveys, therefore, indicated that the mouse brain-derived vaccine did not correlate with the reported ADEM cases in Japan.

The facts that the number of mosquito vectors and the incidence of JE is declining, and that there is an increasing risk of post-vaccination events, lead us to question the necessity for routine JE vaccination. To address this issue, it is essential to understand the current activity of JEV in Japan. Serological surveys of swine have been conducted as part of a national project to monitor JEV activity in each prefecture. Seroconversion of sentinel swine has been observed in most of the southern and western areas of Japan [123]. High seroconversion rates in swine have also been observed in Taiwan [137]. These surveys indicated that JEV activity still exists in areas where JE-related diseases have been well controlled. Next, it is important to consider the natural infection rate of humans. Natural infection rates in Japan have been evaluated by detecting anti-nonstructural protein (NS)1 antibodies, since individuals who have received the inactivated vaccine fail to develop anti-NS1 immune responses [138]. Surveys utilizing this method revealed that the natural infection rates around the Kobe area (west–central Japan) in the 1980s ranged from 5 to 10% [139]. Similarly, the surveys conducted in other areas in 2001–2004 showed natural infection rates of approximately 2% [140,141]. A more recent survey conducted in 2004–2008 revealed that the average annual infection rates in western and eastern Japan were 1.8 and 1.3%, respectively [142]. In this study, conventional neutralization tests were performed using sera from unvaccinated populations, and a similar prevalence rate of 2.6% was recorded [142]. These data clearly demonstrated that JEV still exists in Japan, and that humans are still at risk of exposure.

The natural infection rates in horses, another incidental dead-end host, have also been tested in Japan. Relatively high natural infection rates (>15%) were observed in several areas [143,144], higher than the rate in humans. This, possibly, reflects the fact that humans live in tightly closed, well-screened houses, while horses are kept in stables under open-air/semi-open-air environments, with greater potential exposure to mosquitoes. Furthermore, the mosquito vector *Cx. tritaeniorhynchus* prefers outdoor environments to rest and feed [145]. These data support the higher natural infection rates in horses compared with humans. For mosquitoes, body temperature, carbon dioxide and odors are key factors in locating potential hosts [146]. The body temperature of horses (37.5–38.5°C) is slightly higher than that of humans, and mosquitoes recognize and prefer specific odors. For example, an anthropophilic mosquito, *Aedes aegypti*, has been known to recognize lactic acid that is produced on human skin, but not on the skin of other mammals [147]. Thus, horses might produce unidentified substances, not produced by humans, which attract *Cx. tritaeniorhynchus*. Such factors may affect the differences in the natural infection rates observed between humans and horses. Nevertheless, this high infection rate in horses supports the natural JEV activity observed in Japan, as demonstrated in swine and humans.

In the past few decades, swine farms have been relocated away from human residential areas. Taking this separation into consideration, the natural infection rate of approximately 2% in humans might be considered high, and suggests that JEV has acquired a new lifecycle that is specific to and maintainable in urban areas. To support this hypothesis, antibodies against JEV have been detected in a variety of mammalian animals, including wild boars, raccoons, raccoon dogs, dogs and bats, all of which live near human residential areas. Recent surveys carried out in Japan showed that 52–86% of wild boars were seropositive [10–12,148]. A more recent survey revealed 66.7% seropositivity in wild boars captured in a small island where there were no swine [149], and JEV has been isolated from a wild boar captured nearby human residential areas [150], suggesting that wild boars could act as an amplifier. JEV has also been isolated from bats [151]. More recently, it was reported that JEV can be transmitted by mosquitoes from bats, which did not exhibit detectable viremia [152]. This finding is important, as it indicates that animals in which JEV has not been isolated could be potential amplifiers. Such animals could be candidate JEV amplifiers in the JEV urban lifecycle.

As described, several animals could play a potential role as an amplifier in an urban lifecycle. However, new mosquito vectors must be involved in this urban lifecycle, since the major mosquito vector species, *Cx. tritaeniorhynchus*, does not generally exist in urban areas. *Aedes albopictus* and *Culex pipiens pallens* have been known to inhabit and breed in urban areas [153–155], and *Culex pipiens molestus* is also known to breed in underground environments, such

as subway tunnels and the basements of houses [156]. In fact, a recent survey conducted in Japan revealed that a larger number of *Ae. albopictus* were caught in urban residential areas than *Cx. tritaeniorhynchus* [207]. These mosquito species have been experimentally confirmed to be capable of transmitting JEV [157,158]. Furthermore, JEV has been isolated from Ae. *albopictus* in nature [159]. Thus, these mosquitoes may play a role as a vector in the urban lifecycle of JEV.

A possible explanation for the dramatic decrease in the number of JE cases observed in Japan, Korea and Taiwan is attenuation of the currently circulating strains of JEV. In fact, the JEV genome has been isolated from meningitis patients in Japan [160], suggesting that there may be more potential cases that have gone unreported owing to their nonencephalitis manifestations. As described previously, the predominant genotype of recent isolates has changed from genotype III to genotype I in Japan [43,45]. If the genotype III viruses were more pathogenic than the genotype I viruses, this could account for the drastic decrease in the number of JE cases. However, there is currently no evidence to suggest pathogenic differences between genotypes [38]. Furthermore, the pathogenicity of JEV varied even among genotype I viruses [161]. Thus, the genotype shift is not likely to be the reason for the dramatic decrease in the number of JE cases in these countries. However, virus strains examined in these studies were mostly isolated from swine and mosquitoes. As suggested, JEV may acquire a new urban lifecycle, utilizing different amplifiers and vector mosquitoes. Thus, it could be speculated that JEV maintained in the new lifecycle is attenuated. This possibility needs to be investigated.

In conclusion, JEV is still circulating in Japan, based on the serological surveys of humans and animals, despite the recommendation for routine vaccination being suspended by the government, and mouse brain-derived vaccine production being stopped in 2005. During the past 5 years, the susceptible population to JEV has increased in Japan, especially among school-aged children (Figure 1). In 1950 (before vaccination was started), approximately 900 cases in 0–4-year-old infants were reported (prevalence 9 per 100,000) [208]. Although environmental and virological factors were changed, vaccine effectiveness has been shown by no reported cases in infants between 1990 and 2006. However, two infant JE cases who did not receive JE vaccination (3 and 1 years old) were reported in 2006 and 2009, respectively [209]. Finally, in 2009, a new JE vaccine derived from Vero cell cultures was approved for use in Japan. Then, in April 2010, the Japanese government (the Ministry of Health, Labour and Welfare) restarted their recommendation for routine immunization of a limited population with the new Vero cell-derived vaccine [209].

Future perspective

Japanese encephalitis disease, resulting from JEV infection, is characterized by a high mortality rate and a high incidence of neurological sequelae in survivors. Thus, JEV infection is a serious public health threat in endemic countries, and for those traveling to endemic countries. There are currently no approved specific antiviral drugs against JE. Since approval of the mouse brain-derived inactivated vaccine worldwide, and the live-attenuated vaccine in a few countries, JE vaccination has been proven to be a successful strategy in preventing this disease.

Despite many antiviral drug candidates proving effective *in vitro*, several clinical trials have failed to demonstrate their efficacy in humans. Many candidate antiviral drugs, including IFN and ribavirin, are effective against a broad spectrum of viruses. However, the mechanisms behind these antiviral effects remain unclear.

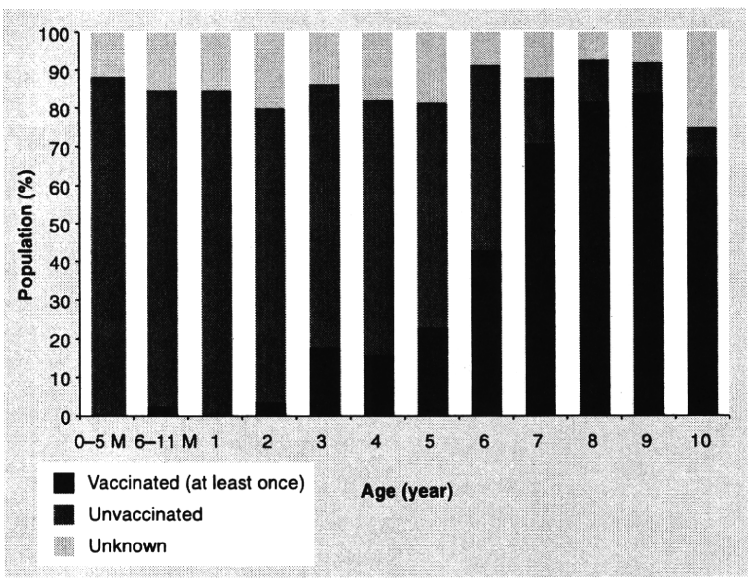

Figure 1. Accumulation of Japanese encephalitis (JE)-susceptible populations in Japan. The percentage of the population who received the JE vaccine at least once is shown in red, the percentage that did not receive the vaccine is shown in blue and the percentage whose vaccine history was unknown is shown in gray, for each age group.
Data were modified from the reports available at [210], which represent part of the national surveillance on JE virus activity carried out in 2008.

Recent advances in our understanding of viral replication in host cells, and the mechanisms by which viruses evade the host immune system, will help in the development of new types of antiviral drugs that may be designed to specifically target JEV. Moreover, it may be possible to develop novel technologies to deliver antivirals to specific tissues/cells where JEV replicates.

A new type of inactivated vaccine derived from cell culture has been developed, which is expected to be safer than the mouse brain-derived inactivated vaccine in terms of the associated neurological adverse events. However, a few cases of ADEM following vaccination with the new Vero cell-derived vaccine have already been reported. In Japan, approximately 500,000 doses of the Vero cell-derived vaccine were administered in 2009. Among these, 18 cases of adverse events, including a single meningitis case, were reported [209]. As mentioned, a survey conducted in Japan revealed that vaccination with mouse brain-derived vaccine was not related to the occurrence of ADEM. Since ADEM is induced independently from JE vaccination, severe ADEM might develop coincidently shortly after JE vaccination. This may also be the case with the new Vero cell-derived vaccine. Therefore, vaccinees should be aware of this issue, and the government should not repeat the decision to stop strongly recommending JE vaccination based on the possible occurrence of post-vaccination ADEM cases.

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Executive summary

Characteristics of Japanese encephalitis virus (JEV) infection

- High mortality rate (up to 40%).
- High neurological sequelae rate in survivors (up to 50%).
- Children and young adults represent a high-risk group.
- No specific antiviral drugs exist.

Current status of JEV infection

- JEV distributes throughout Southeast Asia and parts of Australia.
- It is estimated that there are 30,000–50,000 cases worldwide, with 10,000 deaths annually.

Vaccines to prevent JE

- Mouse brain-derived formalin-inactivated vaccine has been extensively used worldwide; however, its production ceased in 2005.
- Live-attenuated vaccine is mainly used in China.
- Cell culture-derived formalin-inactivated vaccine has been available in Europe, the USA, Australia, Canada and Japan since 2009.

Recent issues concerning vaccines in Japan

- The Japanese government (Ministry of Health, Labour and Welfare) stopped recommending the use of the mouse brain-derived vaccine for routine vaccination between 2005 and 2010.
- The Japanese government, in 2010, reinstated the strongly recommending the Vero cell-derived vaccine for routine vaccination.
- The frequency of acute disseminated encephalomyelitis (ADEM) in children remained unchanged before and after the recommendation was stopped by the government.

Necessity for vaccination in Japan

- Less than ten cases per year have been reported since the 1990s.
- Serological surveys of human and horses showed that JEV still circulates in Japan.
- There is no evidence that the currently circulating virus strains are attenuated.
- Vaccination is still an effective method to prevent JEV-related diseases in Japan.

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Short Communication

Complete genome constellation of a caprine group A rotavirus strain reveals common evolution with ruminant and human rotavirus strains

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This study reports the first complete genome sequence of a caprine group A rotavirus (GAR) strain, GO34. The VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of strain GO34, detected in Bangladesh, were assigned to the G6-P[1]-I2-R2-C2-M2-A11-N2-T6-E2-H3 genotypes, respectively. Strain GO34 was closely related to the VP4, VP6–7 and NSP4–5 genes of bovine GARs and the NSP1 gene of GO34 to an ovine GAR. Strain GO34 shared low nucleotide sequence identities (<90 %) with VP2–3 genes of other GARs, and was equally related to NSP3 genes of human, ruminant and camelid strains. The VP1, VP6 and NSP2 genes of strain GO34 also exhibited a close genetic relatedness to human G2, G6, G8 and G12 DS-1-like GARs, whereas the NSP1 of GO34 was also closely related to human G6P[14] strains. All these findings point to a common evolutionary origin of GO34 and bovine, ovine, antelope, guanaco and human G6P[14] GARs, although phylogenetically GO34 is not particularly closely related to any other rotavirus strains known to date.

Group A rotaviruses (GARs) are a major cause of acute viral gastroenteritis in the young of humans and animals (Estes & Kapikian, 2007). The GAR genome consists of 11 segments of double-stranded RNA, encoding six structural and six non-structural proteins (Estes & Kapikian, 2007). Recently, the 11 GAR gene segments (VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4 and NSP5 genes) have been classified into at least six R, six C, seven M, 31 P, 13 I, 23 G, 16 A, six N, eight T, 12 E and eight H genotypes, respectively, based on specific nucleotide sequence identity cut-off percentages for each gene segment (Matthijnssens *et al.*, 2008a, b, 2009, 2010a; Schumann *et al.*, 2009; Solberg *et al.*, 2009; Trojnar *et al.*, 2009; Ursu *et al.*, 2009). Applying this classification scheme, the full genomes of GAR strains from antelope, birds, cattle, cats, dogs, guanacos, humans, monkeys, pigs, rabbits and sheep were successfully analysed, providing vital insights into the complex genetic diversity of GARs (Ghosh *et al.*, 2010; Heiman *et al.*, 2008; Matthijnssens *et al.*, 2008a, b, 2009, 2010a, b; Schumann *et al.*, 2009; Trojnar *et al.*, 2009; Tsugawa & Hoshino, 2008).

GARs have been associated with diarrhoea in goats from different parts of the world (Kaminjolo & Adesiyun, 1994; Lee *et al.*, 2003; Mendes *et al.*, 1994; Muñoz *et al.*, 1996; Pratelli *et al.*, 1999; Takahashi *et al.*, 1979; Scott *et al.*, 1978). Moreover, in rural areas, caprine GARs might pose a threat to humans living in close proximity to livestock. However, to date, few caprine GAR strains have been molecularly characterized. Among them, the VP7, VP4 and NSP4 gene sequences of a Korean caprine strain, GRV, were assigned to G3, P[3] and E3 genotypes, respectively, and this strain was believed to be derived from reassortment events and/or interspecies transmission of canine, feline and/or simian GARs (Lee *et al.*, 2003). The full-length VP7 and partial VP4 gene sequences (GenBank accession nos AY128708 and AY128709, respectively) of a South African GAR strain, Cap455, exhibited maximum genetic relatedness to those of human G6P[14] strains. In addition, by RT-PCR-based genotyping assays, the VP7 and VP4 genes of two caprine strains from Italy were assigned to G6 and P[1] genotypes, respectively (Pratelli *et al.*, 1999). Therefore, our present knowledge of the

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the VP1–4, VP6–7 and NSP1–5 genes of caprine strain GO34, the VP7, VP4, VP6 and NSP4–5 genes of caprine strains GO100 and GO102, and the NSP1 and NSP5 genes of bovine strain NCDV are GU937877–GU937887, GU937888–GU937891, HM015929–HM015934, GU808570 and GU937876, respectively.

A supplementary table showing sequences of the primers used in this study is available with the online version of this paper.

caprine GAR genome is limited to only three of the 11 gene segments. Full genomic analyses of GAR strains from different host species are essential to obtain conclusive data on (i) the true origin of a strain and its evolutionary relationship to other GARs; (ii) complex gene reassortment events involving strains from different host species; and (iii) interspecies transmission of GARs (Matthijnssens *et al.*, 2008a, b). In the present study, we report for the first time the complete genome sequence of a caprine GAR strain, GO34.

Between June and October 1999, 259 faecal samples were collected from goat kids (aged <3 months) with diarrhoea from villages in the district of Mymensingh, Bangladesh. The caprine faecal samples were screened for GARs by RNA electrophoresis in polyacrylamide gels as described by Herring *et al.* (1982). In total, eight samples were positive for GARs, and of these, three samples (designated GO34, GO100 and GO102) were available in sufficient quantities for further work. Caprine GAR strains GO34, GO100 and GO102 were successfully propagated in MA104 cells as described previously (Wang *et al.*, 2007) and stored at −80 °C until further use. For RT-PCR assays, viral RNA was extracted from the cell culture fluid using a QIAamp Viral RNA Mini kit (Qiagen). Multiplex PCR-based genotyping of VP4 and VP7 genes was carried out using genotype-specific primers reported previously (Das *et al.*, 2004; Ghosh *et al.*, 2006; Isegawa *et al.*, 1993; Paul *et al.*, 2008). Full-length VP1, VP2, VP7, NSP2 and NSP3 genes and partial-length VP3, VP4 and NSP1 genes were

Table 1. Genotype nature of the 11 gene segments of caprine GAR strain GO34 sequenced in this study with those of selected human and animal GAR strains with known genomic constellations

Bold indicates the gene segments with a genotype identical to that of strain GO34, whilst italic indicates the genome segments with a different genotype. An, antelope; Bo, bovine; Cap, caprine; Gu, guanaco; Hu, human; Ov, ovine. A '–' indicates that no sequence data or only a short stretch of sequence was available in GenBank, and therefore a genotype could not be assigned.

Strain/host	Genotype										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
GO34/Cap	**G6**	**P[1]**	**I2**	**R2**	**C2**	**M2**	**A11**	**N2**	**T6**	**E2**	**H3**
GRV/Cap	*G3*	*P[3]*	–	–	–	–	–	–	–	*E3*	–
Cap455/Cap	**G6**	*P[14]*	–	–	–	–	–	–	–	–	–
OVR762/Ov	*G8*	*P[14]*	**I2**	**R2**	**C2**	**M2**	**A11**	**N2**	**T6**	**E2**	**H3**
Lamb-NT/Ov	*G10*	*P[15]*	*I10*	**R2**	**C2**	**M2**	**A11**	**N2**	**T6**	**E2**	**H3**
NCDV/Bo	**G6**	**P[1]**	**I2**	**R2**	**C2**	**M2**	*A3**	**N2**	**T6**	**E2**	**H3***
UK/Bo	**G6**	*P[5]*	**I2**	**R2**	**C2**	**M2**	*A3*	**N2**	*T7*	**E2**	**H3**
WC3/Bo	**G6**	*P[5]*	**I2**	**R2**	**C2**	**M2**	*A3*	**N2**	**T6**	**E2**	**H3**
RUBV319/Bo	**G6**	*P[11]*	**I2**	–	–	–	–	–	–	**E2**	**H3**
RUBV3/Bo	*G3*	*P[3]*	**I2**	–	–	–	–	–	–	**E2**	**H3**
RC-18/08/An	**G6**	*P[14]*	**I2**	**R2**	**C2**	**M2**	**A11**	**N2**	**T6**	**E2**	**H3**
Arg/chubut/99/Gu	*G8*	*P[14]*	**I2**	*R5*	**C2**	**M2**	*A3*	**N2**	**T6**	*E12*	**H3**
IS2/Hu	*G2*	–	**I2**	–	–	–	–	**N2**	*T2*	–	*H2*
NR1/Hu	*G2*	*P[4]*	**I2**	–	–	–	*A2*	**N2**	*T2*	**E2**	*H2*
B1711/Hu	**G6**	*P[6]*	**I2**	**R2**	**C2**	**M2**	*A2*	**N2**	*T2*	**E2**	*H2*
PA169/Hu	**G6**	*P[14]*	**I2**	**R2**	**C2**	**M2**	*A3*	**N2**	**T6**	**E2**	**H3**
Hun5/Hu	**G6**	*P[14]*	**I2**	**R2**	**C2**	**M2**	**A11**	**N2**	**T6**	**E2**	**H3**
111/05-27/Hu	**G6**	*P[14]*	**I2**	**R2**	**C2**	**M2**	*A3*	**N2**	**T6**	**E2**	**H3**
MG6/Hu	**G6**	*P[14]*	**I2**	**R2**	**C2**	**M2**	**A11**	**N2**	**T6**	**E2**	**H3**
B10925-97/Hu	**G6**	*P[14]*	**I2**	**R2**	**C2**	**M2**	*A3*	**N2**	**T6**	**E2**	**H3**
BP1879/03/Hu	**G6**	*P[14]*	**I2**	**R2**	**C2**	**M2**	**A11**	**N2**	**T6**	**E2**	**H3**
MP409/Hu	*G8*	**P[1]**	–	–	–	–	**A11**	–	**T6**	**E2**	–
DRC86/Hu	*G8*	*P[6]*	**I2**	**R2**	**C2**	**M2**	*A2*	**N2**	*T2*	**E2**	*H2*
DRC88/Hu	*G8*	*P[8]*	**I2**	**R2**	**C2**	**M2**	*A2*	**N2**	*T2*	**E2**	*H2*
L26/Hu	*G12*	*P[4]*	**I2**	**R2**	**C2**	*M1*/**M2**†	*A2*	*N1*	*T2*	**E2**	*H1*
RV176-00/Hu	*G12*	*P[6]*	**I2**	**R2**	**C2**	**M2**	*A2*	**N2**	*T2*	*E6*	*H2*
RV161–00/Hu	*G12*	*P[6]*	**I2**	**R2**	**C2**	**M2**	*A2*	**N2**	*T2*	*E1*	*H2*
N26-02/Hu	*G12*	*P[6]*	**I2**	**R2**	**C2**	**M2**	*A2*	*N1*	*T2*	*E6*	*H2*

*The NSP1 and NSP5 genes of strain NCDV were sequenced in the present study.

†Two different nucleotide sequences with GenBank accession numbers EF583035 and AY277918 were available for the VP3 gene of strain L26.

amplified using primers described previously (Gentsch *et al.*, 1992; Ghosh *et al.*, 2010; Taniguchi *et al.*, 1992). Additional primers required for amplification of full-length VP3, VP4, VP6, NSP1, NSP4 and NSP5 genes were designed from conserved stretches of cognate genes of several published GAR strains (see Supplementary Table S1, available in JGV Online). Nucleotide sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing Reaction kit (Applied Biosystems) on an automated sequencer (ABI PRISM 3100; Applied Biosystems). Sequence comparisons and phylogenetic analyses were carried out as described previously (Ghosh *et al.*, 2010).

Caprine GAR strains GO34, GO100 and GO102 exhibited identical RNA migration patterns, as revealed by electrophoresis in polyacrylamide gels. By PCR-based G- and P-genotyping assays and sequence analysis, all three strains were assigned to G6P[1] specificities. The full-length VP7 genes and partial-length VP4 (nt 12–794), VP6 (nt 248–868), NSP4 (nt 121–663) and NSP5 (nt 101–548) genes of strains GO34, GO100 and GO102 exhibited absolute to nearly absolute nucleotide sequence identities (99.7–99.9 % for VP7 and 100 % for the other genes) among themselves. Therefore, in the present study, only one caprine strain (GO34) was sequenced for the full genome. In addition, the NSP1 and NSP5 genes of prototype bovine GAR G6P[1]

Table 2. Nucleotide sequence identities (%) of the VP1–4, VP6–7 and NSP1–5 genes of GAR strain GO34 compared with those of antelope, bovine, guanaco, human, ovine and other caprine GAR strains

Reference strains Wa, DS-1 and AU-1 representing the three major GAR genogroups were also included in the analysis. An, antelope; Bo, bovine; Cap, caprine; Gu, guanaco; Hu, human, Ov; ovine. A '–' indicates that no sequence data were available in GenBank.

Strain/host/G-P combination	Nucleotide sequence identity (%)										
	VP1	VP2	VP3	VP4	VP6	VP7	NSP1	NSP2	NSP3	NSP4	NSP5
GRV/Cap/G3P[3]	–	–	–	76.5	–	77.5		–	–	78.3	–
Cap455/Cap/G6P[14]	–	–	–	66.2	–	86.9	–	–	–	–	–
OVR762/Ov/G8P[14]	86.8	87.1	88.5	68.0	92.7	76.7	94.2	88.4	91.0	91.3	94.5
Lamb-NT/Ov/G10P[15]	89.2	88.9	86.6	74.4	85.5	76.9	86.4	91.1	92.1	88.5	95.2
NCDV/Bo/G6P[1]	85.7	87.3	83.5	80.5	89.8	84.4	74.4	88.2	92.8	89.6	94.8
UK/Bo/G6P[5]	86.0	86.3	83.4	71.0	93.7	85.4	75.3	89.6	84.7	93.5	93.1
WC3/Bo/G6P[5]	85.9	86.6	82.7	70.0	93.6	84.3	74.2	88.5	92.9	84.2	94.9
RUBV319/Bo/G6P[11]	–	–	–	54.7	96.1	91.2	–	–	–	97.1	97.5
RUBV3/Bo/G3P[3]	–	–	–	74.4	96.1	77.5	–	–	–	95.4	97.3
RC-18/08/An/G6P[14]	85.6	87.6	83.6	68.7	93.4	87.1	89.1	88.8	90.8	92.8	94.6
Arg/chubut/99/Gu/G8P[14]	81.9	86.8	83.9	68.8	92.6	76.8	73.7	87.4	92.6	89.6	95.2
IS2/Hu/G2P[?]	–	–	–	–	95.6	75.0	–	95.0	78.8	–*	84.0
NR1/Hu/G2P[4]	–	–	–	69.7	95.3	–	66.4	94.8	77.0	91.3	79.2
B1711/Hu/G6P[6]	91.7	85.7	83.3	70.3	95.4	82.6	67.3	94.7	79.2	92.0	70.3
PA169/Hu/G6P[14]	86.0	85.6	83.4	68.3	93.4	87.2	74.4	89.1	93.1	91.5	94.3
Hun5/Hu/G6P[14]	87.3	87.4	89.9	68.3	88.1	85.8	88.9	88.1	93.4	91.1	95.2
111/05-27/Hu/G6P[14]	86.6	87.4	89.3	68.5	94.2	86.6	75.2	88.6	91.1	91.5	94.9
MG6/Hu/G6P[14]	85.8	87.2	84.2	68.4	92.9	87.2	89.9	87.3	92.1	90.7	94.2
B10925-97/Hu/G6P[14]	86.9	87.3	89.3	68.6	93.7	87.6	75.2	88.7	91.1	91.1	95.2
BP1879/03/Hu/G6P[14]	87.5	87.0	83.6	68.6	93.4	87.9	88.7	88.7	93.5	93.6	94.9
MP409/Hu/G8P[1]	–	–	–	96.6	–	77.0	95.1	–	91.9	94.1	–
DRC86/Hu/G8P[6]	95.9	85.7	88.0	70.3	95.0	77.1	66.7	94.8	79.6	92.7	70.5
DRC88/Hu/G8P[8]	95.8	85.6	88.0	70.4	95.0	77.0	66.7	94.7	79.5	92.3	70.5
L26/Hu/G12P[4]	87.5	85.7	88.9/76.7†	69.8	87.2	75.9	67.0	83.5	80.5	94.6	87.4
RV176-00/Hu/G12P[6]	96.4	85.7	88.1	70.4	95.4	76.3	67.2	94.6	79.6	82.4	70.3
RV161-00/Hu/G12P[6]	96.4	85.8	88.1	70.3	95.4	76.3	67.3	94.7	79.5	83.5	70.5
N26-02/Hu/G12P[6]	96.4	85.7	87.9	70.6	95.0	76.4	67.3	83.2	79.3	82.7	69.9
Wa/Hu/G1P[8]	80.2	79.3	76.4	70.5	79.4	76.5	68.1	81.8	82.9	83.2	86.1
DS-1/Hu/G2P[4]	90.8	85.7	89.7	70.7	87.8	79.8	66.8	87.2	78.8	91.4	84.7
AU-1/Hu/G3P[9]	81.1	80.8	76.7	68.9	80.7	79.4	73.1	80.3	79.6	82.8	95.7

*Only a partial nucleotide sequence (nt 258–566) for the NSP4 gene of strain IS2 (GenBank accession no. FJ487578) was available in GenBank and therefore this was not included in the analysis.

†Two different nucleotide sequences with GenBank accession numbers EF583035 and AY277918 were available for the VP3 gene of strain L26.

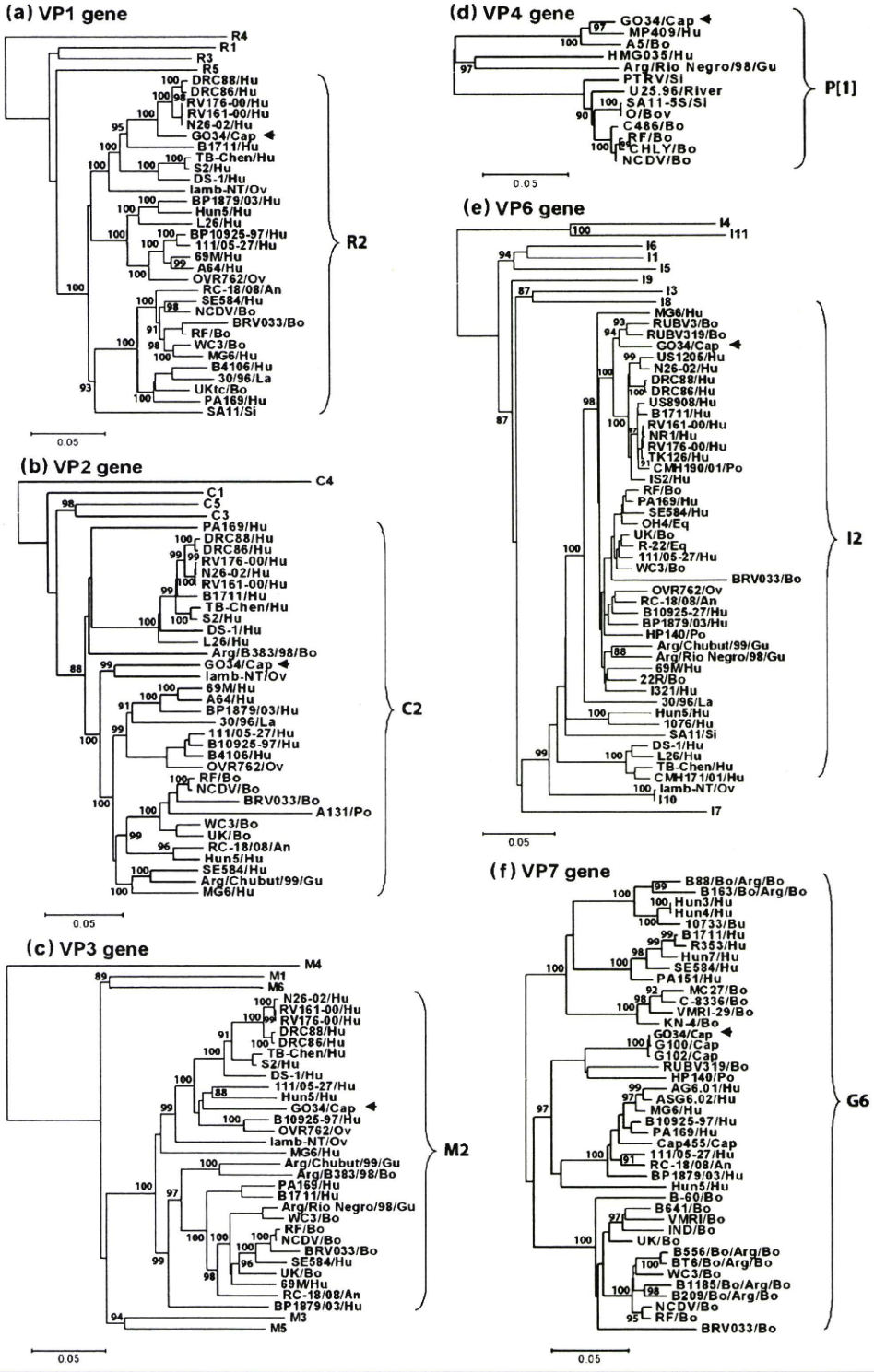
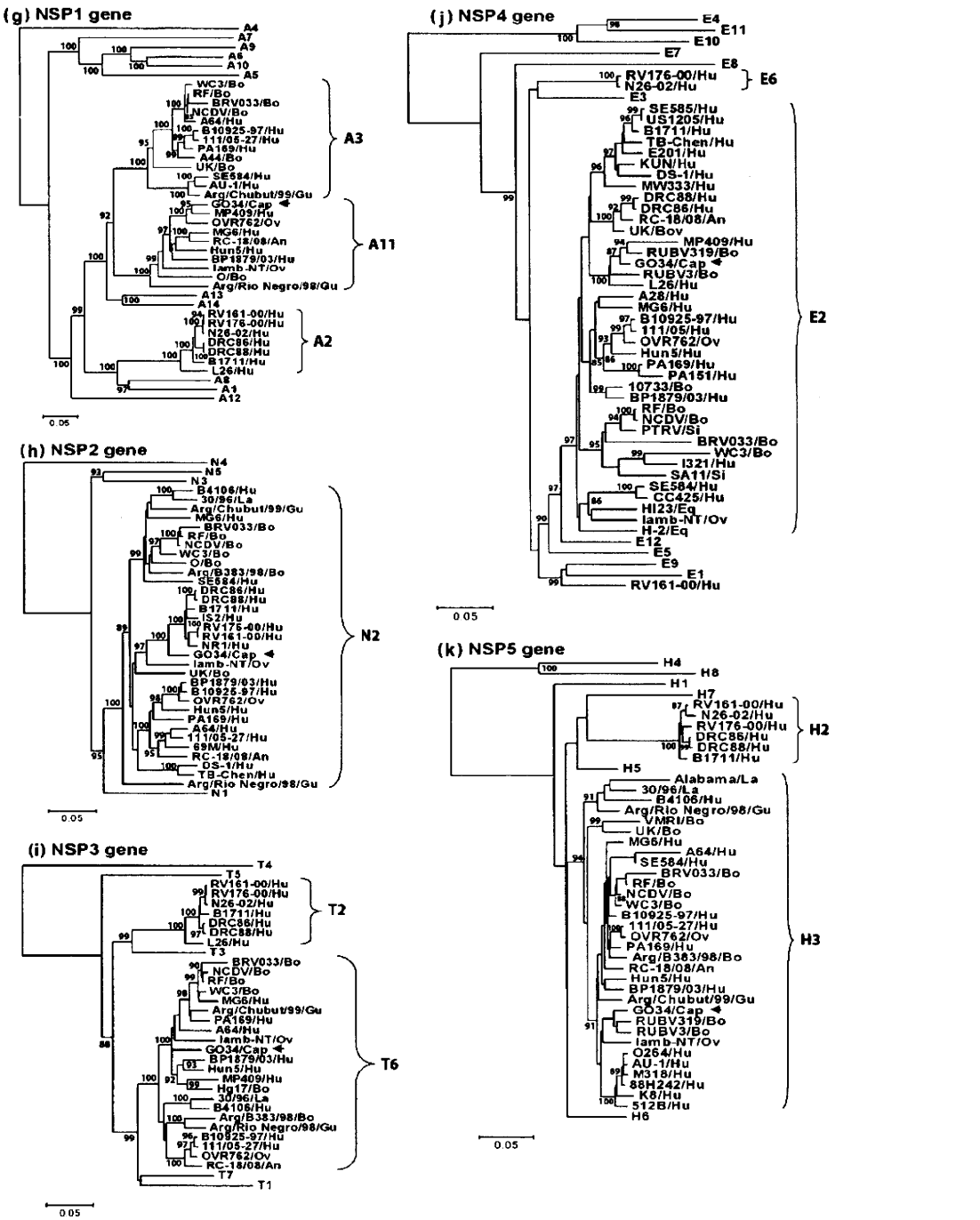


Fig. 1. Phylogenetic trees constructed from the nucleotide sequences of VP1 (a), VP2 (b), VP3 (c), VP4 (d), VP6 (e), VP7 (f), NSP1 (g), NSP2 (h), NSP3 (i), NSP4 (j) and NSP5 (k) genes of caprine GAR strain GO34 with those of GAR strains representing five R, five C, six M, P[1], 11 I, G6, 14 A, five N, seven T, 11 E and eight H genotypes, respectively. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using MEGA software (version 4.1). Phylogenetic distances were measured by the Kimura two-parameter model and the trees were statistically supported by bootstrapping with 1000 replicates. In all trees, the position of strain GO34 is indicated by an arrow. Bootstrap values $\geqslant$85 % are shown. Bar, 0.05 substitutions per nucleotide. An, Antelope; Bo, bovine; Bu, buffalo; Cap, caprine; Eq, equine; Gu, guanaco; Hu, human; La, lapine; Ov, ovine; Po, porcine; Si, simian.



strain NCDV were sequenced, as, to our knowledge, information on these gene sequences is not available in GenBank.

The full genome of caprine GAR strain GO34 was 18 503 bp in size. By nucleotide sequence identities and phylogenetic analyses, the full-length VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of strain GO34 were assigned to the G6-P[1]-I2-R2-C2-M2-A11-N2-T6-E2-H3 genotypes, respectively (Tables 1 and 2; Fig. 1a–k). In a previous study, by comparative analysis of the full genomes of GARs from antelope, cattle, guanacos and sheep, Matthijnssens *et al.* (2009) suggested that the overall genotype constellation of GAR strains circulating among ruminants and camelids might be conserved. Detailed analysis of the first complete caprine GAR genome of strain

GO34 corroborated this observation. The overall genotype constellation of GO34 was similar to that of ovine, camelid and bovine strains. Moreover, within their respective genotypes, caprine strain GO34 was closely related to (i) the VP4 gene of G8P[1] bovine strain A5 from Thailand (nucleotide sequence identity of 95.6 %); (ii) the VP6, VP7, NSP4 and NSP5 genes of bovine G6P[11] strain RUBV319, and the VP6, NSP4 and NSP5 genes of bovine G3P[3] strain RUBV3 from eastern India; and (iii) the NSP1 gene of ovine G8P[14] strain OVR762 from Spain (Table 2; Fig. 1d–g, j–k). On the other hand, the VP2 and VP3 genes of GO34 exhibited low nucleotide sequence identities (<90 %) compared with those of GAR strains from other host species (Table 2). However, by phylogenetic analysis, the caprine VP2 gene clustered near ovine strain lamb-NT (Fig. 1b), whilst its VP3 gene clustered near human G6P[14] strains 111/05-27 and Hun5 (Fig. 1c). The NSP3 gene of caprine strain GO34 appeared to be equally related to a number of other GAR strains isolated from humans, ruminants and camelids (Table 2; Fig. 1i). The NSP1 and NSP5 genes of bovine GAR strain NCDV, sequenced in this study, exhibited maximum nucleotide sequence identities of 99.6 and 99 % with those of bovine GAR strain RF, respectively, and by phylogenetic analysis clustered with bovine GAR strains within genotypes A3 and H3, respectively (Fig. 1g, k).

Full genomic analysis of strain GO34 revealed genetic relatedness in different genes between the caprine and several human GAR strains. By phylogenetic analysis, the NSP1 genes of GO34 and ovine strain OVR762 clustered close to those of human G6P[14] strains (Fig. 1g). Close genetic relationships were observed in the VP4, NSP1 and NSP4 genes between strains GO34 and MP409, a human G8P[1] strain from southern India believed to have a ruminant origin (Rao *et al.*, 2003) (Table 2; Fig. 1d, g, j). The NSP4 gene of human G12 strain L26, detected from the Philippines (Pongsuwanna *et al.*, 2002), was closely related to those of caprine strain GO34 and bovine strains RUBV3 and RUBV319 (Table 2; Fig. 1j), pointing towards its origin from a ruminant GAR, possibly through one or multiple reassortment events. The VP1 gene of caprine strain GO34 exhibited maximum nucleotide sequence identities with those of human G12 strains RV161-00, RV176-00 and N26-02 from Bangladesh (Rahman *et al.*, 2007), followed by human G8 strains DRC86 and DRC88 from the Democratic Republic of Congo (Matthijnssens *et al.*, 2006), and G6P[6] strain B1711 from Belgium (Matthijnssens *et al.*, 2008c) (Table 2), and by phylogenetic analysis, clustered close to strain B1711 and the cluster comprising strains RV161-00, RV176-00, N26-02, DRC86 and DRC88 (Fig. 1a). Similarly, the NSP2 nucleotide sequence identities of GO34 with strains RV161-00, RV176-00, DRC86, DRC88 and B1711, and human G2 strains IS2 and NR1 from eastern India were higher than those observed with other GARs (Table 2), and by phylogenetic analysis, the caprine NSP2 gene clustered close to the cluster consisting of these human strains (Fig. 1h). Although the VP6 gene of caprine strain GO34 exhibited maximum nucleotide sequence identities of 96.1 % with those of bovine strains RUBV3 and RUBV319, nucleotide sequence identities of 95.0–95.6 % were also observed with human G2 strains IS2 and NR1, G6P[6] strain B1711, G8 strains DRC86 and DRC88 and G12 strains RV161-00, RV176-00 and N26-02 (Table 2), and by phylogenetic analysis, the VP6 genes of GO34 and bovine RUBV strains clustered close to the cluster formed by these human strains (Fig. 1e). Taken together, these observations corroborated the hypothesis that DS-1-like human and ruminant GARs are genetically rather closely related and might have a common ancestor in a distant past (Matthijnssens *et al.*, 2008a).

In conclusion, full genomic analysis of GAR strain GO34 has provided important insights into the complete genetic makeup of a caprine GAR strain and its genetic relatedness to GARs from other host species. Moreover, evidence was obtained in support of the hypothesis of a common origin of DS-1-like human and ruminant GARs (Matthijnssens *et al.*, 2008a). Therefore, the present study reasserts the significance of full genomic analyses of GAR strains from different host species. Considering the complex nature of the GO34 genome, full genomic analyses of several GAR strains from goats in different parts of the world might be required to understand properly the genomic nature and genetic diversity of caprine GARs.

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