Scientific review of variola virus research, 1999–2010

December 2010

GLOBAL ALERT AND RESPONSE

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Contents

Background ..... **vii**

1 Smallpox vaccines **1**

Executive summary 2

1.1 Introduction 3

1.2 The history of smallpox vaccination 3

1.3 The WHO Smallpox Global Eradication Programme 4

1.4 The origin of vaccinia virus 5

1.5 The vaccine used to eradicate smallpox 6

1.6 Tissue culture and clonal smallpox vaccines 6

1.7 Smallpox vaccines attenuated by passage in tissue culture or through genetic recombination 7

1.8 Protein subunit and DNA vaccines for smallpox 8

1.9 Vaccination after exposure to smallpox 9

1.10 Future challenges of smallpox vaccination 9

Abbreviations 11

References 12

2 Laboratory diagnostics for smallpox (variola virus) **17**

Executive summary 18

2.1 Introduction 19

2.2 Collection and handling of specimens 19

2.3 Virus isolation 21

2.4 Electron microscopy 22

2.5 Genome or genome element–based diagnostic assays 22

2.5.1 Working with variola virus DNA 23

2.5.2 Restriction fragment length polymorphism 23

2.5.3 Polymerase chain reaction 23

2.5.4 Polymerase chain reaction–restriction fragment length polymorphism 24

2.5.5 Real-time PCR 24

2.5.6 Oligonucleotide microarray analysis 28
2.5.7 Sequencing 29
2.6 Protein-based diagnostic assays 29
2.7 Serology-based diagnostic assays 30
2.8 Summary thoughts 31
Abbreviations 32
References 33

3 Variola genomics 37

Executive summary 38
3.1 The variola virus genome 39
3.2 Variola evolution 43
3.3 Poxvirus genome technologies 44
3.4 Guidelines for variola virus genomes 45
Abbreviations 49
References 50

4 The status of WHO collaborating centre repositories of variola virus and nucleic acid 53

Executive summary 54
4.1 Introduction 55
4.2 The status of strains of variola virus and nucleic acid repositories at the WHO collaborating centre in the Russian Federation 55
4.2.1 The state of the variola virus strains collection and its repository 55
4.2.2 The state of the VECTOR variola virus DNA collection and its repository 60
4.3 The status of strains of variola virus and nucleic acid repositories at the WHO collaborating centre in the United States 67
Abbreviations 82
References 83

5 Animal models and pathogenesis 85

Executive summary 86

5.1 Introduction 87

5.2 Animal models 87

5.3 Conclusion 95

Abbreviations 97

References 98

6 Antiviral drug development for the treatment of smallpox – status of small-molecule therapeutics 103

Executive summary 104

6.1 Introduction 106

6.2 Drug discovery 108

6.2.1 Thiosemicarbazones 108
6.2.2 Evaluation of approved and investigational new drugs 108
6.2.3 Acyclic nucleoside phosphonates 109
6.2.4 Inosine monophosphate dehydrogenase inhibitors 109
6.2.5 S-adenosylhomocysteine hydrolase inhibitors 110
6.2.6 Orotidine-5'-monophosphate decarboxylase inhibitors 110
6.2.7 Thymidylate synthase inhibitors 110
6.2.8 Compounds that interfere with virus assembly 110
6.2.9 Abl-family kinases 111
6.2.10 Immunobiological preparations 111
6.2.11 Miscellaneous compounds 111
6.2.12 Compounds whose structure will not be revealed until late in development 111
6.2.13 Molecular biology–based approach to inhibiting viral replication, such as RNA interference 112

6.3 Variola and monkeypox primate animal models 112

6.4 Human data to validate animal models 113

6.5 Intravenous cidofovir 113

6.6 Oral CMX001 115

6.7 Oral ST-246 116

6.8 Effect of antiviral administration on vaccine protection 118

6.8.1 Cidofovir and Dryvax 118
6.8.2 ST-246 (tecovirimat), and Dryvax and ACAM2000 118

6.9 Drugs under clinical development 119
6.10 Time lines for development of smallpox therapeutics 120
6.11 Concluding discussion 121

Abbreviations 123

References 125

Background

The destruction of stocks of variola virus, the etiological agent of the disease smallpox, has been discussed at the World Health Assembly since 1986, following the unprecedented declaration of smallpox eradication in 1980. Several committees have held intensive discussions on whether the remaining live variola virus material should be retained for further critical public health research and to define the nature of that research using live virus. The World Health Organization (WHO) Advisory Committee on Variola Virus Research (ACVVR), which was established in 1999, oversees all research using live variola virus, based on the World Health Assembly decisions contained in WHA49.10, WHA52.10 and WHA 55.15. WHO-sanctioned repositories of live variola virus are currently maintained only at two WHO collaborating centres: the Centers for Disease Control and Prevention, Atlanta, United States of America, and the State Research Center of Virology and Biotechnology VECTOR laboratory, Novosibirsk, the Russian Federation.

In May 2007, the Sixtieth World Health Assembly in resolution WHA60.1 requested the WHO Director-General to undertake a major review in 2010 of the results of the research undertaken, research currently under way, and plans and requirements for further essential research for global public health purposes, taking into account the recommendations of the ACVVR, so that the Sixty-fourth World Health Assembly may reach global consensus on the timing of the destruction of existing variola virus stocks.

In November 2007, the ACVVR proposed drafting written summaries of the research for discussion by the 2011 World Health Assembly. In November 2008, the ACVVR decided to use the following methods for the major review:

- preparation of a comprehensive review of the literature and of unpublished data concerning live variola virus research, in six distinct chapters (detailed below), by a group of scientists endorsed by the ACVVR and representing all areas of research and development on variola virus and relevant work with other orthopoxviruses;
- consideration of the scientific review by selected members of the ACVVR (December 2009 to April 2010);
- consideration of the scientific review by an external panel of independent experts from outside the variola virus field – the Advisory Group of Independent Experts to review the Smallpox programme (AGIES) (September 2010 to November 2010);
- presentation of the scientific review and the AGIES report for final consideration by the ACVVR (November 2010);
- consideration by the Executive Board of WHO of the scientific review and the AGIES report mandated by resolution WHA60.1, including the recommendations of the ACVVR (WHA60.1 OP4(1)) (January 2011);
- consideration of that report and the Executive Board's comments by the World Health Assembly (May 2011).

The first part of this process, the comprehensive scientific review and summary, was initiated by a group of scientists endorsed by members of the ACVVR at its meeting in

2008 and representing all areas of research on variola virus. Under the supervision of the ACVVR, the group of scientists with specific expertise in variola virus or other orthopoxviruses started writing the current document, *Scientific review of variola virus research, 1999–2010*. The document has six chapters, covering smallpox vaccines, laboratory diagnostics, variola genomics, status of WHO repositories, animal models and antiviral drugs.

In November 2009, the ACVVR considered and discussed the six chapters of the document. From November 2009 to October 2010, the document was edited and discussed several times.

In October 2010, AGIES reviewed these six chapters and provided an independent assessment of the needs for live variola virus.

Collectively, the chapters and the independent review demonstrated the tremendous progress that has been made under the auspices of WHO to:

- characterize many different strains of variola virus;
- develop two excellent candidate antiviral drugs with distinct mechanisms of action;
- develop new, less reactogenic smallpox vaccines (both licensed vaccines and candidate vaccines);
- develop diagnostic tests for variola virus and other orthopoxviruses;
- develop animal models for variola virus and other orthopoxviruses.

A previous version of the *Scientific review of variola virus research, 1999–2010*, dated 10 November 2010, was presented at the 12th meeting of the ACVVR on 17–18 November 2010. The current version, dated December 2010, includes revisions suggested by AGIES and the members of the ACVVR.

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Geneva, December 2010

1 Smallpox vaccines

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Executive summary

Public health importance

Smallpox is the only human disease that has been eradicated by a global vaccination campaign. This accomplishment remains one of the great triumphs of medical science. The smallpox vaccine, which consists of live vaccinia virus, was highly effective. However, it has a history of severe complications, particularly in individuals with an immunodeficiency or with eczema. As well, since it was made in live animals under non-sterile conditions, it would not meet current manufacturing guidelines. There is therefore a clear public health interest in developing a new, efficacious and safe vaccine.

Progress to date

Smallpox vaccines made in tissue culture cells have been produced and licensed. However, these vaccines are likely to induce a rate of adverse effects similar to the original vaccines. Consequently, several approaches have been taken to produce safer vaccines. Progress has been greatest with strains of vaccinia virus that are more attenuated – namely, modified vaccinia virus Ankara (MVA) and LC16m8, which have been produced by repeated tissue culture passage. MVA is more highly attenuated than LC16m8; it has usually been given intramuscularly or subcutaneously, and so does not produce the typical skin lesion that provides evidence of a "take". LC16m8 can be administered by skin scratch, like the conventional smallpox vaccine, but produces a milder take than the parental virus, vaccinia virus strain Lister. MVA and LC16m8 have been shown in non-human primates to be safe and to produce good immunogenicity, including protection against monkeypox virus, a close relative of variola. New generation vaccines consisting of live vaccinia virus with specific gene mutations, DNA (deoxyribonucleic acid) encoding poxvirus genes and purified proteins have all shown promise in animal models, but none have reached clinical testing.

Outcomes and implications

Licensing of smallpox vaccines grown in tissue culture has been a useful step forward; however, use of these vaccines would be medically contraindicated for individuals with immunodeficiency and certain dermatological conditions. Since smallpox has been eradicated, the efficacy of new generation vaccines will need to be tested using poxviruses related to variola virus in animal protection studies, and safety and immunogenicity studies in humans. However, confidence in the ability of these vaccines to protect against smallpox would be increased by use of live variola virus for in vitro neutralization tests and non-human primate studies.

1.1 Introduction

Smallpox was declared eradicated in 1980 as a result of the World Health Organization (WHO) Smallpox Global Eradication Programme. In response to the risk of a deliberate release of smallpox as an act of global terrorism, some countries have manufactured smallpox vaccines to replenish their stocks. The smallpox vaccine, vaccinia virus (VACV), is the only vaccine so far to have led to the eradication of a human infectious disease. However, its safety record does not meet current vaccine standards: use of the vaccine was associated with the risk of transmitting other infections (due to the vaccine's production in animals) and a number of adverse effects. Smallpox vaccines have therefore been produced in tissue culture in recent years; they consist of plaque-purified or non-clonal VACV strains grown in tissue culture or embryonated chicken eggs. Some of these smallpox vaccines are based on non-replicating or highly attenuated VACV strains that have both improved safety and good immunogenic properties.

Information gained from research on VACV allows the development of attenuated live vaccines, constructed using genetic recombinant technology, to modify genes involved in host range or immune evasion. Another strategy that avoids the use of infectious VACV is immunization with viral proteins that induce a protective immune response against infection. New generation vaccines can be tested in surrogate animal models and compared with the vaccines used to eradicate smallpox, using characteristics such as virus neutralization or the induction of specific immune responses. The new generation vaccines are safer than the traditional smallpox vaccines – a property that can be tested in human clinical trials – and retain immunogenic properties. However, because smallpox has been eradicated, a major limitation in testing is the inability to demonstrate that the new smallpox vaccines induce protective immunity against smallpox in humans.

1.2 The history of smallpox vaccination

Smallpox is the only human disease that has been eradicated as a result of a global vaccination campaign, and this accomplishment remains one of the greatest triumphs of modern medical science (Fenner et al., 1988; Smith & McFadden, 2002; Henderson, 2009).

Variolation was the first measure used to control smallpox. Variola virus (VARV) isolated from the smallpox pustules of an infected individual was administered, either by insufflation or scarification, to a non-immune person. Although this process had a high mortality rate (0.5–2%), it was beneficial compared with the death rate from natural smallpox transmitted by the respiratory route (up to 40%). Variolation was carried out in India and China for centuries before being introduced in western Europe in 1723, and was the only means of protection from smallpox until Edward Jenner introduced vaccination in 1796.

Jenner, who was a medical doctor in the small town of Berkeley in England, noticed that milkmaids were sometimes infected on their hands with cowpox virus (CPXV), suffering a local infection that appeared to confer protection from smallpox. Jenner was the first to test this hypothesis, when he took material from the lesion of the milkmaid Sarah Nelmes and vaccinated a boy, James Phipps. When Jenner subsequently challenged the boy by variolation, he resisted the infection. After additional study, Jenner published his *Inquiry* on the subject, marking the beginning of the vaccination era.

Although others had noticed the correlation between CPXV infection and resistance to smallpox, Jenner demonstrated efficacy of vaccination by challenging inoculation with VARV, recommended maintenance of the virus by serial passage in humans and promoted vaccination. These actions justify giving Jenner full credit for the discovery of vaccination. The practice of vaccination rapidly replaced variolation, and its success led Jenner to predict in 1801 "… that the annihilation of the smallpox, the most dreadful scourge of the human species, must be the result of this practice" (Jenner, 1801).

The discovery was timely, since smallpox was then a major scourge in Europe and around the world. Within five years, Jenner's *Inquiry* was translated into most European languages, vaccination institutes were established in many countries and the vaccine had been transferred to all continents. However, widespread vaccination was limited due to technical problems and short supplies of the vaccine. Cowpox was a rare disease in Europe, and was absent in the Americas. Human-to-human vaccination was practised, but it caused the transmission of other pathogens and was eventually banned.

Subsequently, CPXV and then the closely related VACV were produced in the skin of live animals. A further advance was the development of freeze-dried vaccine in 1950, which allowed the vaccine to be maintained, transported and used in the field without refrigeration or loss of potency. Finally, development of the bifurcated needle allowed unskilled personnel to administer the vaccine successfully.

1.3 The WHO Smallpox Global Eradication Programme

In 1959, the Twelfth World Health Assembly adopted a resolution, proposed by the Soviet Union, to achieve global eradication of smallpox (Fenner et al., 1988). Progress from 1959 to 1966 was slower than anticipated, but in 1967 the Intensified Smallpox Eradication Programme started. The worldwide vaccination policy under the programme emphasized surveillance of smallpox, and adopted the method of ring vaccination to prevent human-to-human transmission and control smallpox epidemics. New cases of smallpox were thereby identified and quarantined, and close contacts of infected people were vaccinated and quarantined. This policy led to the eradication of smallpox, with the last naturally occurring case reported in Somalia in 1977. Following extensive surveillance throughout all continents, WHO confirmed the global eradication of smallpox in 1979, and the Thirty-third World Health Assembly declared on 8 May 1980 that smallpox eradication had been achieved.

The eradication of smallpox is the most important success of WHO to date, and demonstrated that prophylaxis by mass vaccination may lead to the eradication of infectious diseases. Success relied on six key properties of both the vaccine and the disease:

- Smallpox infections are restricted to humans. There is no animal reservoir in which the virus may persist and from which it may be reintroduced to the human population.
- VARV cannot establish latent or persistent infections, because individuals recovering from the disease clear all virus.
- Smallpox was a severe disease, and the signs were easily noticed. Infected individuals were therefore readily identified, and potential contacts could then be vaccinated.

- The vaccine induced long-lasting protective immunity and was effective against all strains of VARV.
- No variants of VARV could escape protective immunity by antigenic variation, due to the high fidelity of the viral DNA (deoxyribonucleic acid) polymerase and the presence of multiple antigens.
- The vaccine was easy to prepare, cheap and stable without refrigeration, which facilitated its effective transport during the global eradication campaign.

It is impressive that the eradication of smallpox took place before the advent of molecular biology, and with limited knowledge of the replication cycle of VACV, the viral proteins that are targets of neutralizing immunity or the immune mechanisms of protection.

To avoid the reintroduction of smallpox into the human population, under the direction of WHO, all known stocks of VARV in laboratories around the world were either destroyed or sent to two smallpox repositories in high-security laboratories in the United States and the Soviet Union, now the Russian Federation. These smallpox repositories – respectively at the Centers for Disease Control and Prevention in Atlanta, and the State Research Centre of Virology and Biotechnology VECTOR in Koltsovo – are now the only official places where infectious VARV stocks are maintained. Research on these samples is closely monitored by WHO.

1.4 The origin of vaccinia virus

Initially, CPXV was used for smallpox vaccination. This virus is infrequently found in cattle, and causes sporadic infections in humans and a number of animals, but its natural reservoir is probably wild rodents. In 1939, Downie showed that contemporary CPXV preparations used as smallpox vaccines contained a different virus that was not found in nature; this was named VACV, after the vaccination procedure (Downie, 1939). Over the years, VACV replaced CPXV as the smallpox vaccine. Following the extensive use of VACV for smallpox vaccination during the 20th century, the virus has come to infect domestic animals, notably buffaloes in India and cattle in Brazil. These animals can, in turn, transmit the virus to humans. Despite this, VACV is not considered a natural human pathogen.

Based on analyses of the viral genome sequences, it is unlikely that VACV is derived from either a CPXV or a VARV. The favoured hypothesis for the origin of VACV is that it is a species of orthopoxvirus (OPV) that previously infected animals in which it is no longer endemic. Horsepox virus has been suggested as the origin of VACV, as earlier vaccinators also obtained supplies of vaccine from poxvirus infections of horses, and at least one strain of VACV (Ankara) was isolated from a horse (Mayr, Hochstein-Mintzel & Stickl, 1975; Baxby, 1981). In addition, an OPV whose closest relative is VACV has been isolated from diseased Mongolian horses (Tulman, 2006). The reasons for VACV, rather than CPXV, becoming the 20th century smallpox vaccine are not recorded. Possibly, VACV had a higher prevalence when vaccines were established, or vaccinators selected VACV because CPXV produced a more severe reaction and VACV has lower virulence. Although the origin and natural host of VACV remain mysteries, this virus is the most intensively studied of the poxviruses.

1.5 The vaccine used to eradicate smallpox

Because of the long history of VACV as a vaccine, a wide variety of VACV strains have been used in different regions of the world (Fenner et al., 1988). The New York City Board of Health (NYCBH) strain was used in North America and west Africa. Wyeth Laboratories commercialized the Dryvax vaccine, which was prepared from the lymph fluid of the skin of calves infected with the NYCBH strain; it was available in the United States after the smallpox eradication campaign. The EM-63 strain was derived from the NYCBH strain, and was used in Russia and India, while the Lister/Elstree strain, developed at the Lister Institute in the United Kingdom, became the most widely used vaccine worldwide (Rosenthal et al., 2001). The Temple of Heaven/Tian-Tan strain (China) was also widely used. Other VACV strains used during the eradication programme include the Copenhagen strain (Denmark), the Bern strain (Switzerland), the Dairen strain (Japan), the Ankara strain (Turkey), the Tashkent strain (Uzbekistan) and the Paris strain (France). Most of the vaccines used during the smallpox eradication programme were grown on the skin of live animals – mainly calves, but also sheep, buffaloes and rabbits.

Although the smallpox vaccine is the only vaccine ever to have led to the eradication of a human infectious disease, its safety record was not perfect. Production of the vaccine in animals led to the risk of transmitting other infections, and a number of adverse effects were associated with vaccination. Accidental infections occurred when virus was transmitted from the inoculation site in vaccinees or from contact persons; ocular and generalized infections were of greatest concern. The severe infection in individuals with eczema or immunological deficiency was a major complication. Both conditions are considered contraindications to smallpox vaccination. A small percentage of vaccinees had severe neurological adverse effects, such as encephalitis, and these cases were unpredictable.

Post-vaccine adverse effects were more frequently associated with some vaccine strains than with others (Lane et al., 1969; Fenner et al., 1988) – the limited epidemiological data available suggest that the NYCBH and Lister strains were associated with lower frequencies of adverse effects, while the Copenhagen and Tashkent strains were more virulent. Some modelling studies have estimated that the number of deaths after vaccination with the NYCBH strain was one per million vaccinations (Halloran et al., 2002; Kaplan et al., 2002; Porco et al., 2004). A recent study reviewing the available epidemiological data estimates the number of deaths during a mass vaccination campaign in tens per million for the NYCBH strain, and up to two hundred per million for the Lister strain (Kretzschmar et al., 2006). During a smallpox vaccination campaign in the United States, involving more than 700 000 individuals, the frequency of vaccination-related myopericarditis cases was higher than anticipated, leading to controversy about the programme (Arness et al., 2004; Eckart et al., 2004). Even without adverse effects, less serious side-effects such as a skin lesion, low-grade fever and headaches are common. This can make people reluctant to be vaccinated.

1.6 Tissue culture and clonal smallpox vaccines

Preparation of smallpox vaccines in live animals is currently unacceptable because of concerns about quality control for microbial contamination. Next generation vaccines are instead prepared in tissue culture or embryonated chicken eggs. Although the smallpox vaccine was grown occasionally in embryonated chicken eggs during the eradication

campaign, experience with large-scale production of vaccine in tissue culture is limited, and the effectiveness of this vaccine in the field is not well documented.

Genetic heterogeneity has been documented for the Lister and Dryvax vaccines used during the eradication programme (Li et al., 2006; Osborne et al., 2007; Garcel et al., 2009). The next generation vaccines are plaque-purified viral clones with properties similar to their parental strains. Although they are believed to be as effective as the first vaccines used to eradicate smallpox, it is possible – although unlikely – that they have lost viral clones important for their efficacy in humans.

A non-clonal vaccine using the Lister strain grown in cell culture has been produced by Sanofi Pasteur, and is currently being clinically tested. Another next generation clonal smallpox vaccine, ACAM2000, was licensed for use in the United States in August 2007 (Frey et al., 2009). This vaccine was derived by plaque purification from Dryvax, and has been grown in the Vero monkey cell line. Although these vaccines are manufactured to current standards, they may be associated with the same adverse effects as the standard Dryvax or Lister vaccines used in the smallpox eradication campaign; a significant minority of the population has contraindications that prevent the use of these vaccines.

Because of the infrequent but significant post-vaccine adverse effects observed with smallpox vaccines based on fully replicating VACV, research has been directed towards the development of smallpox vaccines based on non-replicating or highly attenuated VACV strains with improved safety that retain good immunogenic properties. A common method to attenuate VACV is by multiple passages in tissue culture, leading to genetic alterations, lower virulence and restricted host range.

1.7 Smallpox vaccines attenuated by passage in tissue culture or through genetic recombination

Modified vaccinia Ankara (MVA) is a VACV strain derived from chorioallantois vaccinia Ankara (CVA) in the late 1950s by 570 passages in chick embryo fibroblasts (Mayr, Hochstein-Mintzel & Stickl, 1975). This passaging resulted in a virus of restricted host range that is unable to replicate efficiently in human cells but expresses most of the viral proteins (Sutter & Moss, 1992; Mayr, 2003). The virus was licensed as a vaccine in Germany, and safely used to vaccinate more than 100 000 people, but its effectiveness against smallpox was not tested. MVA has large deletions in the terminal regions of the genome, which contain non-essential genes that are often involved in evasion of the host immune response or in maintaining the broad host range of VACV (Antoine et al., 1998). As a result of these deletions, MVA replicates well in chick embryo fibroblasts and baby hamster kidney cells, but is restricted in human cells (Carroll & Moss, 1997). In most types of cells, MVA produces most of the viral antigens, but only immature virus particles are formed, and cell-to-cell spread is restricted. As a vaccine, MVA is considered effective because it provides a nearly complete antigenic dose, and safe because it does not replicate fully in human and most other mammalian cells (as borne out by studies with immunodeficient monkeys [Stittelaar et al., 2001]). It induces an antibody profile similar to that induced by Dryvax and protective immunity against monkeypox virus (MPXV) in non-human primates (Earl et al., 2004, 2008). However, a higher dose or multiple doses of MVA are required to achieve the immune protection produced using a single dose of replicating VACV. Phase I and II clinical trials with MVA have been completed, and phase III clinical trials are expected to start in 2011 (Vollmar et al., 2006; Wilck et al.,

2010); together with appropriate animal studies, these could lead to licensing of the vaccine.

LC16m8 is a VACV strain that was developed by passaging VACV Lister strain through primary kidney epithelial cells at low temperature (30 °C) and was licensed in Japan in 1975 (Hashizume et al. 1985, Kenner et al., 2006). The virus has a take rate similar to the Lister strain; it differs from the Lister strain in being temperature restricted, having a limited host range and showing greatly reduced adverse effects (in terms of both severity of adverse effects and number of people suffering adverse effects). LC16m8 does not have large deletions in its genome, and most of the open reading frames appear to be functional. The small-plaque phenotype of LC16m8 was attributed to a mutation in the *B5R* gene, encoding a protein with homology to complement regulatory proteins. This protein is essential for the formation of extracellular enveloped virus and is an important target antigen for antibodies that neutralize the virion (Putz et al., 2006). Since this mutation may easily revert, a stabilized version of LC16m8 has been developed, with a deletion of the entire *B5R* gene (Kidokoro, Tashiro &Shida, 2005). Other mutations responsible for the temperature restriction and in vivo attenuation are likely to be found elsewhere in the viral genome. LC16m8 has been shown to protect monkeys against MPXV (Saijo et al., 2006).

The Dairen I (DI) strain of VACV was derived from the parental Dairen vaccine strain after 13 passages in eggs (Tagaya, Kitamura & Sano, 1961). It contains a large deletion in the left terminal region of the genome, including genes involved in virus host range and interferon resistance (Ishii et al., 2002).

The information available to date on MVA, LC16m8 and VACV DI strain is encouraging, and this research needs to be continued and expanded.

Recombinant technology allows genes to be inserted, deleted or interrupted in specific genomic sites, to generate safer and more immunogenic vaccines (Moss, 1996; Jacobs et al., 2009). Viruses can be attenuated by the deletion of genes involved in immune modulation, host range or nucleotide metabolism. One of the best characterized attenuated mutants of VACV is NYVAC, which has 18 open reading frames deleted (Tartaglia et al., 1992); another is an *E3L* deletion mutant, which can still be given by scarification (Jentarra et al., 2008). Highly attenuated NYVAC has a lower immunogenicity than vaccine strains (Midgley et al., 2008), and higher virus doses may need to be administered. Protective immunity after vaccination may be increased by more selective inactivation of genes involved in immune evasion, or by the expression of cytokines that potentiate specific aspects of the immune response.

1.8 Protein subunit and DNA vaccines for smallpox

Purified viral proteins produced by recombinant organisms, or DNA expressing such proteins, is another approach to inducing protective immunity against smallpox. Progress has been made in recent years in identifying the viral proteins that induce protective immunity against OPV (Moss, 2010). Antibodies against virion proteins neutralize virus infectivity in tissue culture, and some studies have shown that animals immunized with combinations of membrane proteins – including A33, B5, L1 and H3 – are protected from subsequent challenge with a virulent poxvirus (Fogg et al., 2004; Davies et al., 2005; Heraud et al., 2006; Xiao et al., 2007; Buchman et al., 2010). Immunization with the viral

type I interferon binding protein also protected mice from lethal mousepox; thus viral immunomodulatory proteins may be another option for protein-based vaccines (Xu et al., 2008).

Subunit vaccines are safer than infectious VACV. However, this alternative approach to vaccination is limited by the current lack of a demonstration that these vaccines would be effective in a smallpox outbreak.

1.9 Vaccination after exposure to smallpox

Vaccination after exposure to smallpox virus infection may be effective in minimizing casualties from smallpox (Mortimer, 2003). Several studies have evaluated this possibility in animal models. Most have concluded that the vaccine has to be administered no later than 1–2 days after exposure to a virulent poxvirus in order to protect against death (Staib et al., 2006; Samuelsson et al., 2008; Paran et al., 2009). Interestingly, MVA elicited a more rapid protective response than the NYCBH strain in a monkeypox challenge model, probably due to the high dose used in the trial (Earl et al., 2008).

1.10 Future challenges of smallpox vaccination

In response to the risk of a deliberate release of smallpox as an act of terrorism, some countries have manufactured smallpox vaccines to replenish their stocks (Rosenthal et al., 2001), and WHO has established stocks of smallpox vaccine. Generally, these vaccines have been prepared in tissue culture rather than in animals, to meet current vaccine standards. Given their similarity to the traditional smallpox vaccine, it is likely that these manufactured vaccines will have the same efficacy and the same rate of adverse effects. The production of vaccinia immune globulin, or the development of alternative approaches such as monoclonal antibodies against specific viral components, will potentially be important in treating any adverse effects of vaccination.

Safer smallpox vaccines are needed because of the unacceptable frequency of post-vaccine adverse effects and the significant proportion of the human population that has contraindications for smallpox vaccination. Highly and moderately attenuated VACVs that are moving toward licensure, such as MVA and LC16m8, may go some way towards fulfilling this need. In addition, the information gained from research on VACV can be used to create engineered virus strains that are attenuated rationally, such that they have diminished replication, spread or modulation of the host immune response. The new vaccines can be tested for protection in animal models, and for safety and immunogenicity in human clinical trials.

As well as high cost, a major difficulty for licensure is the inability to demonstrate that newly developed smallpox vaccines induce protective immunity against smallpox in humans. Because smallpox has been eradicated, the new vaccines cannot be tested for efficacy against the natural disease. Instead, the vaccines must be tested and compared with traditional smallpox vaccines in surrogate models of smallpox, such as mousepox in mice, or monkeypox in primates. Another approach is to use reactivity to VARV antigens and neutralization of VARV infectivity in tissue culture as markers (Damon et al., 2009). Our knowledge of the immunological parameters that correlate with protection from virulent poxviruses, although still limited, has increased in recent years, and may provide benchmarks to compare traditional vaccines with the new generation smallpox vaccines

(Putz, et al., 2006; Kennedy et al., 2009). However, in vitro neutralization and non-human primate studies with live VARV would increase confidence in the ability of these vaccines to protect against smallpox.

Abbreviations

CPXV	cowpox virus
CVA	chorioallantois vaccinia virus Ankara
DNA	deoxyribonucleic acid
MPXV	monkeypox virus
MVA	modified vaccinia virus Ankara
NYCBH	New York City Board of Health
OPV	orthopoxvirus
VACV	vaccinia virus
VARV	variola virus
WHO	World Health Organization

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2 Laboratory diagnostics for smallpox (variola virus)

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Executive summary

Public health importance

Variola virus is the causative agent of smallpox, a disease that was declared eradicated by the World Health Assembly in 1980. The virus is considered a potential biowarfare agent or terrorist weapon due to the high morbidity and mortality it can cause, and because much of the human population is now susceptible due to routine smallpox vaccination being largely discontinued in the 1970s (Henderson et al., 1999). Taking into account the serious consequences of a smallpox diagnosis or even the consequences of a misdiagnosis, there is a need to be able to identify smallpox unambiguously, rapidly and reliably. This includes an equally reliable differentiation from other similar clinical entities. The predictive value of a positive diagnostic result (also referred to as predictive value positive) is exceedingly low in a low-prevalence disease; diagnostic strategies that improve predictive value positive need to be used.

Progress to date

Between 2000 and 2010, there have been remarkable advances in the clinical and laboratory diagnostic capacities for smallpox. This chapter reviews historical methods for smallpox diagnosis, and summarizes the advances in nucleic acid diagnostic assays, serological assays and protein detection assays developed for smallpox since 2000. Newer technologies have driven the approaches taken by many investigators. Specifically, nucleic acid detection strategies are increasingly using high-throughput real-time polymerase chain reaction technologies and, in some cases, array platforms.

Outcomes and implications

Many nucleic acid–based assays have been developed, but only a few immunology or protein-based diagnostic techniques. All smallpox and poxvirus assays, including these new assays, are research based; none have completed regulatory review and approval processes. The possible need for live variola virus for regulatory review of assays is being discussed at the time of writing. One nucleic acid–based diagnostic kit is available commercially; however, it is for research purposes only and not diagnostic use.

2.1 Introduction

This chapter focuses on laboratory procedures for the clinical diagnosis of smallpox. It does not review approaches for environmental detection. It presents methods of sample collection and handling, provides a cursory overview of previous diagnostic assays, and focuses on nucleic acid tests and serologic assays. Discussion of a recently developed matrix-assisted laser desorption/ionization (MALDI)-based system is not included in this review; this technology is limited to use in very few laboratories and is under additional development.

Variola virus (VARV) is the causative agent of smallpox. The virus is considered a potential biowarfare agent or terrorist weapon, due to the high morbidity and mortality that it can cause, and because much of the human population is now susceptible to it, due to routine smallpox vaccination being largely discontinued in the 1970s (Henderson et al., 1999). Taking into account the serious consequences of a smallpox diagnosis or even the consequences of a misdiagnosis, there is a need to be able to identify smallpox unambiguously, rapidly and reliably. This includes the need to differentiate VARV in an equally reliable manner from other similar clinical entities. Before its eradication in 1980, smallpox was clinically relatively easy to recognize, although it was sometimes confused with other exanthematous illnesses (Damon & Esposito, 2003; Shchelkunov, Marennikova & Moyer, 2005). For example, the severe chickenpox rash caused by varicella zoster virus (VZV) was often misdiagnosed as that of smallpox. Other diseases confused with vesicular-stage smallpox included monkeypox, generalized vaccinia virus infection, disseminated herpes zoster virus infection, disseminated herpes simplex virus (HSV) infection, drug reactions (eruptions), erythema multiforme, enteroviral infections, secondary syphilis, scabies, insect bites, impetigo and molluscum contagiosum. Diseases confused with haemorrhagic smallpox included acute leukaemia, meningococcaemia and idiopathic thrombocytopenic purpura. As a step to counter this type of diagnostic error, a World Health Organization (WHO) collaborating centre – the Centers for Disease Control and Prevention (CDC) – in collaboration with numerous other professional organizations, has developed an algorithm for evaluating patients for smallpox.[1]

Predictive value positive – the proportion of true positives among those testing positive – varies with disease prevalence; this is different from sensitivity and specificity, which are independent of the prevalence of the disease and reflect the properties of the assay itself. Therefore, for diagnostic confirmation of suspect cases, predictive value positive can be improved by testing samples from patients with clinically appropriate symptoms and using several diagnostic approaches that have independent sources of error and multiple targets of recognition.

2.2 Collection and handling of specimens

A suspected case of smallpox should be reported immediately to the appropriate local or state health department.

[1] See http://emergency.cdc.gov/agent/smallpox/diagnosis/riskalgorithm/ for the algorithm and additional information.

Current international recommendations[2] state that work with VARV is to be done using biosafety level 4 laboratories sanctioned by WHO. As of 2010, two WHO collaborating centres have the capability to handle live VARV specimens – one at CDC in Atlanta, United States, and the other at the State Research Center of Virology and Biotechnology VECTOR (SRC VB VECTOR) in Koltsovo, the Russian Federation. Information about the safe collection and handling of specimens is available on the CDC web site, currently located within "Guide D – specimen collection and transport guidelines" of the smallpox response plan.[3]

At least two to four scabs or material from vesicular lesions (or both) are considered suitable specimens for laboratory testing. Scabs can be separated from the underlying intact skin with a scalpel or a 26-gauge needle, and each specimen should be stored in a separate container to avoid cross-contamination. Coexistent infectious rash illnesses, including chickenpox and monkeypox infections, have been noted. Lesions should be sampled so that both the vesicle fluid and the overlying skin are collected. Once the overlying skin is lifted off and placed in a specimen container, the base of the vesicle should be swabbed vigorously with a wooden applicator, or a polyester or cotton swab. The viscous material can be applied onto a clean glass microscope slide and air dried. A "touch prep" can be prepared by pressing a clean slide onto the opened lesion, using a gradual pressing motion. If available, a series of three electron-microscope grids can be applied to the lesion (shiny side to the unroofed vesicle), using successively minimal, moderate and firm pressure (Hazelton & Gelderblom, 2003). Glass slides and electron-microscope grids should be allowed to air dry for about 10 minutes, and then be placed in a slide holder or a grid carrier box for transport to the laboratory.

Alternative lesion sampling processes, including storing material on appropriate filter paper types, are being evaluated. Sample storage in transport medium (e.g. as done with herpesviruses) is discouraged, largely because the medium dilutes the specimen. Specific recommendations for electron-microscopy sampling and specimen processing are available.[4]

Lesion biopsies may also provide material suitable for direct viral evaluation. A 3–4 mm punch biopsy can be done and the specimen bisected, with half placed in formalin for immunohistochemical testing and the remainder placed in a specimen collection container. Blood and throat swabs obtained from suspected smallpox patients during the prodromal febrile phase and early in the rash phase are also a potential source of virus. In addition, patient serum can be obtained for serologic assays to substantiate a viral diagnosis or to infer a retrospective diagnosis. Five to ten millilitres of serum should be obtained as early as possible in the disease course, and then again three to four weeks later.

Virus-containing specimens should be stored at –20 °C or on dry ice until samples reach their transport destination. The exceptions are electron-microscopy grids and formalin-

[2] http://www.who.int/csr/disease/smallpox/SummaryrecommendationsMay08.pdf
[3] http://emergency.cdc.gov/agent/smallpox/response-plan/#guided
[4] http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf

fixed tissues, which should be kept at room temperature. Standard refrigerator temperatures (4 °C) are acceptable for less than seven days' storage.

Packaging and transport of clinical samples

Packaging and transport of clinical samples should follow international standards for packaging and international regulations for the transport of infectious substances.

A triple packaging system must be used for transport of all clinical samples (WHO, 2008a). Clinical samples should be considered as infectious substances from Category A and should be assigned to United Nations number UN 2814. Practical guidance on complying with regulations for all modes of transport of infectious substances and patient specimens, both nationally and internationally, can be found on the WHO web site.[5]

2.3 Virus isolation

The use of chick embryos for poxvirus diagnostics was first described in 1937 and has since become a valuable diagnostic tool. The only known poxviruses that produce human infection and pocks on the chorioallantoic membrane (CAM) of chicken eggs are four orthopoxviruses (OPVs): VARV, monkeypox virus (MPXV), cowpox virus (CPXV) and vaccinia virus (VACV). Differences in the pock morphology seen in 12-day-old embryos incubated at 34.5–35 °C were useful in differentiating the OPV species; as a consequence, the CAM assay was widely and successfully used during the smallpox eradication campaign.

Despite the availability of new diagnostic techniques, virus isolation remains the gold standard. In addition, virus culture is the only existing method by which to produce a supply of live virus for further examination. Although VARV grows satisfactorily in chick embryos, cell culture is generally the simpler option. VARV can be grown in a variety of established cell culture lines, including:

- Vero, BSC-1 and CV-1 (African green monkey kidney cells)
- LLC-MK2 (rhesus monkey kidney cells)
- human embryonic lung fibroblast cells
- HeLa (human ovarian cancer cells)
- chick embryo fibroblast cells
- MRC-5 (human diploid fibroblast cells).

A cytopathic effect is seen within one or two days, depending on the amount of infectious material in the initial inoculum; if there is little infectious material, individual plaques may not be visualized for three to four days.

[5] http://www.who.int/ihr/biosafety/publications/en/index.html

2.4 Electron microscopy

Electron microscopy is regarded as a first-line method for laboratory diagnosis of poxvirus infections because of the typical morphology of the virion, the high number of particles usually present in poxvirus-induced lesions and the relative ease of acquiring samples. After transmission electron microscopy became a standard method in diagnostic virology in the 1950s, it was widely used during the smallpox eradication era. Clinical diagnosis of a poxvirus infection in humans is now infrequent, so electron microscopy observations may provide one of the first clues to the cause of an unknown rash illness (Hazelton & Gelderblom, 2003).

OPVs share a brick-shaped virion morphology, which is irregularly covered by short, tubular elements resembling small stretches of tape. The size may vary from 250 nm × 290 nm up to 280 nm × 350 nm. Although individual OPV species cannot be distinguished morphologically, they are easily separated from herpesviruses, which are important differential diagnoses in affected humans (e.g. to differentiate from chickenpox caused by VZV). Because poxviruses are tightly associated with the cellular matrix, samples have to be properly prepared so that the virus can be examined by electron microscopy. In the past, this was done by grinding scabbed or minced material from lesions in a mortar with sterile sand or pulverizing the sample after flash-freezing it in liquid nitrogen. Now, commercially available tube systems (lysing matrices in combination with bead beaters or mixing mills) are more advantageous because they allow standardization of the procedure and avoid cross-contamination. Two freeze–thaw cycles or sonication (or both) facilitate disruption of cells in a closed tube system. Using a cup-horn shaped sonicator will allow the release of even more virions from the cell matrix. A concentration of 10^5 viral particles/ml is required for successful diagnosis by the visualization of virions.

Preparation and examination of samples requires patience and experience. Even when brick-shaped poxvirus particles are found rather quickly, it is worthwhile further scanning the sample because additional viruses might also be present. Depending on the number of particles, a sample may take 30 minutes to examine, so electron microscopy can take up to two hours to yield results after the samples are received. Descriptions of methods for negative-stain evaluation and pictures of negative-stained particles are available on the Internet.[6]

2.5 Genome or genome element–based diagnostic assays

The rapid development of nucleic acid research in recent years has yielded many options for DNA (deoxyribonucleic acid)-based detection methods. Nucleotide sequencing techniques have become automated and affordable and this means that techniques like polymerase chain reaction (PCR), real-time PCR, microarrays and – to a lesser extent – genome sequencing are no longer restricted to a few dedicated laboratories.

[6] http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf

2.5.1 Working with variola virus DNA

The distribution, synthesis and handling of VARV DNA are governed by a series of rules (WHO, 2008b). Other than at the two WHO Collaborating Centres for Smallpox and Other Poxvirus Infections, it is strictly forbidden to hold clones containing more than 20% of the VARV genome at any one time. A request to handle VARV DNA greater than 500 nucleotides in length must be submitted through WHO headquarters, and the receiving laboratory may not distribute VARV DNA to third parties. In addition, VARV DNA cannot be used for insertion into VACV or any other poxvirus, and no other OPV can be handled in laboratory rooms where VARV DNA is present. However, VARV DNA not exceeding 500 base pairs can be used as a positive control in diagnostic PCR kits without prior permission, although notifying WHO is desirable in these instances. Likewise, the production of DNA microarrays, on which oligonucleotides (less than 80 base pairs) are covalently bound and therefore difficult to reassemble through ligations, can be performed without permission of WHO. These oligonucleotides could – in aggregate – span the entire genome.

2.5.2 Restriction fragment length polymorphism

The restriction fragment length polymorphism (RFLP) approach is based on the fact that the genomes of even closely related pathogens are defined by variations in sequence. In practice, the RFLP method consists of isolating the target virus, extracting the DNA and then digesting the DNA with one restriction endonuclease enzyme or a panel of several restriction endonucleases. The digested DNA fragments are then individually separated by size, using gel electrophoresis, and visualized. Ideally, each strain will reveal a unique pattern, or fingerprint. For a new protocol setup, many different restriction enzymes may be used. This produces several molecular fingerprints that can be analysed and this determines the best combination of enzymes to differentiate between strains or isolates.

RFLPs generated with the restriction enzyme HindIII have been used to differentiate OPV species (Mackett & Archard, 1979; Esposito & Knight, 1985); however, the RFLP methodology requires a lengthy virus culture to generate a sufficient amount of high-quality DNA.

2.5.3 Polymerase chain reaction

PCR produces large quantities of a desired sequence of DNA from a complex mixture of heterogeneous sequences. Any PCR product generated has, by definition, a characteristic size; its identity is generally confirmed using DNA hybridization probes or restriction endonuclease digests or – more commonly – direct sequencing. The sensitivity of a PCR reaction may be improved by the use of a second set of primers to amplify a subfragment of the first PCR product; however, the use of this nested PCR technique is time consuming and can lead to false-positive results, and thus should be avoided for routine diagnostics.

PCR does not differentiate between viable and non-viable viruses, or complete and incomplete pieces of genomic DNA, which may complicate the interpretation of results. Also, when using PCR, it is important to include both positive and negative controls to validate the PCR results. Using a positive control will help to eliminate false-negative results (i.e. if the whole PCR reaction itself has not worked properly), and using a negative control will help to eliminate false-positive results (i.e. if the samples all become

contaminated by viral DNA or template). Use of these precautions allows PCR to become a realistic option for the diagnostician. Its advantages in terms of speed, sensitivity and specificity now far outweigh the costs of the equipment needed, and procedures are in place to prevent the danger of contamination leading to false-positive results. Currently, PCR is the method of choice in VARV diagnostic identification.

PCR protocols to identify and differentiate OPV species are available, based on sequences of the haemagglutinin (HA) (Ropp et al., 1995), the cytokine response modifier B (CrmB) (Loparev et al., 2001) and the A-type inclusion protein genes (Meyer, Ropp & Esposito, 1997). In these assays, PCR is done by using primers anticipated to amplify a segment of DNA that would be present in any OPV. The PCR amplicon is digested with an appropriate restriction endonuclease and then separated by gel electrophoresis to discriminate species by comparing the fragment profiles with reference virus RFLP profiles. However, when a large set of isolates from an OPV species was analysed, heterogeneity of the resulting restriction fragment patterns became apparent, making interpretation of results rather ambiguous (Meyer et al., 1999).

In order to discriminate OPV species in a one-step assay, a multiplex-PCR assay was recently developed. This method uses unique oligonucleotide primers to identify OPVs at the species level. Four pairs of primers (three pairs for VARV, MPXV and CPXV, respectively, and one genus-specific pair) were used, producing amplicons of various lengths specific for each OPV species (Shchelkunov, Gavrilova & Babkin, 2005). The genus-specific pair serves as an internal PCR control for the presence of OPV DNA in the sample. The specificity and sensitivity of this method were evaluated using DNA of 57 OPV strains, including the DNA prepared from scabs derived from skin lesions of smallpox cases infected in 1970–75, which were deposited with the Russian collection of VARV.

2.5.4 Polymerase chain reaction–restriction fragment length polymorphism

To get a general knowledge of the entire viral genome without sequencing it all, a modified RFLP approach has been applied. PCR is used as a preliminary step to amplify regions spanning the genome. The amplicons are then used as the template DNA for the RFLP technique. This modified technique, known as PCR–RFLP, offers greater sensitivity for the identification of pathogens. PCR–RFLP has been used to discriminate between several OPV species, including VARV (Li et al., 2007). PCR–RFLP analysis was applied to 45 VARVs from CDC and 21 VARVs from SRC VB VECTOR (Babkina et al., 2004a, b; Li et al., 2007) that were selected based on varied geographic distribution and years of isolation. Twenty consensus primer pairs were used to produce 20 overlapping amplicons, covering 99.9% of the VARV genome. A composite dendrogram of all amplicon RFLP profiles differentiated VARV major from VARV minor strains, and strains were generally clustered according to their geographic location or epidemiological history (or both). Despite the impressive advances made in RFLP techniques, the pace of progress in DNA sequencing may circumvent its usefulness in the near future.

2.5.5 Real-time PCR

Conventional PCR methods are now being replaced with real-time PCR assays. In contrast to conventional PCR, real-time PCR combines amplification and detection of target DNA in one vessel, which eliminates time-consuming post-PCR procedures and decreases the

risk of cross-contamination. In addition, real-time PCR provides quantitative information. The recent development of portable real-time PCR machines and lyophilized reagents (Aitichou et al., 2008) raises the exciting prospect that rapid diagnosis (i.e. diagnosis in less than two hours) of disease outbreaks in the field could become a reality.

The many advantages of real-time PCR led to its introduction into the field of poxvirus diagnostics, where it can be used to identify smallpox rapidly and unambiguously, and to differentiate it from other rash-causing illnesses. However, the screening of large OPV strain collections is essential to demonstrate the utility of the assays developed using these methods and to establish their performance characteristics. Generally, in any one assay, less than 0.1% of the genome is sampled to provide the result. One report stated that mismatches in the probes enabled discrimination of VARV from other OPVs by melting curve analysis (Espy et al., 2002), but with the addition of new OPV sequences in GenBank, the probes display identity for camelpox virus (CMLV) and some CPXV strains, which means that a reliable identification of VARV is not possible. It will be important, as additional sequence information becomes available from related viruses, that the various PCR primers and probes are periodically reviewed – in silico, if not via practical laboratory testing – for their actual specificity and sensitivity.

A screening assay for real-time LightCycler PCR identification of VARV DNA was developed and compiled in a kit system under good manufacturing practice conditions, with standardized reagents (Olson et al., 2004). A single nucleotide mismatch, resulting in a unique amino acid substitution in a total of 64 VARV strains, was used to design a hybridization probe pair with a specific sensor probe that allows differentiation of VARV from other OPVs via melting curve analysis. The applicability of this method was demonstrated by amplification of 180 strains belonging to various OPV species (VARV, MPXV, VZV, VACV, CMLV and ectromelia virus [ECTV]), and the VARV melting temperature differed significantly from that of the other OPV strains. In spiked blood donor samples (Schmidt et al., 2005), an assay with a detection limit of 11 DNA copies per procedure enables reliable detection of OPV DNA in viraemic samples. Thus, by early detection of VARV, the sensitivity of this assay could potentially help prevent the dispersion of viral agents by blood transfusion after an act of bioterrorism.

There is another highly sensitive and specific assay for the rapid detection of VARV DNA, using both the SmartCycler and LightCycler platforms (Kulesh et al., 2004). The assay is based on TaqMan chemistry, with the OPV HA gene used as the target sequence. The assay was evaluated in a blind study with 322 coded samples that included genomic DNAs from 48 different isolates of VARV and 25 different strains other than VARV. Another approach (Nitsche, Ellerbrok & Pauli, 2003) uses simultaneous detection of orthopoxvirus-generic and variola-specific genomic regions, which might be beneficial for the analysis of viral mixtures that include VARV. In this assay, VARV shows the highest melting temperature, and any variant displays a lower melting temperature. Additional real-time PCR assays have been published, and other assays are in development in various international laboratories. It should be noted that PCR inhibition can occur, which causes false-negative results; however, this can be checked by proper internal controls. For a summary of recently published VARV real-time PCR assays and their validations, see Table 2.1.

It does need to be stressed that a positive VARV PCR result must be confirmed by amplifying other parts of the genome. The use of multiple assays that target various

portions of the genome, in addition to non–nucleic acid detection and diagnostic assays, will increase confidence in a laboratory-based diagnosis of smallpox. This is especially true in the current absence of naturally occurring disease, when predictive value positive is necessarily near zero.

Table 2.1 Real-time PCR assays for the detection of variola virus

Reference	Target gene (VACV – Copenhagen nomenclature)	Method	Validation with genomic VARV?	Comments
Espy et al., 2002	HA/A56R	LightCycler with hybridization probes; melting curve analysis differentiates VARV from other orthopoxviruses	No Uses cloned VARV DNA fragment to validate	Several CPXV and CMLV strains have identical melting temperatures to VARV
Ibrahim et al., 2003	HA/A56R	TaqMan, VARV-specific probe cleavage	Yes	—
Panning et al., 2004	HA/A56R	LightCycler with hybridization probes; melting curve analysis differentiates VARV from other orthopoxviruses	No An artificial construct was used	Several CPXV strains have identical melting temperatures to VARV
Nitsche, Ellerbrok & Pauli, 2004	Assay 1: Rpo 18 Assay 2: VETF Assay 3: A13L (VARV) Assay 4: A13L (nVAR-OPX)	LightCycler with hybridization probes; melting curve analysis differentiates VARV from other orthopoxviruses	Yes However, data presented uses artificially synthesized VARV fragments	—
Kulesh et al., 2004	Assay 1: B10R Assay 2: B9R Assay 3: HA/A56R	TaqMan, VARV-specific probes	Yes	Assay 1 and 2: some CPXV strains are amplified Assay 3: target nucleic acid is the same as in the assay described by Ibrahim et al. (2003), but with a slightly shortened probe
Olson et al., 2004	14kD/A27L	LightCycler with hybridization probes; melting curve analysis differentiates VARV from other orthopoxviruses	Yes	The assay is commercially available: RealArt Orthopoxvirus LC Kit (Qiagen, Hilden, Germany)
Carletti et al., 2005	CrmB	LightCycler with hybridization probes; melting curve analysis differentiates between orthopox and herpes viruses	Yes However, data presented uses synthetic VARV fragments	Specific identification of VARV has to be performed by restriction enzyme analysis of PCR amplicons
Fedele et al., 2006	CrmB	Two TaqMan probes; one probe is VARV-specific	No An artificial construct was used	—
Scaramozzino et al., 2007	14 kDa/A27L	Two TaqMan probes; one probe is VARV-specific	Yes	
Aitichou et al., 2008	HA/A56R	Two TaqMan probes; one probe is VARV-specific	No Uses cloned VARV DNA fragments to validate	—
Putkuri et al., 2009	HA/A56R	LightCycler with hybridization probes; melting curve analysis differentiates VARV from other orthopoxviruses	No Uses artificially derived VARV DNA fragments to validate	—

Table 2.1 Real-time PCR assays for the detection of variola virus *continued*

Reference	Target gene (VACV – Copenhagen nomenclature)	Method	Validation with genomic VARV?	Comments
Loveless et al., 2009	B9R/B10R	LightCycler with hybridization probes; melting curve analysis differentiates VARV from variola minor virus	No Uses artificially derived VARV DNA fragments to validate	—

CMLV, camelpox virus; CPXV, cowpox virus; CrmB, cytokine response modifier B; HA, haemagglutinin; nVAR-OPX, non-variola orthopoxvirus; PCR, polymerase chain reaction; VACV, vaccinia virus; VARV, variola virus; VETF, virus early transcription factor.

2.5.6 Oligonucleotide microarray analysis

Many of the previously mentioned problems that arise during species-level detection of the viruses can be solved using hybridization of DNA molecules on oligonucleotide microarrays, frequently called microchips. The first method is based on hybridization of a fluorescently labelled amplified DNA specimen with oligonucleotide DNA probes immobilized on a three-dimensional polyacrylamide-gel microchip (microarrays of gel-immobilized compounds [MAGIChip]). The probes identify species-specific sites within the viral CrmB gene. Overall, 59 samples of OPV DNA were analysed, representing six different species, and there was no discrepancy between hybridization and conventional identification results (Lapa et al., 2002).

An alternative oligonucleotide microarray was developed using plain glass slides and the VARV India strain. The target gene is *G3R*, which encodes a chemokine binding protein (Laassri et al., 2003). This microarray-based method simultaneously detects and discriminates between four OPV species pathogenic for humans and distinguishes them from VZV. The authors tested 49 known and blinded samples of OPV DNAs, representing different OPV species and two VZV strains. The oligonucleotide microarray identified all the samples correctly and reliably.

To ensure redundancy and robustness, the microchip contains several unique oligonucleotide probes specific for each virus species. This new procedure takes only three hours and it can be used for parallel testing of multiple samples. Simultaneous analysis of multiple genes can further increase the reliability of the assay.

Another microarray-based method for simultaneous detection and identification of six OPV species (VARV, MPXV, CPXV, CMLV, VACV and ECTV) has been developed, which also allows the discrimination of OPV species from VZV, HSV-1 and HSV-2 (Ryabinin et al., 2006). The sequences of genes *B29R* and *B19R* from VACV strain Copenhagen were used to help identify the corresponding genes in different OPV strains. These were then used to design species-specific microarray oligonucleotide probes. *B29R*, which encodes a CC-chemokine–binding protein, was identified for 86 OPV strains. *B19R*, which encodes a type I interferon–binding protein, was identified for 72 OPV strains. The microarray also contained several oligoprobes for the identification of VZV, HSV-1 and HSV-2.

2.5.7 Sequencing

Sequencing of various PCR amplicons in a diagnostic setting enables allocation of a sample to known relatives after comparison with the respective database. Sequences of the HA gene of more than 200 OPVs are available and have proven useful for phylogenetic studies. These studies confirm the current concept of established OPV species, which, historically, was based on the different phenotypes of the respective species.

A total of 45 epidemiologically varied VARV isolates from 1940 to 1977 have been sequenced (Esposito et al., 2006). The genome is a linear DNA of approximately 186 kilobase pairs, with covalently closed ends. A low degree of sequence diversity suggests that there is probably very little difference in the isolates' functional gene content. This increases the likelihood that sequence-based detection methods will efficiently identify a re-emergent VARV strain. In addition, the low sequence diversity is reassuring and important from a biodefence perspective, because it suggests a high probability of identifying VARV infections if tracking single or multisource outbreaks. The ability to track the virus might be a deterrent to deliberate use in its own right. In addition to the methods described above, a bio-barcode assay using sequencing technologies has been developed for pathogen detection, including VARV; this was evaluated using a synthesized VARV fragment of 30 nucleotides in length (He et al., 2008).

Concern does exist that biotechnology enables the construction of dangerous pathogens from the genetic material of naturally occurring organisms. Based on sequence comparison, CMLV and taterapox virus are the closest relatives of VARV, and just a few thousand mutations could convert such OPV DNA into VARV DNA (Sanchez-Seco et al., 2006).

Poxvirus genome sequences are accessible on the Internet.[7] All eight genera of the subfamily *Chordopoxvirinae* are represented, including 49 VARV sequences.

Progress in sequencing technologies will certainly make them a valuable forensic tool should smallpox re-emerge, since sequencing is one way to verify the virus strain with clarity. Interpretation of such sequence-based forensic analysis will need to be understood in the context of the mutations that may be accumulated via various virus propagation techniques (e.g. animal growth, CAM or tissue culture).

2.6 Protein-based diagnostic assays

Although a number of laboratories are evaluating various antibody preparations for use in antigen-capture detection of OPVs (Czerny, Meyer & Mahnel, 1989), CDC has developed one monoclonal antibody that appears specific for VARV. Currently, only one protein-based diagnostic assay, using polyclonal anti-VACV sera, is available for the detection of OPV. The assay has had limited characterization, but may be of interest for

[7] http://www.poxvirus.org

use and evaluation in field detection of OPV infections in order to evaluate its clinical sensitivity and specificity.

Members of the OPV genus are the only poxviruses that produce an HA antigen, which is detectable by haemadsorption or haemagglutination assays using suitable chicken erythrocytes. Inhibition of haemagglutination and haemadsorption by patient serum is an indicator of OPV infection. These assays, along with gel-diffusion and complement-fixation assays, were classic components of methodologies used to diagnose smallpox in the pre-eradication era (i.e. pre-1970s). These methodologies are not widely or routinely used today, but may be valuable adjuncts to re-evaluate.

2.7 Serology-based diagnostic assays

When virus specimens are not available, antibody assessment by the neutralization test (NT) or other methods may be the only way to define the disease etiology. Another type of test, frequently requested in bioterrorism response awareness, is for the evaluation of residual protection from previous vaccination. However, there is no single routine immunologic test that defines an individual's degree of protection against a poxvirus infection. Protection requires a concert of cell-mediated and humoral immune responses. The presence of neutralizing antibodies generally indicates recovery from an infection, not always protracted protection from future infection.

Neutralizing antibodies against VARV, MPXV, CPXV or VACV may be detectable as early as six days after infection or vaccination; NT efficacy using sera of infected animals or humans ranges from 50% to 95%. Neutralizing antibodies have been detected more than 20 years after vaccinia vaccination or natural infection with other human OPVs (Putz et al., 2005). In the NT, a four-fold rise in antibody titre between serum samples drawn during the acute and convalescent phases is usually considered diagnostic of poxvirus infection. More recently, the neutralizing effects of antibodies against the two infectious forms of the virus have been better characterized. The two forms of infectious virus (mature virus – MV, and extracellular virus – EV) have different membrane structures and different surface membrane proteins that are recognized by the immune system. Neutralizing responses to a number of proteins of the MV are characterized; in contrast, only one protein of the EV (B5) is known to be recognized by a neutralizing antibody response.

Serologic methods currently in use for antibody detection include assays of antibodies against human OPVs. These assays have included the virus NT, the haemagglutination inhibition assay, the enzyme-linked immunosorbent assay (ELISA) and western blots (Putz et al., 2006). The recent description of an OPV immunoglobulin M (IgM) assay could improve investigations of OPV outbreaks, often semiretrospectively (Karem et al., 2005). This technique offers the advantage of measuring recent infection or illness with an OPV. Its sensitivity and specificity when diagnosing recent OPV infection (as assessed during the monkeypox outbreak in the United States) are both approximately 95% when assayed between days 4 and 56 after onset of rash. With appropriate epidemiologic surveillance, these assays may be valuable in evaluating disease incidence; however, antibodies cross-react among members of each poxvirus genus, rendering serology nonspecific for a given virus species (Trojan et al., 2007). Additional tissue culture–based enzyme immunoassays, microplaque reduction assays, and Cellomics-based and fluorescence-activated cell sorting (FACS)–based analyses are also available for the

serologic virus neutralization responses (Eyal et al., 2005; Earl, Americo & Moss, 2003; Borges et al., 2008; Johnson et al., 2008).

Although cell-mediated immune responses play an important role in poxvirus infections and are believed to be crucial for long-term immunity, current routine testing for T cell response is not reliable and reproducible. A recent real-time PCR assay that assesses CD8+ T cell response post-VACV vaccination could be an additional approach to measure variola infection. However, at this time, there is no specificity for this assay, even for OPV, as it simply measures an interferon-gamma response.

2.8 Summary thoughts

There has been a remarkable expansion in the number of nucleic acid diagnostic assays to identify OPVs, including VARV, and a very limited expansion in diagnostic techniques based on immunology, proteins or whole virus. All assays developed to date are research based; none have completed regulatory review and approval processes. The possible need for live VARV for regulatory review of assays is currently being discussed. One nucleic acid–based diagnostic kit is available commercially; however, it is for research purposes only and not for diagnostic use. One commercially available generic OPV antigen capture kit has been developed, also for research purposes only. Research tools include standard PCR assays, followed by OPV species identification via sequencing or RFLP. Serological assays evaluating humoral immune responses (IgG, IgM, neutralization and others) are all research based, and reagents may not be widely available. A defined number of WHO collaborating centres for poxviruses and smallpox, and other specialty laboratories (academic and government) may currently have various capabilities and expertise for poxvirus diagnosis and smallpox identification.

Rigorous comparative review of the assay approaches that have been developed is advised. The assays also need to be reviewed before they can attain clinical diagnostic status. VARV-derived nucleic acid will be needed for assessment of the sensitivity and specificity of nucleic acid tests, especially as current diagnostic platforms become obsolete. The best material (e.g. full genome, amplicons, plasmids) for this needs to be assessed. Review and development of clinical diagnostics will require studies with infectious virus to determine the best preparation and extraction techniques (especially in the case of antigen capture and nucleic acid tests); some of these studies may be done with other infectious OPVs.

Abbreviations

CAM	chorioallantoic membrane
CDC	Centers for Disease Control and Prevention
CMLV	camelpox virus
CPXV	cowpox virus
CrmB	cytokine response modifier B
DNA	deoxyribonucleic acid
ECTV	ectromelia virus
HA	haemagglutinin
HSV	herpes simplex virus
MPXV	monkeypox virus
NT	neutralization test
OPV	orthopoxvirus
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SRC VB VECTOR	State Research Center of Virology and Biotechnology VECTOR
VACV	vaccinia virus
VARV	variola virus
VZV	varicella zoster virus
WHO	World Health Organization

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3 Variola genomics

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Executive summary

Public health importance

New technologies have radically improved our understanding of the genomics of variola virus. This has led to new ways of detecting and diagnosing smallpox, and insight into the evolutionary history of smallpox infections and the reasons for their severity. However, new technologies in synthetic biology have also created unanticipated problems for controlling access to variola genetic materials. This chapter provides an overview of the latest discoveries in variola virus genomics, and discusses how new technologies in genome synthesis could confound existing strategies for containment of the virus.

Progress to date

The complete DNA (deoxyribonucleic acid) sequence of two closely related variola virus genomes was first published in the early 1990s. As a result of an intensified smallpox research agenda, which was approved by the World Health Organization (WHO) Secretariat and begun in 2000, near-complete genome information is now publicly available for 48 geographically distinct isolates of variola virus. These data can be used to improve understanding of variola virus evolution, to develop improved diagnostics, and (with biostructural studies) to provide insights into drug target sensitivities. Working with cloned variola virus genes, researchers have also increased their understanding of interactions and activities of individual variola virus proteins. This provides further important insights into how the virus causes disease in humans.

This chapter summarizes the available genomic information for variola virus, and shows how it has been applied to study the relatedness of the virus to other animal poxviruses, to study virus evolution during human epidemics and to develop diagnostic tests. The chapter discusses the future use of variola virus genomic material in light of new synthetic DNA technologies.

Outcomes and implications

Publicly available genomic information has been used by many international scientists to design highly sensitive virus diagnostics. New information about the relationship between variola virus and other orthopoxviruses is also important for understanding the value and limitations of animal models for human smallpox. As a result of the remarkable expansion in the technologies of DNA synthesis, sequencing and cloning, it is now technically possible to synthesize the entire variola virus genome from scratch, using only publicly available sequence information, and to reconstitute infectious virus using currently available techniques of molecular biology.

Future biodefence strategies need to incorporate new thinking about how best to control the application of these synthetic biology technologies.

3.1 The variola virus genome

The complete DNA (deoxyribonucleic acid) sequence of two closely related variola virus (VARV) genomes was published in the early 1990s. In 2006, a systematic study compared the full genomic sequences from a number of geographically distinct VARV isolates, collected from around the world (Esposito et al., 2006). The general features of the generic VARV genome are illustrated in Figure 3.1, and an analysis of the clade relationship between the sequenced isolates is shown in Figure 3.2. Technically, the genome of only one strain of VARV (Bangladesh-1975) has been sequenced completely; this is the only strain for which the terminal sequences of the ends of the VARV genome have been formally reported (Massung et al., 1994). The terminal sequences, which have a hairpin configuration, are closely related to the orthologous hairpin sequences from vaccinia virus (VACV). We refer to "complete VARV genomic sequences" as those that include all of the genome apart from any strain differences that might be associated with the terminal hairpins and adjacent sequence. Although it has never formally been demonstrated, it is assumed that the one published VARV terminal hairpin sequence is either identical to, or could fully substitute for, the terminal sequences in the other VARV strains.

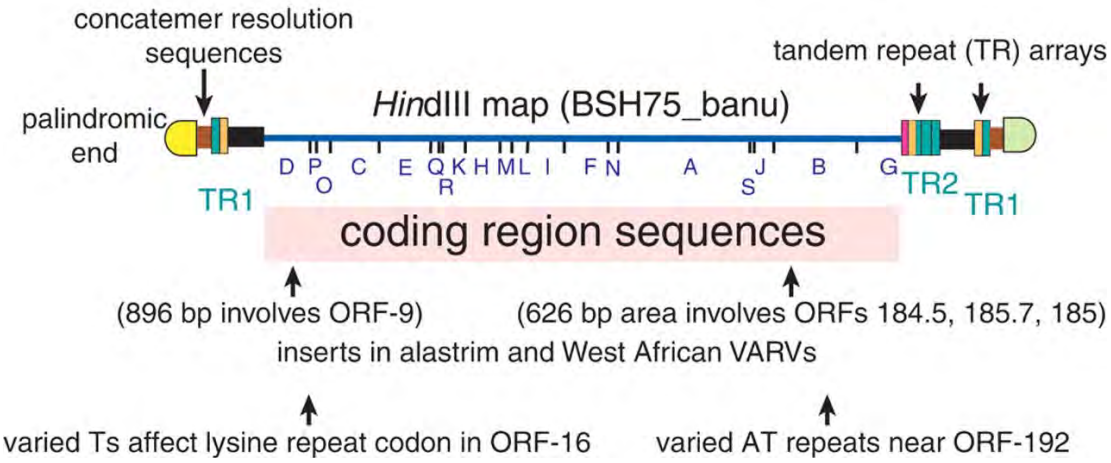

Figure 3.1 Variola virus genome

bp, base pair; ORF, open reading frame; VARV, variola virus.
Source: Esposito JJ et al. (2006). Reproduced with permission.

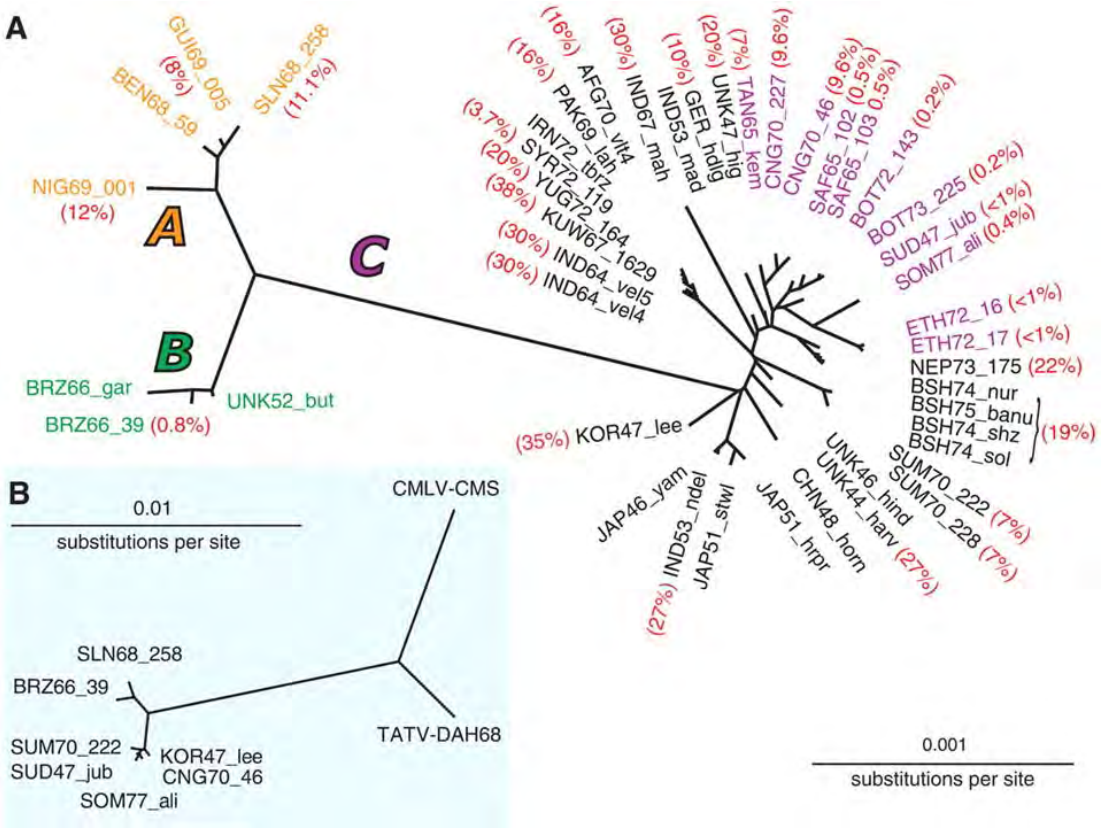

Figure 3.2 Clade relationships between sequenced isolates of variola virus

Phylogenetic relationships between sequenced isolates of VARV. (A) An unrooted consensus phylogram from an alignment of 65 kilobase pairs of the mid–coding region sequences of 45 VARVs reveals three high-level clades that represent clusters of isolates with origins in west Africa (clade A, orange), South America (clade B, green) and Asia (clade C, purple). The Asian clade contains a subgroup of non-west-African African variants (violet) that diverge into viral types of low or midrange case–fatality ratio. Case–fatality ratios (red) associated with some isolates are indicated, and some of these are discussed in the supporting text. (B) A consensus tree rooted using CMLV (camelpox virus)-CMS70 and TATV (taterapox virus)-DAH68 mid–coding region sequences aligned with a subset of VARVs representative of the tree in A.
Source: Esposito JJ et al. (2006). Reproduced with permission.

The database of VARV genomic sequences has expanded rapidly over the past decade because of continued technological advances in DNA sequencing and bioinformatics. One of the first two reported VARV genome sequences was determined using Maxam–Gilbert chemistry (Shchelkunov, Blinov & Sandakhchiev, 1993a, b; Shchelkunov et al., 1993), but all the other sequences were determined using automated Sanger shotgun sequencing (Massung et al., 1993, 1994) or primer walking techniques (Esposito et al., 2006). Gene chips have also been useful tools for rapidly resequencing and identifying VARV strains (Sulaiman et al., 2007; Sulaiman, Sammons & Wohlhueter, 2008). These technological advances have greatly improved the accuracy and speed of sequencing, and have reduced the associated costs dramatically. For example, using current technologies, any genome can be sequenced with about 25-fold read redundancy, in a few days, for a cost of less than US$ 1000.

These genomic sequences provide a rich source of new insights into VARV genetics, evolution, relationships with other orthopoxviruses (OPVs) and co-evolutionary interactions with the human host (Gubser et al., 2004; Shchelkunov, Marennikova & Moyer, 2005). They have also been used to develop diagnostic tools to distinguish VARV from other OPVs that can be detected in biological samples (Li et al., 2007b; Sulaiman et

al., 2007; Sulaiman, Sammons & Wohlhueter, 2008). The sequences, together with the known gene sequences and drug sensitivities of other OPVs, provide evidence that all VARV strains should be sensitive to drug therapeutics such as ST-246 and the cidofovir derivative CMX001. Diagnostic and antiviral advances are covered in Chapter 6 of this report.

As a result of the recent remarkable developments in DNA sequencing, cloning and gene synthesis technologies, it is now technically possible, as described below, to synthesize the entire VARV genome from scratch, using only publicly available sequence information, and to reconstitute an infectious VARV using standard techniques of molecular biology. Thus it may no longer be possible to eradicate the threat of re-emergence of live VARV as a biologic entity, even if the existing virus stocks at the World Health Organization (WHO) collaborating centres in the United States and the Russian Federation are destroyed.

The genomes of all the VARV isolates that have been sequenced to date are closely related to each other, in part because evolutionary drift occurs more slowly in poxviruses than in many other viruses (particularly RNA [ribonucleic acid] viruses such as human immunodeficiency virus and influenza virus). The sequenced VARV genomes, which are approximately 185 kilobases in length, all contain approximately 200 open reading frames (ORFs). These ORFs express proteins with varying degrees of similarity to proteins expressed by other OPVs, such as camelpox virus (CMLV), taterapox virus (GBLV), monkeypox virus (MPXV), cowpox virus (CPXV) and VACV (the vaccine used to eradicate smallpox). Like all poxviruses, the viral genome is double-stranded DNA, with hairpin termini and terminal inverted repeat (TIR) sequences. In contrast to most other poxviruses, the TIR sequences of VARV do not encode any viral proteins, and so all the VARV ORFs are present in a single copy. The function of the hairpin termini is thought to relate strictly to genomic replication, ensuring complete synthesis of all the viral DNA sequences during the virus life cycle. Thus the hairpin termini are likely to be functionally interchangeable between poxviruses.

Within the central region of the VARV genome are clustered approximately 90 highly conserved genes that are orthologues of genes found in other chordopoxvirus genomes (Gubser et al., 2004). These conserved genes are believed to encode the essential elements for poxvirus replication, gene expression and virion morphogenesis. Genes that encode the unique aspects of VARV biology, such as virulence, anti-immune determinants and disease pathogenesis markers, tend to cluster more towards the ends of the genome. The greatest variation in DNA sequences between VARV strains with different case fatality rates appears in the genomic regions closest to the TIR sequences (Shchelkunov, Massung & Esposito, et al., 1995; Massung et al., 1996; Shchelkunov et al., 2000; Esposito et al., 2006). Approximately 90% of VARV genes have clear orthologues in other poxvirus genomes, while truncated versions of the remainder can be found in at least one other OPV. Genetically, the two OPVs most closely related to VARV, with about 98% nucleotide identity over the central 110 kilobases of the genomes, are CMLV and the gerbil pathogen GBLV. The genetic distances between VARV and MPXV, CPXV and

VACV are considerably greater (Shchelkunov et al. 2001; Gubser et al., 2004; Meyer et al., 2005; Shchelkunov, Marennikova & Moyer, 2005).

Among the 48 VARV isolates that have been sequenced and recorded in public databases,[8] individual pairs of VARV genomes can differ by as many as 700 single nucleotide polymorphisms (SNPs) and up to 90 insertion/deletions (indels). The full spectrum of VARV genomic variation – more than 1700 SNPs and 4800 indels – has been documented; overall, however, the sequences are most notable for their close similarities. These sequenced isolates (one of which was sequenced twice, for a total of 49 database entries) are listed at the end of this chapter (Table 3.1).

The VARV isolates selected for genomic sequencing were chosen to represent a broad cross-section of archived strains, with different geographic origins and clinical properties. Further genetic variations would probably be uncovered if the remaining archived VARV isolates were sequenced; approximately 550 such isolates are held at the United States Centers for Disease Control and Prevention and the Russian Federation State Research Center of Virology and Biotechnology VECTOR. This additional information on sequence variation may be of benefit in finer molecular epidemiologic or "forensic" studies of the virus. However, it is unlikely that such additional sequencing would be of value for the specific development of vaccines or diagnostics. If the sequences of specific viral proteins that are targeted by the drugs and therapeutics currently being developed are more diverse than those in the current database, additional sequence information might indicate whether drug-resistant polymorphisms could exist in the VARV stocks that have not yet been sequenced. However, drug development has largely targeted viral genes that are relatively well conserved across VARV and other OPVs, and thus additional sequence information would probably only be of modest intrinsic scientific value.

Further insights into VARV biology have come from study of individually expressed VARV proteins, based on DNA sequence information (Dunlop et al., 2003). For example, proteins encoded by several VARV genes, which have been expressed or synthesized in the laboratory, interact with specific elements of the human immune system, such as serum complement, interleukin-18, interferon-gamma, tumour necrosis factor, chemokines and various cell signalling pathways (Seregin et al., 1996; Rosengard et al., 2002; Esteban, Nuara & Buller, 2004; Kim et al., 2004; Alejo et al., 2006; Gileva et al., 2006; Liszewski et al., 2008; Yadav et al., 2008). Most recently, to gain insights into how many different VARV proteins might physically interact with the complete set of human proteins, researchers used a yeast two-hybrid system to systematically screen all the unique VARV proteins that are not found in VACV against the complete human proteome. This study revealed many new interactions between human and VARV proteins, including a new family of VARV inhibitors of an important human inflammatory signalling cascade that is mediated by nuclear factor kappa B (Mohamed et al., 2009). It is highly likely that further study of individually expressed VARV proteins will provide additional insights into fundamental processes of innate immune response signalling in human cells and lead to the discovery of additional interactions with the host.

[8] http://www.poxvirus.org; http://www.ncbi.nlm.nih.gov/Genbank/

3.2 Variola evolution

All poxviruses continue to diverge from each other by genetic mechanisms that include point mutations, insertions, deletions, various recombination events, and the loss or acquisition of entire genes (Smith, Chan & Howard, 1991; Shchelkunov & Totmenin, 1995; McLysaght, Baldi & Gaut,, 2003; Gubser et al., 2004; Babkin & Shchelkunov 2008). The available genomic sequencing data show several distinctive groups of VARV. Depending on the method of analysis, these groupings can be considered either as three distinct clades, or as two major clades, one containing two subgroups (Esposito et al., 2006; Li et al., 2007a). Members of the first group include isolates of variola major from Asia that are associated with high mortality, and isolates from Africa (especially eastern, central and southern Africa) that are associated with varying mortalities. The second group consists of variola minor (alastrim) from South America, which has lower fatality rates. Closely related isolates from the third group are from western Africa, and are associated with intermediate levels of disease severity. These sequencing studies provide important clues about the spread of smallpox around the world, and about how variola major and minor were beginning to diverge from each other until they were eradicated by the WHO vaccination campaign in 1977.

Depending on how the sequence information is correlated with case studies and historical records, it has been estimated that VARV diverged from an ancestral poxvirus, probably from rodents in Africa, at some time between 16 000 and 68 000 years ago (Li et al., 2007a). Clinical cases were first reported in China, and possibly India. The disease later spread into the Mediterranean basin and Europe, then finally appeared in the New World in the 16th century. Although the historical records do not provide an exact dating for the first smallpox outbreaks in human populations, an analytical approach to VARV evolution has been used to estimate the time at which VARV arose, using cases for which emergence dates and places are documented (Babkin & Shchelkunov 2006; Shchelkunov, 2009). In this approach, the extended central conservative region of the OPV genome (about 102 kilobases) was analysed, along with eight genes of multisubunit RNA polymerase of various genera from the *Poxviridae*. Using the known dating of the smallpox introduction from west Africa to South America (16th–18th centuries) and the data on the close phylogenetic relationships between the modern west African and South American VARV isolates, it was calculated that the average rate of accumulation of mutations in these DNA viruses was 10^{-6} nucleotide substitutions per site per year. Assuming that this rate has been relatively constant over time, OPVs diverged from an ancestral virus into the currently recognized genera at least 130 000 years ago. By this measurement, VARV started its independent evolution 3400 (±800) years ago, most likely around the time it first made the leap from an unknown rodent host into humans (Babkin & Shchelkunov, 2008).

The original ancestral virus that was the evolutionary parent to the current OPVs remains unknown. The best evidence suggests that existing strains of CPXV are more closely related to the original ancestor virus from which the current OPVs are presumably derived, because CPXV contains the largest number of the variable genes, some of which are closely related to orthologues in VARV (Shchelkunov et al., 1998). It is also interesting that all other known OPV genomes, including VARV, contain a number of genes that have been fragmented or inactivated compared with the larger CPXV genome. Whether the first strain of VARV to appear in human populations was variola major or minor is

unknown, but it is clear that these strains were in the process of evolving away from each other by the time of eradication of smallpox (Esposito et al., 2006; Li et al., 2007a).

The rates of evolution of poxviruses tend to be much slower than for many other viruses, probably because of the high fidelity of the poxviral DNA polymerase enzyme that is responsible for copying the viral genetic information during the viral replication cycle. In general, RNA viruses evolve more rapidly, because their mutational frequencies are much higher than for DNA viruses or eukaryotic organisms, and they lack a proofreading exonuclease activity. However, calculations of the rates of genetic drift of VARV over time are difficult, and today we rely more on phylogenetic relationships derived from similarities between nucleic acid and amino acid sequences of viruses that were isolated during the 20th century. We still understand only poorly how VARV evolved into a human-specific pathogen. Its closest genetic cousins, GBLV and CMLV, also exhibit narrow host ranges, whereas poxviruses with the largest genomes (like CPXV) tend to exhibit the widest host ranges in nature.

3.3 Poxvirus genome technologies

Although genome comparisons provide important insights into which poxvirus-encoded gene products could potentially account for the virulence of VARV, these studies alone cannot explain the causes of smallpox disease. Newer technologies of gene manipulation and gene synthesis have provided more recent insights into the biological properties of VARV and VARV-encoded genes. For example, the small complement control proteins of VACV and VARV differ by only 11 amino acid residues, a sufficiently small difference to allow the VACV gene to be converted into its VARV counterpart using site-directed mutagenesis (Rosengard et al., 2002). However, this is a more labour-intensive approach than chemical synthesis of the VARV gene, which is accessible to any laboratory able to pay a modest fee. A particular advantage of gene synthesis is that the DNA sequence can be altered in any desired way; the most common alteration is optimization of the codon choices in the altered gene to improve the efficiency of expression of recombinant proteins in commonly used expression systems. This activity has had the unanticipated consequence of creating DNA clones that may fall outside the purview of national regulations regarding the possession and manipulation of VARV DNA. This is because, while these genes still encode a VARV protein, the DNA is formally (and probably legally as well) no longer VARV DNA.

The cost of gene synthesis continues to decline rapidly, reflecting continuing improvements in the capacity to assemble long, error-free constructs (Czar et al., 2009). By some estimates, the cost is halving, and the achievable length is doubling, every two to three years. Gene synthesis has been used, for example, to synthesize infectious poliovirus de novo, and to resurrect the 1918 pandemic strain of influenza virus (Cello, Paul & Wimmer, 2002; Tumpey et al., 2005). The ability to synthesize any gene to order raises the concern that anyone skilled in the art of DNA synthesis could reconstruct a live OPV using the same approaches. This would not be as straightforward a task as it was for poliovirus (which has a single-stranded positive-sense RNA genome), since naked poxvirus DNA is not infectious and OPV genomes are about 25 times larger; however, all the necessary technical methods exist for synthesizing an intact poxvirus genome and using it to create a live virus. In 2002, infectious VACV was recovered from a full-length viral genome cloned into a bacterial artificial chromosome (Domi & Moss, 2002). Furthermore, it was possible to assemble and reactivate VACVs using mixtures of DNA

fragments transfected into cells that had previously been infected with a helper leporipoxvirus (Yao & Evans, 2003). In the latter study, the DNA comprised a mix of fragments generated using the polymerase chain reaction and VACV restriction fragments; however, while several technical problems complicate these experiments (especially accidental mutations), there is no compelling reason to believe that wholly synthetic fragments could not be used to resurrect live VARV. The estimated expense of synthesizing all of the necessary clones would currently be less than US$ 200 000, and this cost is likely to drop in the future.

De novo synthesis of an intact VARV is not the only way VARV or a VARV-like virus could be created. Although current WHO recommendations prohibit the genetic engineering of VARV, there is no doubt that the many methods employed for genetic modification of poxviruses could be used to modify the virulence of any OPV, including VARV. For example, OPV genomes are readily altered using homologous recombination, and drug-resistance markers are easily introduced into normally drug-sensitive strains. Similarly, the insertion of host immunoregulatory genes has the potential to alter poxviral virulence or the sensitivity of the infection to previous vaccination. Hybrid viruses also represent a potential hazard, and would be much easier to assemble than wild-type VARV. Nearly 50 years ago, it was shown that recombinant OPVs are viable, and that a recombinant can be produced by coinfecting cells with VARV and rabbitpox virus (a VACV strain) (Bedson & Dumbell, 1964a) or CPXV (Bedson & Dumbell, 1964b). Whether these laboratory hybrids would be pathogenic in humans is unknown and untestable. Malignant rabbit virus, a recombinant between harmless (Shope fibroma) and virulent (myxoma) leporipoxviruses, retains much of the virulence of its myxoma virus parent (Opgenorth et al., 1992). One analysis, in fact, suggests that VARV strain alastrim could be a natural hybrid derived from recombination between west African and Asian VARV strains (Esposito et al., 2006). No obvious technical or biological barrier would prevent the substitution of one OPV gene for another, the incorporation of a gene unique to pathogenic OPVs into VACV, the replacement of homologous parts of one genome with synthetic segments copied from another virus, or the generation of hybrid viruses (for example, a hybrid of MPXV and CMLV) with potentially novel virulence patterns that could mimic those of VARV. Certainly, the ability to mix and match virus genes within recombinant OPVs is a sobering thought, especially if drug resistance alleles of target genes were to be engineered into a reconstructed virus.

3.4 Guidelines for variola virus genomes

The advances in genomic technologies discussed above require a reappraisal and updating of current VARV containment strategies. These strategies were designed in the 1980s, and have been revised frequently since then. The possibility that poxviruses could be recovered from cloned DNA using reactivation methods is why no single laboratory (other than the two WHO collaborating centres) is permitted to retain more than 20% of the VARV genome, and why any manipulation of VARV DNA must be geographically isolated from work involving the storage or propagation of other poxviruses. The existing controls focus sensibly on physical and administrative control of access to live virus or cloned fragments of the variola genome; they certainly remain relevant and need to be retained, along with prohibitions on performing activities such as the deliberate introduction of VARV genes into other poxviruses. However, when these procedures and guidelines were developed, nobody anticipated that, 25 years later, advances in genome sequencing and gene synthesis would render substantial portions of VARV accessible to

anyone with an internet connection and access to a DNA synthesizer. That "anyone" could even be a well-intentioned researcher, unfamiliar with smallpox and lacking an appreciation of the special rules that govern access to VARV genes.

This problem has been discussed by a number of authors, and particularly by researchers in the field of "synthetic biology". In 2007, many of the issues associated with advances in DNA synthesis technologies were described, and a proposal was outlined for managing biological security (Bügl et al., 2007). As far as the authors of this chapter are aware, these proposals have not been adopted by any Member States as official policy; however, they have been adopted as operating principles by some of the commercial companies engaged in these activities. For example, GENEART, an industry leader in the field of large-scale gene synthesis, uses BLAST searches to filter all requests for its services against the lists of controlled pathogens – including VARV and MPXV – that have been identified by the Australia Group.[9] Customers requesting synthetic services that match with these lists are required to identify themselves and provide the necessary import and export documents.

The authors of this chapter strongly recommend that companies and institutions offering such services adhere to surveillance guidelines. However, this type of surveillance would not cover scientists carrying out gene synthesis with their own equipment. It might be worth examining the possibility of hardwiring technology into commercial gene synthesis devices to prevent such activities; just as many paper copy printers in use today bear pre-emptive chips that prevent the reproduction of currency.

Finally, genome technologies have also greatly changed our understanding of the evolutionary relationships among OPVs, with implications for containment of viruses closely related to VARV. Most aspects of current virus containment policies were devised in an environment in which the genetic relationship between VARV and other zoonotic poxviruses (like MPXV) was still uncertain, and of most practical concern to health authorities. MPXV has long been recognized as a human zoonotic pathogen deserving special regulatory attention; however, it is now known that GBLV and CMLV are actually the closest extant relatives of VARV, and genomics permits a precise determination of the gene differences. For example, VARV (strain Congo) encodes approximately 8 genes not found in CMLV, and CMLV encodes approximately 16 genes not found in VARV. There is little evidence to suggest that wild-type GBLV and CMLV are major human health hazards per se; however, as research continues to provide new insights into the functions of these genes, some future review of biocontainment and research policies for OPVs closely related to VARV may be wise.

[9] http://www.australiagroup.net

Table 3.1 Variola virus strain genomic sequences in the public database

Variola virus	Repository description	Year isolated	Sample origin	Sequences determined	Coding region sequences	Putative ORFs	GenBank accession number
BEN68_59	V68-59, Dahomey	1968	Benin	187 070	185 591	205	DQ441416
BOT72_143	V72-143	1972	Botswana	185 931	184 186	203	DQ441417
BOT73_225	V73-225	1973	Botswana	185 931	184 126	201	DQ441418
CNG70_46	V70-46 Kinshasa	1970	Congo region	186 553	184 140	203	DQ437583
CNG70_227	V74-227 Gispen Congo 9	1970	Congo region	186 652	184 093	200	DQ441423
ETH72_16	Eth16 R14-1X-72 Addis	1972	Ethiopia	186 648	184 152	202	DQ441424
ETH72_17	Eth17 R14-1X-72 Addis	1972	Ethiopia	186 648	184 152	201	DQ441425
GUI69_005	V69-005 Guinea	1969	Guinea	186 883	185 579	204	DQ441426
NIG69_001	Import from Nigeria	1969	Niger	186 942	185 707	205	DQ441434
SAF65_102	102 Natal, Ingwavuma	1965	South Africa	186 050	184 315	200	DQ441435
SAF65_103	103 T'vaal, Nelspruit	1965	South Africa	185 881	184 148	202	DQ441436
SLN68_258	V68-258	1969	Sierra Leone	187 014	185 763	204	DQ441437
SOM77_ali	V77-2479 last case	1977	Somalia	186 231	184 155	202	DQ437590
SOM77_1252	V77-1252	1977	Somalia	184 191	—	—	DQ441438
SOM77_1605	V77-1605	1977	Somalia	184 170	—	—	DQ441439
SUD47_jub	Juba (alastrim phenotype)	1947	Sudan	186 284	184 208	201	DQ441440
SUD47_rum	Rumbec	1947	Sudan	186 415	—	—	DQ441441
TAN65_kem	Kembula	1965	Tanzania[a]	185 826	184 085	198	DQ441443
AFG70_vlt4	Variolator-4	1970	Afghanistan	185 855	184 062	203	DQ437580
BSH74_nur	Nur Islam	1974	Bangladesh	186 293	183 534	196	DQ441420
BSH74_shz	Shahzaman	1974	Bangladesh	186 293	183 534	197	DQ441421
BSH74_sol	Solaiman	1974	Bangladesh	186 293	183 534	197	DQ441422
BSH75_banu	V75-550 re-sequence	1975	Bangladesh	185 976	183 562	201	DQ437581
CHN48_horn	China Horn Sabin lab	1948	China	186 668	184 188	204	DQ437582
IND53_mad	Kali-Muthu-Madras	1953	India	186 108	184 173	201	DQ441427
IND53_ndel	New Delhi	1953	India	186 662	184 178	201	DQ441428
IND64_vel4	7124 Vellore	1964	India	186 677	184 051	205	DQ437585
IND64_vel5	7125 Vellore	1964	India	186 127	184 058	202	DQ437586
IND67_mah	Vector Maharastra E6	1967	India	185 578	184 151	198	NC_001611

Table 3.1 Variola virus strain genomic sequences in the public database *continued*

Variola virus	Repository description	Year isolated	Sample origin	Sequences determined	Coding region sequences	Putative ORFs	GenBank accession number
JAP46_yam	Yamada MS-2A Tokyo	1946	Japan	186 662	184 178	203	DQ441429
JAP51_hrpr	Harper Masterseed	1951	Japan	186 180	184 179	202	DQ441430
JAP51_stwl	Stillwell Masterseed	1951	Japan	186 115	184 798	201	DQ441431
KOR47_lee	Lee Masterseed	1947	Korea[b]	186 383	184 102	203	DQ441432
KUW67_1629	K1629	1967	Kuwait	185 853	184 060	199	DQ441433
NEP73_175	V73-175	1973	Nepal	185 654	183 517	202	DQ437588
PAK69_lah	Rafiq Lahore	1969	Pakistan	185 865	184 072	203	DQ437589
SUM70_222	V70-222	1970	Sumatra	185 449	184 197	202	DQ437591
SUM70_228	V70-228	1970	Sumatra	185 405	184 564	199	DQ441442
SYR72_119	V72-119	1972	Syria[c]	185 853	184 060	203	DQ437592
GER58_hdlg	Heidelberg, from India	1958	Germany	184 900	184 168	201	DQ437584
UNK44_harv	Harvey Middlesex	1944	United Kingdom	185 771	184 184	203	DQ441444
UNK46_hind	Hinden	1946	United Kingdom	186 096	184 093	198	DQ441445
UNK47_hig	Higgins Staffordshire	1947	United Kingdom	185 026	184 225	200	DQ441446
UNK52_but	Butler alastrim	1952	United Kingdom	188 251	185 845	207	DQ441447
YUG72_164	Yugoslavia from Iraq	1972	Yugoslavia[b]	185 851	184 058	201	DQ441448
BRZ66_39	V66-39 alastrim	1966	Brazil	188 062	185 725	207	DQ441419
BRZ66_gar	Garcia alastrim	1966	Brazil	186 986	185 846	207	Y16780

ORF, open reading frame.
[a] Now the United Republic of Tanzania.
[b] Current name of country unknown.
[c] Now the Syrian Arab Republic.

Abbreviations

CMLV	camelpox virus
CPXV	cowpox virus
DNA	deoxyribonucleic acid
GBLV	gerbilpox virus (taterapox virus)
indels	insertion/deletions
MPXV	monkeypox virus
OPV	orthopoxvirus
ORF	open reading frame
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
TIR	terminal inverted repeat
VACV	vaccinia virus
VARV	variola virus
WHO	World Health Organization

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4 The status of WHO collaborating centre repositories of variola virus and nucleic acid

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Executive summary

This chapter summarizes the status (as of January 2010) of live variola virus (VARV) stocks and VARV DNA (deoxyribonucleic acid) stocks, and – where appropriate – use and distribution of VARV gene fragments, as per World Health Organization (WHO) recommendations.

In 1976, as efforts to eradicate smallpox met with increased success, the WHO Smallpox Eradication Unit initiated attempts to reduce the number of VARV stocks held in laboratories. As a result, the number of laboratories self-reporting VARV stocks to the Global Commission for Smallpox Eradication decreased from 75 to 7 by December 1979, and subsequently to 4 by 1981. The remaining stocks were located in the Russian Federation, South Africa, the United Kingdom and the United States.

In 1982, VARV stocks from Porton Down in the United Kingdom were transferred to the United States, to the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia. The virus stocks in South Africa, which were maintained at the National Institute for Virology in Sandringham, were destroyed in 1983 (although South Africa still retains cloned, non-infectious VARV fragments).

In May 1996, resolution WHA 33.4 of the World Health Assembly endorsed recommendations for the post-smallpox eradication era. The resolution specified that the remaining repositories of VARV should be held at a limited number of sites. The collection has since been reduced, and is currently restricted to two laboratories: the WHO Collaborating Centre on Smallpox and Other Poxvirus Infections at the CDC, and the WHO Collaborating Centre for Orthopoxvirus Diagnosis and Repository for Variola Virus Strains and DNA at the Russian State Research Centre of Virology and Biotechnology VECTOR (SRC VB VECTOR) in Koltsovo, Novosibirsk Region, the Russian Federation.

Annual reports from these two laboratories are submitted to a WHO Secretariat. The reports cover use of live VARV and the status of the repositories. Since 2000, these reports have also been made in person at the annual meetings of the WHO Advisory Committee on Variola Virus Research, which are convened to review work with live VARV. Abstracts of these presentations are available online, via the WHO web site.[10]

[10] http://www.who.int/csr/disease/smallpox/research/en/index.html

4.1 Introduction

In 1976, as the success of smallpox eradication efforts became increasingly apparent, the World Health Organization (WHO) Smallpox Eradication Unit initiated attempts to reduce the number of variola virus (VARV) stocks held in various laboratories. The number of laboratories self-reporting VARV stocks to the Global Commission decreased from 75 in 1976 to 7 by December 1979, and subsequently to 4 by 1981. These remaining stocks were held in the Russian Federation, South Africa, the United Kingdom and the United States.

In 1982, VARV stocks from Porton Down in the United Kingdom were transferred to the site of the WHO Collaborating Centre on Smallpox and Other Poxvirus Infections in the United States, at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia.

In 1983, the VARV stocks maintained at the National Institute for Virology in Sandringham, South Africa, were destroyed (although South Africa still retains cloned non-infectious VARV fragments).

In May 1996, resolution WHA 33.4 of the World Health Assembly endorsed recommendations for the post-smallpox eradication era. The recommendations specified that remaining repositories of VARV should be held at a limited number of sites. The collection of VARV has since been reduced, and is currently restricted to two repositories:

- the WHO Collaborating Centre on Smallpox and Other Poxvirus Infections at the CDC in Atlanta, Georgia, United States;
- the WHO Collaborating Centre for Orthopoxvirus Diagnosis and Repository for Variola Virus Strains and DNA at the Russian State Research Centre of Virology and Biotechnology VECTOR (SRC VB VECTOR; referred to here as “VECTOR”) in Koltsovo, Novosibirsk Region, the Russian Federation.

4.2 The status of strains of variola virus and nucleic acid repositories at the WHO collaborating centre in the Russian Federation

4.2.1 The state of the variola virus strains collection and its repository

The Russian Federation’s collection of VARV was started in the mid-1950s, at the Mechnikov Research Institute of Vaccines and Sera, in Moscow. It continued at the Research Institute of Viral Preparations (RIVP), where the WHO Collaborating Centre for Smallpox and Related Infections started work in 1967. The collection has been used for diagnostic studies for the Global Smallpox Eradication Programme throughout its existence. More than half of the 120 VARV strains and isolates available in the collection were studied in detail by classical biological markers from 1960 to 1975; the rest were identified as VARV at isolation, but were not studied in detail.

The transfer of the VARV strain collection from RIVP to VECTOR was organized and carried out under the Joint Order of the Ministry of Health and Medical Industry of the Russian Federation, the Ministry of Education and Science of the Russian Federation, the State Committee for Sanitary and Epidemiological Surveillance (Goskomsanepidnadzor) of the Russian Federation, and RAMS N 187/123/105/71, dated 8 September 1994. It was

confirmed by the *Joint statement on the transfer of the variola virus collection from the Research Institute of Viral Preparations RAMS to the Russian Federation SRC NPO VECTOR*, dated 27 September 1994. According to the Decree of the Government of the Russian Federation N 725–47, dated 24 June 1996, the VECTOR microorganism collection, including the VARV strains collection, was included in the list of official collections of the Russian Federation. This situation was confirmed by Departmental Order N33 of the Ministry of Health and Medical Industry of the Russian Federation dated 21 August 1996.

On 19 June 1997, WHO officially registered the establishment of the WHO Collaborating Centre for Orthopoxvirus Diagnosis and Repository for Variola Virus Strains and DNA at VECTOR (WHO letter dated 19 June 1997 – outgoing N LTS S2/180/4, LTS S2/286/3). This move was executed at Russian Federation national level by the Order of the Ministry of Health of the Russian Federation N 300, dated 9 October 1997. Subsequently, this was confirmed by the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor) N 772, dated 16 November 2005. At the international level, the right to store the collection of VARV strains at VECTOR was acknowledged by the decision of the WHA 49.10, and confirmed by the subsequent decisions of WHA 52.10, 55.15 and 60.1.

Organization of, and experimentation with, the VARV collection at the WHO collaborating centre are in compliance with national and international requirements, and the recommendations of the WHO Global Commission. Instructions regulating research, as well as all maintenance and control procedures, have been developed on the basis of the documents listed above. Plans have been developed for anti-epidemic measures and response to accidents, and emergency teams have been established for activation in case of accidents and emergency situations.

Between 1995 and 2009, WHO experts made six inspection visits to VECTOR to evaluate the conditions for biological and physical safety of work with VARV. The experts confirmed that conditions complied with international requirements.

Officials responsible for the VARV collection at VECTOR were appointed by the VECTOR General Director. The transfer of responsibility for the collection as VECTOR officials replaced one another – as a result of change in command – was carried out through the commission inventory, with Statements of Transfer approved by the General Director. At the time of writing in 2010, the VARV collection comprises 120 strains (Table 4.1), originating from Asia, Africa, the Americas, the Eastern Mediterranean and Europe.

Table 4.1 Geographical distribution of origin of the variola virus strains held at VECTOR

Geographical distribution of strain isolation according to WHO regions	Country of strain isolation[a]	Number of strains
South-East Asia	Bangladesh	3
	India	13
	Indonesia	10
	Nepal	8
Africa	Botswana	7
	Burundi	1
	Congo	6
	Ethiopia	12
	Kenya	4
	Rwanda	6
	Somalia	3
	Sudan	1
	Tanzania[b]	8
	Zaire[c]	1
Americas	Brazil	8
Eastern Mediterranean	Kuwait	3
	Oman	4
	Pakistan	14
Europe	USSR[d]	7
	United Kingdom	1
Total:		120

WHO, World Health Organization.
[a] Names of countries are those used at the time of strain isolation.
[b] Now the United Republic of Tanzania.
[c] Now the Democratic Republic of the Congo.
[d] Now the Russian Federation.

VECTOR carries out research with live VARV in a specialized laboratory building – a detached, four-storey building with a total floor area of 6330 m^2. The building is located on guarded territory, with access controlled around the clock by armed guards, on-duty personnel and engineered security systems. The VARV collection is permanently stored in a guarded repository of the building, designated specifically for work with VARV. Access to the repository is limited by specific instructions and rules, which mandate that two staff members are present at all times. The repository, and the –70 °C freezers in which the collection is stored, are continually monitored and equipped with appropriate alarm signalling systems; levels of redundancy are built into the systems and equipment.

The cultures of VARV strains are stored in different forms:

- frozen
  - embryonated chicken eggs (ECEs) on the chorioallantoic membrane (CAM)
  - ECE CAM homogenates
  - cell lysates infected with VARV virus

- freeze-dried
- scabs from smallpox patients.

Air-tight tubes with the frozen strains, air-tight ampoules of the freeze-dried cultures, and air-tight tubes with the scabs from smallpox patients are all stored in air-tight metal containers that are maintained in freezers at –70 °C. All work with VARV is done in the building designated for this purpose, in biosafety level 4 facilities. In 2009, to increase the safety of working conditions, the number of glass vials containing VARV strains was minimized, and the contents of these vials were transferred into polypropylene cryovials.

The Russian Federation collection comprises 120 strains. Analysis of viability has been performed on 59 VARV strains from this collection, and 32 strains proved to be viable (see Table 4.2). Analysis of the viability of the remaining 61 VARV isolates is proposed. Full-length DNA (deoxyribonucleic acid) is stored from 39 strains – 32 viable strains and 6 non-viable strains from Table 4.2, and an additional strain (Ind-70). DNA of strain Ind-70 was extracted from material stored in a frozen state, without preliminary virus recovery on ECE CAM (see Table 4.3).

VARV was isolated from the stored cultures by diluting the material in nutrient medium, preparing serial 10-fold dilutions and applying these to CAM of 12-day ECE. After three days of incubation at 34.5 °C (±0.5 °C), the ECEs were opened and the number of specific lesions (pocks) on ECE CAM was counted to determine the biological activity (titre) of the culture.

No viable VARV was detected after infecting ECE with material prepared from homogenized CAM stored in the Russian VARV collection in the frozen state. An attempt was made to perform cumulative passages on a monolayer of Vero cell culture. However, no viable VARV was detected, even after three successive “blind” passages followed by inoculation of these cell culture homogenates onto ECE CAM. The initial and working collection materials were nevertheless stored for use in further studies (e.g. using molecular genetics methods). Results suggest that some of the cultures stored in the frozen state could have lost their biological activity (i.e. their infectivity).

Viable VARV was found in all freeze-dried materials (see Table 4.2). These strain cultures were produced in a monolayer of Vero cell culture, to obtain VARV material required for the subsequent work on DNA isolation. This procedure used materials from previous passages stored in the collection, to avoid additional passages of the VARV.

Table 4.2 Viability of variola virus strains held at VECTOR

No.	Variola virus strain	Form of material storage[a]	Titre (pock-forming units/ml)
1	M-Abr-60	Freeze-dried	1.5×10^6
2	Aziz	Freeze-dried	0.9×10^6
3	M-Sok-60	Freeze-dried	1.2×10^6
4	M-Sur-60	Freeze-dried	2.5×10^6
5	Semat	Freeze-dried	1.0×10^6
6	12/62	Freeze-dried	9.6×10^5
7	Ind-4a	Freeze-dried	1.5×10^7
8	Helder	Freeze-dried	1.8×10^6
9	Rw-18	Freeze-dried	1.3×10^5
10	M-N-60	Freeze-dried	8.1×10^4
11	22/62	Freeze-dried	3.0×10^6
12	Mary	Freeze-dried	2.0×10^6
13	M-A-60	Freeze-dried	1.0×10^3
14	M-Bl-60	Freeze-dried	3.0×10^6
15	Butler	Freeze-dried	8.0×10^5
16	6–58	Freeze-dried	7.0×10^5
17	Ngami	Freeze-dried	6.1×10^7
18	Kuw-5	Freeze-dried	2.9×10^6
19	Ind-3a	Freeze-dried	7.2×10^7
20	Congo-2[b]	Freeze-dried	8.8×10^7
21	Congo-9[b]	Freeze-dried	8.7×10^7
22	Taj Barin	Freeze-dried	5.5×10^5
23	Wsim Ahmed	Freeze-dried	4.4×10^5
24	13/62	Freeze-dried	1.3×10^6
25	M-Gavr-60	Freeze-dried	9.6×10^5
26	India 378	Scabs	0.8×10^3[c]
27	Khateen	Scabs	$5–10$[c]
28	India 71	Scabs	$5–10$[c]
29	Brazil 128	Scabs	$5–10$[c]
30	Brazil 131	Scabs	$5–10$[c]
31	Aslam	Scabs	1.5×10^3[c]
32	Zaire 1028	Scabs	Material isolated in the second passage in VERO cell culture
33	Dub-1	Frozen CAM	Material not isolated
34	Dub-3	Frozen CAM	Material not isolated
35	Dub-4	Frozen CAM	Material not isolated
36	Dub-5	Frozen CAM	Material not isolated
37	Kuw-28	Frozen CAM	Material not isolated
38	Kuw-29	Frozen CAM	Material not isolated
39	Indon-1	Frozen CAM	Material not isolated
40	Indon-2	Frozen CAM	Material not isolated
41	Indon-3	Frozen CAM	Material not isolated
42	Indon-4	Frozen CAM	Material not isolated
43	Indon-5	Frozen CAM	Material not isolated
44	Indon-6	Frozen CAM	Material not isolated
45	Indon-7	Frozen CAM	Material not isolated
46	Indon-8	Frozen CAM	Material not isolated
47	Indon-9	Frozen CAM	Material not isolated

Table 4.2 Viability of variola virus strains held at VECTOR *continued*

No.	Variola virus strain	Form of material storage[a]	Titre (pock-forming units/ml)
48	Indon-10	Frozen CAM	Material not isolated
49	Nepal 21	Scabs	Material not isolated
50	India 164	Scabs	Material not isolated
51	India 294	Scabs	Material not isolated
52	Nepal 89	Scabs	Material not isolated
53	Rais	Scabs	Material not isolated
54	Ethiopia 142	Scabs	Material not isolated
55	Ethiopia 182	Scabs	Material not isolated
56	Abd. Jalil	Frozen CAM	Material not isolated
57	Abid	CAM homogenate	Material not isolated
58	Nep-67	Frozen CAM	Material not isolated
59	Nepal-53	Frozen CAM	Material not isolated

CAM, chorioallantoic membrane.
[a] All materials, regardless of their storage form, are stored in hermetic containers at –70 ºC.
[b] The culture was prepared in 1996 after the first passage of the virus isolated from the patients' scabs on ECE CAM.
[c] Biological concentration is given in pock-forming units/mg.

Despite all primary isolates from patients (scabs) being stored under equivalent conditions at –70 °C, viable virus was isolated only from 7 of 14 isolates studied. In contrast to freeze-dried material, cumulative passages of several strains were performed in a monolayer of Vero cell culture, to isolate material from scabs. The scabs were macerated for 12 hours in 0.5 ml of RPMI-1640 medium at a temperature of 4 °C, and crushed. The homogenate thus obtained was used to infect the cell monolayer. Infected monolayers were incubated at 34.5 °C (±0.5 °C) until there were signs of the cytopathic effect (CPE) characteristic of VARV. CPE usually developed on day 2 post-infection. On day 3, the cells were scraped off, resuspended in a small amount of medium, destroyed by freezing and thawing, supplemented with 10% glycerol solution, and then frozen. This procedure generated the necessary amount of VARV material, and DNA preparations of 27 VARV strains were isolated in amounts sufficient for conducting long polymerase chain reaction (LPCR).

The storage of the VARV collection and work with VARV strains at VECTOR was thus in accordance with national and international requirements, and WHO recommendations. In summary, 59 of the 120 VARV strains at the WHO collaborating centre have been studied for viability, and 32 strains proved viable. Viability studies have not yet been performed on the remaining 61 VARV strains. Controlled storage and work with the VARV collection approved by WHO are performed in a building designated specifically for work with VARV.

4.2.2 The state of the VECTOR variola virus DNA collection and its repository

Work on the study of the structural and functional organization of orthopoxvirus (OPV) genomes has been conducted at VECTOR since 1991. Sequencing of the full genome (excluding the terminal hairpins) of India-1967, a highly virulent VARV strain, was completed in 1992. For the first time, the genetic map of VARV had been completed, and a thorough analysis of the genome structure was performed. Between 1993 and 1995, VECTOR, in collaboration with the CDC, sequenced the genome of Garcia-1966, a low-virulence VARV strain. Work on isolating the VARV genomic DNA based on the VARV

strains collection has been undertaken since 2001. In recent years, a collection of 39 DNA preparations of different VARV strains has been created at VECTOR.

In 1986, the fourth meeting of the WHO Committee for Orthopoxviral Infections decided to eliminate all collections of VARV strains and their genomic DNAs. However, it was then necessary to conserve the genetic material of different VARV isolates in a reliable and biologically safe form, because these are extremely important for future research. At present, the VARV DNA repository consists of three repositories:

- VARV genomic DNA;
- amplicon collections (each of which corresponds to an individual VARV strain, and has a short code name with a serial number);
- recombinant plasmid collections (each of which is assigned a code name with a serial number).

The accounting unit at the VECTOR international repository of VARV DNA is a labelled plastic microvial.

The repository is located in a guarded building designated for work with VARV, and equipped with a guard and an automatic temperature monitoring and alarm system. This repository stores 5438 vials, including:

- 197 vials of full-length VARV genomic DNA (39 different VARV strains) in the form of solution, stored at +4 °C;
- 1446 vials comprising 17 individual collections of amplicons with VARV DNA fragments, stored at –70 °C; each collection contains amplicons with genome fragments of a single VARV strain, in the form of three repeating sets
  - the first set in the form of alcohol precipitate
  - the second set in the form of alcohol precipitate
  - the third set in the form of solution in LPCR mixture;
- 3795 vials comprising 16 individual collections of recombinant plasmids with VARV DNA fragments, stored at –70 °C; each collection contains recombinant plasmids with genome fragments of a single VARV strain in the form of three repeating sets
  - the first set in the form of solution in Tris-EDTA (TE) buffer
  - the second set in the form of alcohol precipitate
  - the third set in the form of alcohol precipitate.

VARV DNA fragments have not been transferred to any external organizations.

Isolation of DNA of variola virus cultivated in cell culture

Preparations of full-length VARV DNA are stored at +4 °C at the VECTOR repository, in labelled plastic microvials. Currently, preparations of 39 VARV DNA strains from different geographical regions are stored in 197 vials, as outlined in Table 4.3.

Table 4.3 Inventory list of full-length variola virus DNA

No.	Strain	Distribution according to WHO regions	Country of strain origin[a]	Year of isolation
1	Brazil 128	AMR	Brazil	Unknown
2	Brazil 131	AMR	Brazil	Unknown
3	Congo-2	AFR	Congo	1970
4	Congo-9	AFR	Congo	1970
5	Butler	EUR	Great Britain[b]	1952
6	Ind-4a	SEAR	India	1967
7	Ind-3a	SEAR	India	1967
8	Ind-70	SEAR	India	1975
9	India 164	SEAR	India	1975
10	India 71	SEAR	India	1975
11	India 378	SEAR	India	1975
12	Indon-3	SEAR	Indonesia	1971
13	Indon-9	SEAR	Indonesia	1971
14	Kuw-29	EMR	Kuwait	1967
15	Kuw-5	EMR	Kuwait	1967
16	Nepal 89	SEAR	Nepal	Unknown
17	6–58	EMR	Pakistan	1958
18	Wsim Ahmed	EMR	Pakistan	1970
19	Rais	EMR	Pakistan	1970
20	Khateen	EMR	Pakistan	1970
21	Taj Barin	EMR	Pakistan	1970
22	Aslam	EMR	Pakistan	1970
23	Aziz	EMR	Pakistan	1970
24	Rw-18	AFR	Rwanda	1970
25	Mary	AFR	Tanzania[c]	1962
26	13/62	AFR	Tanzania[c]	1962
27	Helder	AFR	Tanzania[c]	1962
28	22/62	AFR	Tanzania[c]	1962
29	12/62	AFR	Tanzania[c]	1962
30	Semat	AFR	Tanzania[c]	1962
31	Ngami	AFR	Tanzania[c]	1962
32	M-Gavr-60	EUR	USSR[d]	1960
33	M-Bl-60	EUR	USSR[d]	1960
34	M-Sok-60	EUR	USSR[d]	1960
35	M-Abr-60	EUR	USSR[d]	1960
36	M-N-60	EUR	USSR[d]	1960
37	M-Sur-60	EUR	USSR[d]	1960
38	M-A-60	EUR	USSR[d]	1960
39	Zaire 1028	AFR	Zaire[e]	Unknown

AFR, WHO African Region; AMR, WHO Region of the Americas; EMR, WHO Eastern Mediterranean Region; EUR, WHO European Region; SEAR, WHO South-East Asia Region.

[a] Names of countries are those used at the time of strain isolation.

[b] Now the United Kingdom.

[c] Now the United Republic of Tanzania.

[d] Now the Russian Federation.

[e] Now the Democratic Republic of the Congo.

Conservation of genetic material of different variola virus strains from the Russian Federation collection

Repository of the collections of variola virus DNA amplicons

The LPCR method allowed the creation of the repository of amplicons of full VARV genomes, and the collection of hybrid plasmids carrying VARV DNA fragments. This method was used to conserve VARV genetic material. The VARV genome sequences listed in Table 4.3 can be stored for a long time in a biologically safe form. This allows for study of the genetic organization of VARV strains, and for the development of modern methods for rapid diagnostics of VARV and other OPVs.

Certified collections of VARV DNA amplicons currently include preparations of 17 VARV strains (see Table 4.4). Data on the number of vials in collections of VARV DNA amplicons are summarized in Table 4.5. Degradation of DNA in the amplicon collections has not been studied.

Table 4.4 Variola virus DNAs used to create amplicon collections

Geographical region of strain isolation	Country of strain isolation[a]	Strain	Year of isolation	No. in repository collection	Epidemiological type of the virus
AFR	Rwanda	Rw-18	1970	MA 13	Variola major
AFR	Tanzania[b]	13/62	1962	MA 9	Variola major
AFR	Tanzania[b]	12/62	1962	MA 10	Variola major
AFR	Tanzania[b]	Helder	1962	MA 12	Variola major
AFR	Tanzania[b]	Mary	1962	MA 15	Variola major
AFR	Tanzania[b]	Ngami	1962	MA 16	Variola major
AMR	Brazil	Brazil 131	Unknown	MA 6	Variola minor alastrim
EMR	Pakistan	Aziz	1970	MA 8	Variola major
EMR	Pakistan	6–58	1958	MA 11	Variola major
EMR	Pakistan	Taj Barin	1970	MA 14	Variola major
EMR	Pakistan	Wsim Ahmed	1970	MA 17	Variola major
EUR	USSR[c]	M-A-60	1960	MA 1	Variola major
EUR	USSR[c]	M-Abr-60	1960	MA 2	Variola major
EUR	USSR[c]	M-Bl-60	1960	MA 3	Variola major
EUR	USSR[c]	M-Gavr-60	1960	MA 4	Variola major
EUR	USSR[c]	M-N-60	1960	MA 5	Variola major
SEAR	India	India 71	1975	MA 7	Variola major

AFR, WHO African Region; AMR, WHO Region of the Americas; EMR, WHO Eastern Mediterranean Region; EUR, WHO European Region; SEAR, WHO South-East Asia Region.

[a] Names of countries are those used at the time of strain isolation.

[b] Now the United Republic of Tanzania.

[c] Now the Russian Federation.

Creation of clone libraries of full genomic DNA fragments of variola virus strains

The disadvantages of keeping genetic information in the form of an amplicon collection include the possible risk of degradation of LPCR products in long-term storage, and the impossibility of maintaining a full-size collection of amplicons in the absence of genomic VARV DNA. To solve these problems, genetic information can be stored in the form of collections of recombinant plasmids containing VARV DNA fragments, referred to in this document as "clone libraries".

A scheme was proposed that involved cleavage of LPCR amplicons by certain restriction endonucleases, and the subsequent cloning of resultant DNA fragments in *Escherichia coli* within plasmid vectors. This would enable the creation of clone libraries of VARV DNA fragments in which any plasmid construction could be easily and quickly produced in necessary amounts. The use of *E. coli* strains with a defective repair system minimizes the accumulation of errors during DNA replication. Although more labour-intensive than conventional methods, such an approach would provide safe and reliable long-term storage of VARV DNA fragments. VARV genome sequences could then be stored as recombinant plasmids in a biologically safe form for an indefinite period. Also, as explained above, this would enable investigation of the genetic organization of VARV strains and the development of modern methods for rapid VARV diagnostics. Such plasmids could also be the source of VARV genes. At present, certified collections of plasmids containing DNA fragments include 16 VARV DNA strains (see Table 4.6). Data on the number of vials in collections are summarized in Table 4.5.

Table 4.5 Inventory list of variola virus strains whose DNAs were used for genetic material conservation

Strain	Geographical region of strain isolation	Country of strain isolation[a]	Year of isolation	Epidemiological type of virus	Amplicon repository, no. of vials	Repository of fragments clone libraries, no. of vials
Congo-2	AFR	Congo	1970	Variola major		261
Rw-18	AFR	Rwanda	1970	Variola major	84	288
12/62	AFR	Tanzania[b]	1962	Variola major	84	
13/62	AFR	Tanzania[b]	1962	Variola major	84	273
Helder	AFR	Tanzania[b]	1962	Variola major	84	
Mary	AFR	Tanzania[b]	1962	Variola major	99	
Ngami	AFR	Tanzania[b]	1962	Variola major	87	231
Brazil 128	AMR	Brazil	Unknown	Variola minor alastrim		285
Brazil 131	AMR	Brazil	Unknown	Variola minor alastrim	84	
Garcia-1966	AMR	Brazil	1966	Variola minor alastrim		45
6–58	EMR	Pakistan	1958	Variola major	84	282
Aziz	EMR	Pakistan	1970	Variola major	84	
Taj Barin	EMR	Pakistan	1970	Variola major	84	288
Wsim Ahmed	EMR	Pakistan	1970	Variola major	84	213
Butler	EUR	Great Britain[c]	1952	Variola minor alastrim		213
M-A-60	EUR	USSR[d]	1960	Variola major	84	246
M-Abr-60	EUR	USSR[d]	1960	Variola major	84	
M-Bl-60	EUR	USSR[d]	1960	Variola major	84	288
M-Gavr-60	EUR	USSR[d]	1960	Variola major	84	276
M-Sur-60	EUR	USSR[d]	1960	Variola major		279
M-N-60	EUR	USSR[d]	1960	Variola major	84	
Ind-3a	SEAR	India	1967	Variola major		222
India 71	SEAR	India	1975	Variola major	84	
India-1967	SEAR	India	1967	Variola major		105

AFR, WHO African Region; AMR, WHO Region of the Americas; EMR, WHO Eastern Mediterranean Region; EUR, WHO European Region; SEAR, WHO South-East Asia Region.

[a] Names of countries are those used at the time of strain isolation.

[b] Now the United Republic of Tanzania.

[c] Now the United Kingdom.

[d] Now the Russian Federation.

Table 4.6 List of variola virus DNAs used to create the repository of recombinant plasmids

Geographical region of variola virus isolation	Country of variola virus isolation[a]	Variola virus strain	Year of isolation	No. in repository collection	Epidemiological type of the virus
AFR	Tanzania[b]	Ngami	1962	P 1	Variola major
AFR	Congo	Congo-2	1970	P 7	Variola major
AFR	Rwanda	Rw-18	1970	P 11	Variola major
AFR	Tanzania[b]	13/62	1962	P 16	Variola major
AMR	Brazil	Brazil 128	Not determined	P 4	Variola minor alastrim
AMR	Brazil	Garsia - 1966	1966	P 10	Variola minor alastrim
EMR	Pakistan	Taj Barin	1970	P 3	Variola major
EMR	Pakistan	Wsim Ahmed	1970	P 6	Variola major
EMR	Pakistan	6-58	1958	P 13	Variola major
EUR	Great Britain[c]	Butler	1952	P 8	Variola minor alastrim
EUR	USSR[d]	M-Sur-60	1960	P 2	Variola major
EUR	USSR[d]	M-Bl-60	1960	P 12	Variola major
EUR	USSR[d]	M-A-60	1960	P 14	Variola major
EUR	USSR[d]	M-Gavr-60	1960	P 15	Variola major
SEAR	India	Ind-3a	1967	P 5	Variola major
SEAR	India	India - 1967	1967	P 9	Variola major

AFR, WHO African Region; AMR, WHO Region of the Americas; EMR, WHO Eastern Mediterranean Region; EUR, WHO European Region; SEAR, WHO South-East Asia Region.

[a] Names of countries are those used at the time of strain isolation.

[b] Now the United Republic of Tanzania.

[c] Now the United Kingdom.

[d] Now the Russian Federation.

As a result of this work, a repository was created, comprising repositories of:

- genomic DNA preparations of 39 VARV strains;
- collections of amplicons of 17 VARV DNA strains;
- collections of plasmids containing full genomic DNA fragments of 16 VARV strains of the Russian Federation collection, belonging to two epidemiological types, and isolated in different geographical regions.

The authors of this chapter believe that the plasmid and amplicon collections should each represent at least 6 VARV strains of different biological subtypes (6 strains each of variola major, variola minor and variola minor alastrim, giving a total of 18 strains) to ensure representativeness and coverage of VARV biodiversity. It would also be reasonable to have original full-length or partial DNA samples of all VARV strains from the collection. These could be safely maintained in a bank of genetic VARV material. The rationale for this is that the repositories in both the Russian Federation and the United States comprise nature-derived cultures of VARV whose synthetic analogues will probably not be able to fully reproduce the entire spectrum of properties of the naive viruses.

Certified collections were placed at the International Variola Virus DNA Repository at VECTOR. VECTOR performs an annual inventory of VARV strains and DNA, and submits an annual inventory list of VARV strains and DNA to WHO, in a format agreed with WHO. VECTOR also contributes WHO collaborating centre annual reports to WHO. These reports include sections concerning work with the collections of VARV strains and DNA.

All work with the collections of VARV strains and DNA uses the following methods:

- regular submission of proposals for obtaining permission to work with the collections to the Scientific Subcommittee of the WHO Advisory Committee on Variola Virus Research;
- undertaking of the work proposed, as approved by the Scientific Subcommittee of the WHO Advisory Committee on Variola Virus Research;
- presentation of the annual report, in the form of an oral communication on the work carried out, at the annual meeting of the WHO Advisory Committee on Variola Virus Research;
- submission to the WHO Secretariat of an annual written report on work carried out.

4.3 The status of strains of variola virus and nucleic acid repositories at the WHO collaborating centre in the United States

The United States–maintained WHO collaborating centre repository at the CDC contains VARV collections from Japan, the Netherlands, the United Kingdom and the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) laboratories. It also contains collections previously maintained by the CDC and the American Type Culture Collection. Inventory samples of 451 isolates or specimens are maintained in their original forms, in safe and secure locations. It is believed that all materials were collected within the 50 years before smallpox eradication.

The inventory mostly includes human patient isolates and replicate samples of reference VARV strains. However, the repository also contains a few experimentally derived "hybrid" viruses constructed via cowpox virus, as well as VARV or rabbitpoxvirus and VARV recombination, and non-VARV materials donated by outside laboratories, including samples of vaccinia immune globulin.

In 2000, an electronic database was generated to link information such as year and geographic location of discovery of the VARV isolate, clinical and epidemiologic information about the patient, and the outbreak from whom or which the isolate was derived. The database also links data on passage history of the sample with the labelled name on the specimen container, to guide selection of diverse isolates for further study. Information sources used to gather this information included:

- review of
  - written documentation provided with the specimens
  - previous reports made to WHO on the status of these materials
  - archival WHO records in Geneva
  - publications
- requests for information from the donating country laboratory.

Ultimately, information was available for about 360 non-identical VARV materials derived from the various original collections. A total of 142 specimens could be linked to country of isolation and year of isolation, and included the materials' passage histories; 8 could be linked to country and year of isolation (but did not include passage history of the material provided); and 163 could not be linked to either country or year of isolation.

This latter material included 19 primary scabs from individual patients and 3 variolator stocks.

Since 2000, after approval from the WHO Technical Advisory Committee on Variola Virus Research, a subset of materials from the CDC repository has been further analysed by complete genomic analysis. Forty-six isolates were chosen for genome sequencing; they included specimens from within the same or epidemiologically linked outbreaks, in addition to apparently "unlinked" outbreaks (Esposito et al., 2006; Li et al., 2007). These particular specimens were sequenced because they were felt to represent the greatest source of virus diversity (reflected in country and year of isolation, and low passage history) within the CDC collection. Sequenced material included isolates from cases clinically or epidemiologically described as "major" or "minor", and biologically or epidemiologically described as alastrim. Most of the sequenced material was from isolates described as VARV major. The genetic variability among these isolates correlated with geographic location of the isolate, and did not have a temporal correlation. For example, samples isolated 30 years apart from the Horn of Africa were grouped with the non–west African isolates. Two of the hybrid viruses were sequenced to evaluate the ability of the LPCR/restriction fragment length polymorphism (RFLP) methods to identify recombination events. Subsequently, complementary biologic characterization of VARV isolates was completed (Olson et al., 2009) to provide relevant genetic and biologic information for scientific analysis and evaluation of potential anti-VARV therapeutics or prophylactics.

CDC VARV stocks no more than one passage from the sequenced master seed are those that have been used since 2000 in the WHO-approved essential public health research proposals using live VARV.

Table 4.7 Geographical distribution of origin of variola virus strains held at CDC

Geographical region of strain isolation	Country of strain isolation[a]	Number of strains
Africa	Botswana	6
	Central Africa (unspecified country)	2
	Dahomey[b]	2
	Djibouti	1
	Ethiopia	11
	Gabon	1
	Ghana	2
	Guinea	1
	Kenya	12
	Mali	3
	Niger	2
	Nigeria	5
	Sierra Leone	4
	Somalia	7
	South Africa	9
	Sudan	2
	Tanzania	10
	Togo	2
	Uganda	2
	Upper Volta[c]	1
	West Africa (unspecified country)	1
	Zaire[d]	9
Asia	Bangladesh	20
	China	4
	India	25
	Indonesia	12
	Japan	3
	Korea[e]	3
	Nepal	5
North America	United States	1
South America	Brazil	15
Europe	Germany	1
	Netherlands	2
	Italy	1
	United Kingdom	30
	Yugoslavia[e]	1
Middle East	Iran	3
	Kuwait	3
	Pakistan	12
	Syria[f]	1
Unknown		214
Total		451

[a] Names of countries are those used at the time of strain isolation.
[b] Now Benin.
[c] Now Burkina Faso.
[d] Now Democratic Republic of the Congo.
[e] Current name of country unknown.
[f] Now Syrian Arab Republic.

A minority of the material contained within the repository has been used for experimental work. Table 4.8 lists VARV strains that have been used in studies, and have therefore been grown in tissue culture. Most of the material tested was found to be viable, although some strains required “blind” passaging to rescue viable VARV. Before 2000, most experimental work had used four strains, which had been propagated for seed stocks, working stocks and nucleic acid production for antiviral screening and sequencing efforts. In 2010, the amount of stored seed stock, working material and plaque picks (measured in numbers of “tubes”) has increased 22-fold since 2000. These materials have been, or are being, used for in vitro antiviral screening, animal challenge studies, in vitro vaccine efficacy bridging studies, and sequencing. In addition, there are 4209 stored specimens from six VARV animal challenge studies aimed at evaluating antiviral efficacy. These samples will be retained until all pertinent questions or concerns from regulatory agencies (e.g. the United States Food and Drug Administration) have been satisfactorily addressed. Forty-six per cent of the 22-fold increase in the stored seed stock, working material and plaque picks is from the creation of single-use aliquots for antiviral screening or vaccine efficacy–related studies. These low-titre, single-use aliquots permit an increase in uniformity between experiments, and limit repeated freeze–thaw cycles of virus.

Access to these materials is limited to selected persons at CDC, all of whom have completed appropriate training in biosafety and biosecurity practices. Access to the repository is further restricted. Materials are inventoried annually, and current practice is to safety seal boxes after inventory.

Table 4.8 CDC working stock selection: identification of viable variola virus material

Isolate	Original laboratory	Date of isolation	Country[a]	Region	Original passage history	Current status
Minnesota 124	USAMRIID	5 Feb 1939	United States	North America	123 CAM (Nelson), Deterick 1 CAM = 124 CAM (26 Mar 1958)	Seed stock (E124;BSC40p2)
Yamada MS-2(A) Tokyo strain	USAMRIID	1946	Japan	Asia	2 CAM (Hahon), Detrick 2 CAM = 4 CAM (3 Nov 1958)	Seed stock (E4;BSC40p2)
Kim E-316 (2), Masterseed	USAMRIID	1946	Korea[b]	Asia	2 CAM (Kempe), Detrick 1 CAM = 3 CAM (18 Feb 1958)	Non-viable
Hinden	Porton Down, United Kingdom	1946	United Kingdom	Europe	E2	Seed stock (E2;BSC40p2)
Harvey	Porton Down, United Kingdom	1946	United Kingdom, imported	Europe	E1	Seed stock (E1; BSC40p2)
Lee, Masterseed	USAMRIID	1947	Korea[b]	Asia	2 CAM (Kempe), Detrick 2 CAM = 4 CAM (15 Sept 1958)	Seed stock (E4;BSC40p2)
Juba	Porton Down, United Kingdom	7 Oct 1947	Sudan	Africa	E8	Seed stock (E8;BSC40p2)
Rumbec	Porton Down, United Kingdom	1947	Sudan	Africa	E7	Seed stock (E7;BSC40p2)
Higgins	Porton Down, United Kingdom	5 Apr 1947	Staffordshire, United Kingdom	Europe	Unknown	Seed stock (?;BSC40p2)
Horn (Sabin Lab)	USAMRIID	pre-1948	China	Asia	2 (?) CAM (Shabel), Detrick 2 CAM = 4 CAM (4 Jun 1959)	Seed stock (E4;BSC40p2)
Harper, Masterseed	USAMRIID	pre-1951	Japan	Asia	3 CAM (Hahon) , 2 CAM Detrick = 5 CAM (25 Jul 1958)	Seed stock (E5;BSC40p2)
Stillwell Masterseed	USAMRIID	pre-1951	Japan	Asia	6CAM (Hahon)	Seed stock (E6, BSC40p2)
BUT (Butler)	Porton Down, United Kingdom	1952	United Kingdom	Europe	E8	Seed stock (E8;BSC40p2)
Kali-Muthu M 50	USAMRIID	5 Sept 1953	India	Asia	#2228 orig. material in Locke's 13 Jan 1958 homogenate crust	Seed stock (BSC40p3)

Table 4.8 CDC working stock selection: identification of viable variola virus material *continued*

Isolate	Original laboratory	Date of isolation	Country[a]	Region	Original passage history	Current status
New Delhi	USAMRIID	1953	India	Asia	2 CAM (Kempe), Detrick 3 CAM= 5 CAM (23 Jun 1959)	Seed stock (E5;BSC40p2)
Nigeria – Kudano	Porton Down, United Kingdom	Jun 1961	Nigeria	Africa	Crust	Non-viable
696 Madras DJB	Porton Down, United Kingdom	1963	India	Asia	Crust	Non-viable
7125	Porton Down, United Kingdom	1964	India	Asia	E4	Seed stock (E4; BSC40p2)
7124	Porton Down, United Kingdom	1964	India	Asia	E4	Seed stock (E4; BSC40p2)
102	Porton Down, United Kingdom	12 Apr 1965	South Africa	Africa	E1	Seed stock (E1;BSC40p2)
103	Porton Down, United Kingdom	14 Apr 1965	South Africa	Africa	E1	Seed stock (E1, BSC40p2)
Kembula	Porton Down, United Kingdom	Sept 1965	Tanzania[c]	Africa	Crust	Seed stock (BSC40p2)
Garcia, Brazil (Minor)	CDC	1966	Brazil	South America	E3 1.00E-01	Working stock (E3;BSC40p3)
v66–39	CDC	5 Ju 1966	Brazil	South America	E1 from S Silva/J Noble isol day 4 rash	Seed stock (E1; BSC40p2)
K1629	Porton Down, United Kingdom	6 May 1967	Kuwait	Middle East	E5	Seed stock (E5;BSC40p2)
V68–59	CDC	10 Apr 1968	Dahomey[d]	Africa	E1	Seed stock (E1;BSC40p2)
Rafiq Lahore	Porton Down, United Kingdom	3 Mar 1969	Pakistan	Asia	Crust	Seed stock (BSC40p3)
V68–258	CDC	2 Jan 1969	Sierra Leone	Africa	E4 1.00E-01	Seed stock (E4;BSC40p2)

Table 4.8 CDC working stock selection: identification of viable variola virus material *continued*

Isolate	Original laboratory	Date of isolation	Country	Region	Original passage history	Current status
Congo V70–46	CDC	12 Mar 1970	Zaire[e]	Africa	E1 1.00E-01	Working stock (E1;BSC40p3)
Afghan Variolator 4	Porton Down, United Kingdom	18 Mar 1970	Afghanistan	Middle East	Crust	Seed stock (BSC40p2)
V70–222	CDC	17 Oct 1970	Indonesia	Asia	E1 1.00E-01	Seed stock (E1;BSC40p2)
V70–228	CDC	26 Oct 1970	Indonesia	Asia	E1 1.00E-01	Seed stock (E1; BSC40p2)
V72–119	CDC	6 Apr 1972	Syria[f]	Middle East	E1 1.00E-01	Seed stock (E1;BSC40p2)
V72–143	CDC	26 Apr 1972	Botswana	Africa	E1 1.00E-01	Seed stock (E4; BSC40p2)
Eth16 R14–1X-72	Porton Down, United Kingdom	29 Aug 1972	Ethiopia	Africa	Crust	Seed stock (BSC40p3)
Eth17 R14–1X-72	Porton Down, United Kingdom	29 Aug 1972	Ethiopia	Africa	Crust	Seed stock (BSC40p3)
V73–225	CDC	8 Oct 1973	Botswana	Africa	E1 1.00E-01	Seed stock (E1;BSC40p2)
V73–175	CDC	26 Jul 1973	Nepal	Asia	E1 1.00E-01	Seed stock (E1; BSC40p2)
Nur Islam	Porton Down, United Kingdom	1974	Bangladesh	Asia	Crust	Seed stock (BSC40p2)
Shahzaman	Porton Down, United Kingdom	1974	Bangladesh	Asia	Crust	Seed stock (BSC40p3)
Solaiman	Porton Down, United Kingdom	1974	Bangladesh	Asia	Crust	Seed stock (BSC40p2)
BSH V75–550 ‘Bangladesh’	CDC	24 Nov 1975	Bangladesh	Asia	E1 1.00E-01	Working stock (BSC40p6)

Table 4.8 CDC working stock selection: identification of viable variola virus material *continued*

Isolate	Original laboratory	Date of isolation	Country	Region	Original passage history	Current status
V77–1252	CDC	19 May 1977	Somalia	Africa	E1 1.00E-01	Seed stock (E1, BSC40p2)
V77–1605	CDC	9 Aug 1977	Somalia	Africa	E1 1.00E-01	Seed stock (E1; BSC40p2)
V77–2479 (Ali Maow Maalin) 'Somalia'	CDC	10 Nov 1977	Somalia	Africa	E2 1.00E-02	Working stock (E2; BSC40p3)
AR1, recombinant alastrim/ rabbitpox	Porton Down, United Kingdom	Data published in 1964	Laboratory strain	NA	Unknown	Nucleic acid extracts
VC13, recombinant var /cowpox	Porton Down, United Kingdom	Data published in 1964	Laboratory strain	NA	Unknown	Nucleic acid extracts
Heidelberg	USAMRIID	Unknown	Germany	Europe	Detrick ISOL crust, 1 CAM stock date 24 May 1960	Seed stock (E1;BSC40p2)
Congo 9 (Gispen) V74–227	CDC	1974	Zaire[e]	Africa	E1 1.00E-01	Seed stock (E1;BSC40p2)
Northern Indian, Masterseed	USAMRIID	Unknown	Northern India	Asia	30 CAM (Nelson), Detrick 3 CAM = 33 CAM (14 Jul 1958)	Non-viable
Iran 2602	Porton Down, United Kingdom	Unknown	Iran	Middle East	E5	Seed stock (E5;BSC40p2)

CAM, chorioallantoic membrane; CDC, Centers for Disease Control and Prevention, Atlanta, Georgia; NA, not applicable; USAMRIID, United States Army Medical Research Institute of Infectious Diseases.

[a] Names of countries are those used at the time of strain isolation.

[b] Current name of country unknown.

[c] Now United Republic of Tanzania.

[d] Now Benin.

[e] Now Democratic Republic of the Congo.

[f] Now Syrian Arab Republic.

CDC currently maintains VARV nucleic acid that is representative of those genomes that have been sequenced and a few other viruses. The material, described in Tables 4.9 and 4.10, is maintained as full-length genomic material, and in a subset as plasmid collections or LPCR amplicons. This material is referenced in a secure, password-protected database, and access to material is restricted to a few members of the programme. Currently, plasmids or LPCR amplicons representing nine of the strains sequenced from the WHO collaborating centre collection housed in the United States are archived. Notably, all 47 sequenced viruses in the collection have been used to validate nucleic acid–based diagnostics. Additional plasmid-based, LPCR or other genome fragments representing the genomes may need to be produced in anticipation of the destruction of variola stocks and intact genomic DNAs. Discussion of the best ways to archive this material should consider the mutation rates introduced by PCR strategies, the stabilities of the various approaches to genome archiving, and the work involved in the possible approaches.

Table 4.9 CDC inventory list of full-length variola virus DNA

No.	Strain	Region	Country of origin[a]	Year of isolation
1	102	Africa	Natal, Ingwavuma, South Africa	1965
2	103	Africa	Transvaal, Nelspruit, South Africa	1965
3	66–39	South America	Brazil	1966
4	68–59	Africa	Dahomey[b]	1968
5	68–258	Africa	Sierra Leone	1969
6	69–1	Africa	Niger	1969
7	69–5	Africa	Guinea	1969
8	70–222	Asia	Indonesia	1970
9	70–228	Asia	Indonesia	1970
10	7124	Asia	India	1964
11	7125	Asia	India	1964
12	72–119	Middle East	Syria[c]	1972
13	72–143	Africa	Botswana	1972
14	72–164	Europe	Yugoslavia[d]	1972
15	73–175	Asia	Nepal	1973
16	73–225	Africa	Botswana	1973
17	74–227	Africa	Zaire[e]	1974
18	77–1252	Africa	Somalia	1977
19	77–1605	Africa	Somalia	1977
20	Afghan Variolator 4	Middle East	Afghanistan	1970
21	AR1	NA	Laboratory strain	Published in 1964
22	Ashiq	Middle East	Pakistan	1969
23	Aslam	Middle East	Pakistan	1969
24	BSH	Asia	Bangladesh	1975
25	Bombay	Asia	India	1958
26	Brazil Garcia	South America	Brazil	1966
27	Butler	Europe	United Kingdom	1952
28	Congo V70–46	Africa	Zaire[e]	1970
29	Djib	Africa	Djibouti	1971
30	ETH 16	Africa	Ethiopia	1972
31	ETH 17	Africa	Ethiopia	1972

Table 4.9 CDC inventory list of full-length variola virus DNA *continued*

No.	Strain	Region	Country of origin	Year of isolation
32	Farid	Middle East	Pakistan	1969
33	Harper	Asia	Japan	1951
34	Harvey	Europe	United Kingdom	1946
35	Heidelberg	Europe	Germany	Unknown
36	Higgins	Europe	United Kingdom	1947
37	Hinden	Europe	United Kingdom	1947
38	Horn	Asia	China	pre-1948
39	Iran 2602	Middle East	Iran	Unknown
40	Juba	Africa	Sudan	1947
41	Kembula	Africa	Tanzania[f]	1965
42	K1629	Middle East	Kuwait	1967
43	Kali Mathu	Asia	India	1953
44	A. Mannan	Asia	Bangladesh	1974
45	Minnesota 124	North America	United States	1939
46	Misba	Middle East	Pakistan	1970
47	M.S. Lee	Asia	Korea[d]	1947
48	New Delhi	Asia	India	1953
49	Nigeria Kudano	Africa	Nigeria	1961
50	Nur Islam	Asia	Bangladesh	1974
51	Parker	Europe	United Kingdom	1978
52	Parvin	Asia	Bangladesh	1974
53	Rafiq Lahore	Middle East	Pakistan	1969
54	Ramjan	Middle East	Pakistan	1970
55	Rafiq	Middle East	Pakistan	1969
56	Shahzaman	Asia	Bangladesh	1974
57	Solomain	Asia	Bangladesh	1974
58	Somalia	Africa	Somalia	1977
59	Stillwell	Asia	Japan	1951
60	VC13	NA	Laboratory strain	Published in 1964
61	Yamada	Asia	Japan	1946

NA, not applicable.
[a] Names of countries are those used at the time of strain isolation.
[b] Now Benin.
[c] Now Syrian Arab Republic.
[d] Current name of country unknown.
[e] Now Democratic Republic of the Congo.
[f] Now United Republic of Tanzania.

Table 4.10 CDC list of variola virus strains used to create amplicon and plasmid collections

Geographical region of strain isolation	Country of strain isolation	Strain	Year of isolation	Epidemiological type	Collection type	Number of vials
Asia	Bangladesh	BSH	1974	Variola major	Plasmids	250
Europe	United Kingdom	Butler	1952	Variola minor alastrim	Plasmids	110
Africa	Democratic Republic of the Congo	Congo	1970	Variola major	Plasmids	180
South America	Brazil	Brazil Garcia	1966	Variola minor alastrim	Plasmids	135
Europe	United Kingdom	Harvey	1946	Variola major	Plasmids	100
Asia	China	Horn	pre-1948	Variola major	Amplicons	48
Asia	India	7124	1964	Variola major	Amplicons	134
Asia	Nepal	73–175	1973	Variola major	Amplicons	127
Africa	Somalia	Somalia	1977	Variola major	Plasmids	100

VARV fragments have been provided by CDC to a number of outside investigators, using approved protocols from WHO and the CDC/United States Department of Health and Human Services (HHS) for distribution of VARV nucleic acid, and following the recommendations for use provided by WHO.[11] This provision is outlined in Table 4.11.

[11] http://www.who.int/csr/disease/smallpox/research/en/index.html

Table 4.11 Variola virus fragments provided to outside investigators

Institute, country	WHO approval given (Y/N)	Strain	Gene(s)	Request date	Fully executed	Shipped
Cornell University, United States	Y	BSH75 Horn Heidelberg V73–175 102 Nur Islam Shahzaman Solaiman	RAP94 RPO147	20 Jan 2008	15 May 2008	NA
University of Florida, United States	Y	Bangladesh	G1R	24 Apr 2007	10 Jun 2008	24 Jun 2008
University of Medicine and Dentistry of New Jersey, United States	Y	Bangladesh	D9R	12 Jul 2006	NA	30 Jan 2008
University of California, United States	Y	Bangladesh	D1L E7L A27L A39L B2L B3L B4L B9R B10R B14R B19R B20R B22R	3 Aug 2006	Oct 2006	23 Oct 2006
NIH, United States	Y		A24R	20 Apr 2005	16 Aug 2005	NA
Instituto Cantonale di Microbiologia, Switzerland	Y	Bangladesh	J9R B10R B11R	29 Apr 2005	1 Jun 2006	26 Jul 2006
USAMRIID, United States	Y	Bangladesh Garcia	M1R B6R A36R A31L	2 Aug 2004	1 Jun 2006	Aug 2006
University of Pennsylvania, United States	Y	Bangladesh	A31L A18L A36R B6R F8L I3L M1R C13L	26 Jan 2005	NA	NA

Table 4.11 Variola virus fragments provided to outside investigators *continued*

Institute, country	WHO approval given (Y/N)	Strain	Gene(s)	Request date	Fully executed	Shipped
Myriad Genetics, Inc, United States	Y	Bangladesh	B8L C18L A27L A39L B4L B9R B11R:B10R B12R:B11R B14L B22R:B19R C13L:B20R B22R G3R:G1R, G2R	8 Dec 2004	Jun 2006	23 Oct 2006
Centro Nacional de Biotechnologia, Spain	Y	Bangladesh	B8R B17R D7L G2R G3R A44L P1L D4R D15L A41L	23 Jul 2003	22 Sep 2004	20 Jul 2006
Finland	Y	Bangladesh	D12R B7R	7 Sep 2001	NA	NA
AFIP, Washington, DC, United States	Y	Bangladesh	L2R B8R G2R J7R E9L L6R A25R	Nov 2001	19 Feb 2002	NA

Table 4.11 Variola virus fragments provided to outside investigators *continued*

Institute, country	WHO approval given (Y/N)	Strain	Gene(s)	Request date	Fully executed	Shipped
USAMRIID, United States	Y	Bangladesh	D8L-D10L D16L-D18L	8 Jan 2002	22 Feb 2002	NA
		Bangladesh Garcia	C7L M1R I5R F8L A14L A26L-A28L A34R A36R-A37R A40R A47-A48L J6R-J7R B3L B9L-B10L B19L-B20L B22R G1R G3R-G4R B6R L2R B8R G2R J7R E9L L6R A25R A31L	Jan 2005	Jun 2006	Aug 2006
Dana-Farber Cancer Institute, Harvard Medical School, United States	Y	Bangladesh	D4R C9L A46R J7R B6R	Nov 2001	Jan 2002	NA
San Jose State University, United States	Y	Bangladesh	A41-A44	15 Aug 1996	Aug 1997	NA
AFIP, United States	Y	Bangladesh	J7R E9L G1R	Jan 1999	Feb 1999	NA
DSTL, Porton Down, United Kingdom	Y	V74–227 (Congo) Solaiman Butler	P1L (BSH homolog) C3L (BSH homolog) A14L (BSH homolog) A36R A38R B6R B17R	Oct 2001	NA	29 Apr 2003

Table 4.11 Variola virus fragments provided to outside investigators *continued*

Institute, country	WHO approval given (Y/N)	Strain	Gene(s)	Request date	Fully executed	Shipped
Harvard Medical School, United States	Y	Bangladesh	TK gene	Jan 2003	NA	8 May 2003
INSERM, France	Y	Bangladesh	SacI fragment BstEII D fragment	Apr 2003	Aug 2004	27 Aug 2004
CRSSA Emile Pardé, France	Y	Bangladesh Garcia	A31L K9R C9L	Jul 2003	Oct 2003	Feb 2004
University of Alberta, Canada	Y	Bangladesh	E9L	May 2004	May 2004	24 Aug 2004
23 laboratories in United States	Y	Bangladesh	2 <500 nucleotide inserts	NA	NA	NA

AFIP, Armed Forces Institute of Pathology; DSTL, Defence Science and Technology Laboratory; INSERM, Institut National de la Santé et de la Recherche Médicale; NA, not available; NIH, National Institutes of Health; USAMRIID, United States Army Medical Research Institute of Infectious Diseases.

Formal written reports concerning the repositories, their use and their status were submitted to WHO in writing in June 1997 (report on use from 1979 to 1997) and in 1998. As of 1999, these reports have been made regularly in abstracted form, as electronic databases and in annual oral presentations at the WHO Ad Hoc Meeting on Orthopoxviruses (1999) and subsequently at the annual WHO Technical Advisory Committee Meetings on Variola Virus Research (2000–2008). The more recent abstracted reports are available on the WHO website.[12]

All work with live VARV at CDC is conducted in a biosafety level 4 facility, which is inspected regularly by local and federal authorities (from the HHS Select Agent and Toxins Program at the federal level), and by international (WHO) authorities, to assure the highest standards of biosafety and biosecurity practices.

[12] http://www.who.int/csr/disease/smallpox/research/en/index.html

Abbreviations

CAM	chorioallantoic membrane
CDC	Centers for Disease Control and Prevention, Atlanta, Georgia
CPE	cytopathic effect
DNA	deoxyribonucleic acid
ECE	embryonated chicken egg
LPCR	long polymerase chain reaction
OPV	orthopoxvirus
RIVP	Research Institute of Viral Preparations
VARV	variola virus
VECTOR	Russian State Research Centre of Virology and Biotechnology
WHO	World Health Organization

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5 Animal models and pathogenesis

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Executive summary

Public health importance

The potential for variola virus to be exploited as a bioterrorist weapon is widely understood. In addition, the re-emergence of monkeypox as a public health concern in the Democratic Republic of the Congo has increased the urgency of developing improved countermeasures, including vaccines and antiviral drugs, for these orthopoxviruses. Since it is generally recognized that animal models will be needed to demonstrate efficacy of these countermeasures, this chapter focuses on useful animal models for orthopoxvirus disease.

Progress to date

Small animal models using ectromelia virus (the cause of mousepox), cowpox virus, rabbitpox virus and vaccinia virus have provided insight into the pathogenesis and immunology of poxvirus infections; this knowledge has been used to design critical studies using primates. Primate models using variola virus or monkeypox viruses are the most relevant to the development of safe and effective countermeasures against smallpox in humans.

Current challenges

Various combinations of variola virus doses and routes of exposure in primates (cynomolgus monkeys) lead to predictable disease patterns that replicate some, but not all, features of human smallpox. Although the models require further refinement, they have been adequate to demonstrate the efficacy of several candidate antiviral drugs, including cidofovir and ST-246. It is likely that no single combination of conditions will result in a model that will simultaneously satisfy all of the criteria required under the United States Food and Drug Administration “animal rule” (US 21CRF310.610); different models may be required to assess different indications. Further refinement of the primate models might include pathophysiologic data from studies using telemetry and medical imaging. Special attention should also be paid to finding biomarker patterns that could be used in a clinical setting as triggers for early intervention, thus increasing the likelihood of successful intervention and facilitating the licensing of countermeasures. Although much of this developmental work can be accomplished using surrogate orthopoxvirus in rodents and primates, increased confidence in countermeasures against variola virus can be obtained only by efficacy testing in primate models using variola virus.

5.1 Introduction

Despite the eradication of smallpox, variola virus (VARV) remains a public health concern, because of the possibility that clandestine stocks of VARV may be in the hands of bioterrorists (Henderson et al., 1999). The impact of a VARV attack in the human population now would be even more catastrophic than it was during the previous century: vaccination programmes were abandoned worldwide around 1976, immunosuppressed populations are more prevalent, and people's increased mobility (including by intercontinental air travel) has accelerated the pace of viral spread around the world. For these reasons, considerable investment is being made in developing improved countermeasures against smallpox, including new vaccines and antiviral drugs (LeDuc & Jahrling, 2001).

Development and licensure of such countermeasures will depend on animal models to demonstrate their protective efficacy. These animal models should be faithful to the human disease, and ideally would use the actual etiological agent (VARV) rather than a surrogate. Although much of such developmental work can be accomplished using surrogate orthopoxviruses (OPVs) in rodents and primates, increased confidence in countermeasures against VARV can be obtained only by efficacy testing in primate models using VARV.

5.2 Animal models

The fact that VARV naturally infects only humans frustrates the development of animal models for smallpox using VARV. VARV can infect a variety of laboratory animals experimentally, but this does not result in lethal, systemic disease (Fenner et al., 1988), with the exception of recent studies using monkeys (Jahrling et al., 2004). Further refinement of the VARV primate model is desirable, including the natural aerogenic route of exposure (USFDA, 2009).

This chapter focuses on animal models for OPV disease that promise to be useful for developing countermeasures for smallpox. Primate models using VARV or monkeypox virus (MPXV) are most relevant for this purpose, and could also provide insight into the pathophysiology of smallpox in humans; however, primate studies are expensive, and use of VARV requires the highest level of biosafety (level 4, BSL-4) and biosecurity. The virus is restricted to the two relevant World Health Organization (WHO) collaborating centres – the Centers for Disease Control and Prevention (CDC) in Atlanta, United States, and the State Research Centre of Virology and Biotechnology VECTOR in the Russian Federation. MPXV, while less restricted, still requires biosafety level 3 (BSL-3) biocontainment and is a Select Agent (US Department of Health and Human Services, 2005), meaning there are restrictions on its use. As a result of these restrictions, the use of small animal models for OPV disease, using ectromelia virus (ECTV), cowpox virus (CPXV), rabbitpox virus (RPXV) and vaccinia virus (VACV), has a place in efforts to understand and develop countermeasures for the human pathogens.

Animal models for OPV disease must address the critical balance between direct viral interaction with host target cells and the protective immune response. Poxviruses, more than most other viral pathogens, express a variety of immunomodulatory proteins and apoptosis inhibitors, which can tip the balance toward virulence. Some of these virus–

host interactions may be species specific, and may not reliably generalize to models in other species.

Much of the knowledge about VARV pathogenesis is inferred from studies with ECTV in mice (Buller & Palumbo, 1991). ECTV is a natural pathogen of mice and, after it was discovered in the 1930s, Fenner used the model to elucidate the concept of primary and secondary viraemia, which parallels exanthematous disease in humans (Fenner, 1948). In the 1970s, ECTV–mouse models were used to demonstrate the role of T cells and macrophages in cell-mediated immunity and recovery from acute disease. In the 1980s, ECTV infections of inbred mouse strains were used to identify genetic determinants of resistance and susceptibility (Buller, 1985; Buller & Palumbo, 1991). More recently, the availability of various knockout strains of inbred mice has facilitated the investigation of virus–host relationships.

A detailed description of the immunobiology of OPV infections is beyond the scope of this chapter.

Susceptibility to ECTV is genetically determined, and the genetics are complex. C57BL/6 strain mice are relatively resistant; the LD_{50} is more than 10^6 plaque-forming units (PFU) via footpad inoculation. In contrast, the LD_{50} for A/J mice is less than 0.01 PFU (Buller, 1985). Genetic resistance relates in part to the granule exocytosis pathway of effector T cells (Müllbacher et al., 1999). BALB/c and DBA/2 strain mice are, like A/J mice, highly susceptible via both dermal and aerosol routes. Resistance and susceptibility can also vary with the ECTV strain.

ECTV infections in susceptible mice are initiated by abrasions to the skin. The virus replicates locally, then migrates to internal organs via the afferent lymphatics and draining lymph nodes and the bloodstream (primary viraemia). The virus replicates in major organs, especially the liver and spleen, resulting in secondary viraemia within 4–5 days. Depending on the mouse strain, replication in the skin may lead to exanthema as early as 6 days after exposure. In A/J mice, death occurs before exanthema, as a consequence of severe liver necrosis. Aerosol exposure leads to a severe primary pneumonia.

The model using ECTV in the A/J mouse strain has been used recently to evaluate various analogues of the antiviral drug cidofovir for their effectiveness against lethal infection (Buller et al., 2004). In this study, the octadecyloxyethyl derivative of cidofovir, administered orally, protected 100% of mice challenged via aerosol with a lethal dose of 2.3×10^4 PFU, and completely blocked viral replication in spleen and liver. Under these same conditions, unmodified cidofovir was ineffective.

VACV also infects mice; the outcome depends on mouse genetics, VACV strains, doses and routes of exposure. Infection of C57BL/6 mice by VACV strain Western Reserve (WR) via the intranasal route using doses greater than 10^4 PFU is lethal (Brandt & Jacobs, 2001). This model was used to demonstrate that the VACV gene *E3L*, which provides interferon (IFN) resistance in vitro, is required for pathogenesis in the intact animal. BALB/c mice are somewhat more resistant to VACV, although head-to-head comparisons have not been reported. Lethality in BALB/c mice is dose dependent: in one study, 10^7 PFU of VACV strain WR was 100% lethal, while 10^4 PFU killed 20% of mice (Alcami & Smith, 1992). BALB/c mice were also used to rank VACV strains for virulence. The New

York City Board of Health (NYCBH) strain, with an LD_{50} via the intranasal route of $10^{4.0}$ PFU, was more virulent than the WR strain, with an LD_{50} of $10^{4.8}$ PFU. Neither strain was lethal via tail scarification, subcutaneous or oral routes (Lee et al., 1992). VACV derived from the Wyeth vaccine was less virulent via intranasal exposure (LD_{50} >10^7 PFU).

SKH-1 hairless mice have been used to establish dermal infections using VACV. The number of skin lesions indicates the severity of systemic infection. This model has been used to demonstrate the efficacy of 5% cidofovir, applied topically, in reducing both skin lesions and viral burdens in lung, kidney and spleen (Quenelle, Collins & Kern, 2004).

Intranasal infection of BALB/c mice with VACV WR leads to pneumonia, weight loss and death. Administration of cidofovir (100 mg/kg administered intraperitoneally [IP]) from one day after intranasal exposure protected all treated mice; in contrast, placebo controls all died within 8 days of exposure. Cidofovir markedly improved lung consolidation scores and reduced viral burdens in liver, spleen and brain; peak titres were 30- to 1000-fold lower than in placebo controls (Smee, Bailey & Sidwell, 2001).

Similar studies have been performed using CPXV strain Brighton Red, which is lethal for BALB/c mice under defined conditions (Bray et al., 2000). Disease patterns and lethality following aerosol or intranasal exposure vary with age and weight of the mice: 100% of 4-week-old mice infected with 2×10^6 PFU were killed, with a mean time to death of 8 days, whereas only 50% of 7-week-old mice succumbed. Infected mice died with bilateral viral pneumonitis and viral burdens of more than 10^9 PFU/g in the lungs. This model has been used to test the efficacy of various treatments for protection against systemic disease. Mice treated with a single dose of cidofovir administered IP (100 mg/kg) were 100% protected against an intranasal challenge ($2–5 \times 10^6$ PFU) when the drug was given 4 days before exposure, and as late as 4 days after exposure. Five days or more after exposure, cidofovir was less effective (Robbins et al., 2005). In contrast, vaccinia immune globulin was totally ineffective in reducing mortality. IFN-α B/D (5×10^7 U/kg) was effective before exposure and one day after exposure, but not later. When mice were vaccinated by tail scarification, they were protected when the procedure was begun 8 days before challenge; vaccination was less effective when this interval was reduced and ineffective when it began 2 days after challenge. This observation conflicts with epidemiological data suggesting vaccine efficacy up to 4 days after exposure in humans. The difference may reflect the higher challenge dose in the animal model, and illustrates the danger in extrapolating from rodent models to humans.

Although murine models can provide important insights into virulence and protective immune responses, virus–host interactions must be assessed individually, and cannot be generalized (Müllbacher et al., 2004). For example, ECTV and VACV differ in the requirement for IFN-γ after infection. In ECTV-infected mice, transfer of immune splenocytes from IFN-γ knockout mice is highly effective in reducing the titre of virus in liver and spleen; in an analogous experiment, VACV-immune splenocytes are ineffective (Müllbacher & Blanden, 2004). Thus, despite the apparent similarity of these two model OPV infections, recovery involves diverse and somewhat unpredictable host immune responses. Cytolytic T cell functions can be beneficial, detrimental or neutral (Müllbacher et al., 2004), and this balance will be unique to each virus–host system. This type of difficulty is an important consideration when specialized pathogens like OPVs are studied outside their natural hosts.

Rabbits exposed to RPXV via the aerosol route develop a disease syndrome similar to humans with smallpox (Lancaster et al., 1966; Westwood et al., 1966). In these studies, the Utrecht strain of RPXV was somewhat more virulent than the Rockefeller Institute strain. The Utrecht strain produced a lethal infection in New Zealand White rabbits, with death occurring 7–12 days after exposure; higher doses resulted in a more fulminant disease course, but little more than a single RPXV particle was sufficient to cause infection. Rabbits typically remained healthy for a 4–6 day incubation period, followed by fever, weakness, rapid weight loss, and profuse, purulent discharges from the eyes and nose. A bright erythema appeared on the lips and tongue, coinciding with a generalized skin rash, with the number of lesions varying from a few to confluence. In some cases, death occurred before the rash developed. The lesions started as red papules, converting to pseudo-pustules with caseous contents. Death usually occurred before true scabs could form, and was presaged by a rapid fall in body temperature. High RPXV burdens were detected in all visceral tissues, peaking between days 5 and 8, at titres of 10^8 PFU/g in the lung and 10^7 PFU/g in the spleen and adrenal glands. In some instances, early deaths of the rabbits correlated with a blood coagulation defect (Boulter, Maber & Bowen, 1961), analogous to the haemorrhagic form of human smallpox (Martin, 2002). There is also some evidence that infected rabbits become contagious only in the late stages of disease, despite the presence of virus in nasopharyngeal fluids at earlier stages, as described for human smallpox.

In more recent studies, intradermal inoculation of rabbits resulted in a similar disease pattern (Adams, Rice & Moyer, 2007). A viral dose of 1×10^2 PFU administered intradermally results in systemic infection, but a higher dose (5×10^3 PFU) is required for lethality. Initially, the injection site becomes swollen, leading to necrosis by 5 days after infection. Fever begins by day 3, followed by increased respiration rate by day 4. Secondary lesions, including eye and nasal discharges, occur by day 7, accompanied by weight loss. Shortly before death, respiration rate decreases and heart rate increases, and the animal falls into respiratory distress by day 7 or 8. RPXV infection of the rabbit has parallels to human smallpox.

A lack of reagents and inbred rabbit strains hinders sophisticated analysis of these immunologic events; for these reasons, model studies using mice are preferred. However, virulence genes can now be evaluated by genetic manipulation of RPXV and testing of these modified strains in rabbits (McFadden, 2005a). As well, the RPXV rabbit model could possibly be developed further and used to test candidate therapeutics and vaccines against human smallpox, providing a stepping stone for prioritizing testing of these agents in the available primate models.

It is important to distinguish between RPXV, which is a VACV strain classified in the genus *Orthopoxvirus,* and myxoma virus, which is in a distinct genus, *Lepovipoxvirus*. Although myxoma virus produces a lethal disease in New Zealand White rabbits that has many similarities to the disease caused by RPXV, myxoma virus is more distantly related to the human pathogens, and is therefore less relevant to human smallpox than the animal models for OPVs discussed elsewhere in this chapter.

MPXV is a significant human pathogen that produces many of the signs and symptoms of smallpox, although it has less potential for transmission from person to person (Fine et al., 1988; Jezek et al., 1988). There is evidence that MPXV strains of west African origin are less virulent than those that arise sporadically in central Africa, specifically the

Democratic Republic of the Congo (McFadden, 2005b). The name "monkeypox" may be a misnomer, since the virus is maintained in nature in rodent reservoirs, including squirrels (Khodakevich, Jezek & Kinzanzka, 1986; Charatan, 2003). In 2003, MPXV was inadvertently imported into the United States in a shipment of rodents originating in the Republic of Ghana that included an infected giant Gambian rat (Perkins, 2003; Ligon, 2004). The rat infected a number of prairie dogs held in the same facility, and a chain of transmission ensued that involved hundreds of prairie dogs and spread to more than 75 human cases in 11 states. This outbreak of monkeypox rekindled interest in MPXV, not only as a surrogate for smallpox, but as a disease entity in its own right.

Experimental infection of ground squirrels with the United States strain of MPXV was reported to kill all squirrels exposed to $10^{5.1}$ PFU by the IP route, or to $10^{6.1}$ PFU by the intranasal route, within 6–9 days (Tesh et al., 2004). Systemic infections with high viral burdens were reported; major histologic findings included centrilobular necrosis of the liver, splenic necrosis and interstitial inflammation in the lungs. It is possible that MPXV infection of squirrels might be developed into a useful animal model for testing countermeasures for monkeypox and smallpox. Prairie dogs involved in the United States MPXV outbreak had pulmonary consolidation, enlarged lymph nodes and multifocal plaques in the gastrointestinal wall (Langohr et al., 2004). Recent advances in the development of prairie dog models for human monkeypox have been reported (Knight, 2003; Hutson et al., 2009), as have alternative rodent models, including African dormice (*Graphiurus* sp.) (Schultz et al., 2009).

The commercial availability of strains of mice has further expanded the options for testing countermeasures. Recently, STAT1-deficient C57BL/6 mice were reported to be susceptible to low doses of MPXV via the intranasal route, and these mice were useful for demonstrating the efficacy of two antiviral drugs – CMX001 (or HDP-cidofovir) and ST-246 – administered on the day of infection (Stabenow et al., 2010). Even more recently, the commercially available inbred mouse strain CAST/EiJ has been shown to be susceptible to MPXV infection via the intranasal route, with an LD_{50} of 680 PFU (Americo, Moss & Earl, 2010). These mice were even more sensitive (LD_{50} of 14 PFU) when inoculated via the IP route. CAST/EiJ mice are immunologically competent and provide significant advantages for testing potential countermeasures against OPV infections – they are genetically homogeneous and commercially available, and immunological reagents to assess host response are available.

Primates have been infected with MPXV via the aerosol (Zaucha et al., 2001), intramuscular (Wenner et al., 1969), intratracheal, intrabronchial and intravenous (IV) routes of exposure (Stittelaar et al., 2006). Most of the early reported studies used cynomolgus macaques, either *Macaca iris* or *M. fascicularis* (Hahon, 1961), although rhesus monkeys (*M. mulatta*) may also be suitable (Hooper et al., 2004). Aerosol exposures are most appropriate for modelling primary exposures following a biological warfare attack. Natural transmission of MPXV (and VARV) probably occurs by a combination of aerosol exposure, fomites and mucosal exposure. Aerosol exposure requires BSL-4 biocontainment in a Class III cabinet, and is less controllable than IV exposure.

Experimental MPXV infection of cynomolgus monkeys by the aerosol route (calculated inhaled dose of 3×10^4 PFU) resulted in five of six monkeys dying (one on each of days 9, 11 and 12, and two on day 10), with a mean time to death of 10.4 days. The monkeys

had significant fevers (>39 °C), mild exanthema, coughs, and leukocytosis with an absolute and relative monocytosis (Jahrling, Zaucha & Huggins, 2000). Virus was isolated from buffy coat cells of febrile animals and, at necropsy, high titres of virus (>10^6 PFU/g) were isolated from lungs and spleens (Zaucha et al., 2001). Histopathologic examinations attributed death to severe fibrinonecrotic bronchopneumonia; immunohistochemistry showed abundant MPXV antigen in samples of affected airway epithelium and surrounding interstitium. The clinical parameters measured in monkeys exposed to aerosolized MPXV occur in a sequence similar to that in humans, but at a greater rate (Breman & Henderson, 2002).

IV exposure of cynomolgus macaques to MPXV also resulted in uniform systemic infection; disease severity was related to dose (Stittelaar et al., 2006; Huggins & Jahrling, unpublished observations). Cynomolgus monkeys infected by the IV route with 1×10^7 PFU of MPXV (Zaire 79 strain, CDC V79-I-005) developed a low-grade fever beginning on day 3 and pox lesions from days 4 to 5. Death occurred from day 8, with a mean time to death of 12 days, which was 4–8 days after onset of the rash. This is shorter than the 10–14 days seen with human monkeypox. Mortality occurred in 11 of 12 infected monkeys (92%), compared with 10% in the human disease. Pox lesions were found in all animals, and were graded as "grave" on the WHO scoring system (more than 250 lesions). The hands, feet, mouth and soft palate were fully involved. All monkeys followed this typical pattern of progressing through the stages of lesion development, and those that lived long enough ultimately developed scabs. Weight loss was seen in all animals. Laboratory findings were largely unremarkable, except for a rise in blood urea nitrogen and creatinine shortly before death.

At necropsy, organs were significantly affected by both gross pathological lesions and virus replication. Virus titres in lung, liver and spleen were greater than 10^8 PFU/g, and blood had 10^5 PFU/ml of virus, which was cell associated. Plasma was free of infectious virus. Virus titres in kidney were slightly above those in blood, suggesting that the kidney is a site of viral replication; significant virus burdens (above the contained blood) were not detected in the brain.

To determine the effect of infectious dose on progression of the disease, lower doses of 10^5 and 10^6 PFU were evaluated. The lower doses did not cause death, but all animals became sick and developed lesions. The number of lesions, based on the WHO scoring system, depended on the dose and ranged from mild (at 10^5 PFU), through moderate (at 10^6 PFU), to severe (at 10^7 PFU). Infected animals showed significant increases in white blood cells, but this was not dose dependent. There was a dose-dependent drop in platelets, reaching a low on days 2–8, but platelets then returned to normal ranges. Pulmonary function was not significantly impaired at the lower virus doses. All animals developed low-grade fevers (<38.3 °C) by days 3–4. Poxvirus lesions were first seen between days 4 and 5, continued to increase in size until days 10–12, and then resolved over the next two weeks in surviving animals. Animals infected with lethal doses of 10^7 PFU or greater had more than 1500 lesions. Viral loads in blood, measured as genomes per millilitre of whole blood by quantitative polymerase chain reaction (PCR), could be detected 24 hours after infection, and increased to more than 10^7 genomes/ml before death. Viral loads in surviving animals – either those given lower infectious doses or those treated successfully with antiviral chemotherapy or vaccination – never exceeded 10^6 genomes/ml. Albumin decreased in a dose-dependent manner, falling to

15 mg/ml in monkeys infected with 10^7 PFU, while total serum protein remained within normal limits.

The IV MPXV challenge model was used to test the efficacy of a candidate vaccine for smallpox, the highly attenuated modified vaccinia virus Ankara (MVA). In this study, the MVA vaccine was compared, and used in combination, with the licensed Dryvax vaccine (Earl et al., 2004). Monkeys were vaccinated with MVA or Dryvax. In week 8, the MVA-immunized monkeys were boosted with either MVA or Dryvax and then, in week 16, challenged by the IV route with MPXV. The placebo controls developed more than 500 pox lesions and became gravely ill; two of six died. In contrast, none of the monkeys receiving Dryvax or MVA/Dryvax developed illness; monkeys in the MVA/MVA group remained healthy but developed an average of 16 lesions. None of the vaccinated monkeys developed significant viraemia, as detected by quantitative PCR (Kulesh et al., 2004), in contrast with placebo controls, which developed virus titres greater than 10^8 genomes/ml in blood. When the monkeys were being immunized, it was observed that MVA elicited higher enzyme-linked immunosorbent assay titres within 10 days of immunization than Dryvax. To determine if the immune response to MVA was sufficient to be protective this early, monkeys were immunized with a single dose of MVA or Dryvax, and challenged on day 10. In contrast with controls, which developed more than 500 lesions each and became gravely ill, none of the MVA or Dryvax recipients became ill; both groups developed isolated lesions (three to six per animal). MVA and Dryvax both limited viral replication to titres lower than the artificial viraemia created by IV infection with MPXV. Dryvax provided solid protection against an aerosol challenge with MPXV in vaccine efficacy studies that compared Dryvax with a VACV derived from cell culture (Jahrling, 2002). More recently, MVA provided solid protection against lethal MPXV challenges via the respiratory route in cynomolgus macaques (Stittelaar et al., 2005).

Rhesus monkeys were used in a similar IV challenge model to evaluate a DNA (deoxyribonucleic acid) vaccine strategy with a combination of four VACV genes (*L1R*, *A27L*, *A33R* and *B5R*); results were promising (Hooper et al., 2004). Recently, these genes have been expressed in an alphavirus replicon, with similar promising results (Hooper et al., 2009).

There has been some reluctance to accept the IV challenge model, on the grounds that the challenge should be via the "natural" route. The counterargument to this concern is that protection against an overwhelming IV dose is a very stringent criterion, and may predict efficacy against infection by peripheral routes. However, because of these concerns, and because IV challenge sets the bar too high for antiviral drug evaluations, alternative exposure models, including intratracheal routes, are being explored. Since the dose–response curve is very steep, IV administration of the virus is an advantage in calibrating the inoculum dose. Aerogenic or mucosal routes of exposure would require larger numbers of animals.

Despite these limitations, the IV MPXV model has been used to demonstrate the efficacy of a number of candidate antiviral drugs, including cidofovir (Wei et al., 2009) and ST-246 (Jordan et al., 2009); these studies are outside the scope of this chapter.

Recently, alternative primate models using other OPV challenges have been explored. One promising approach is CPXV infection of marmosets (*Callithrix jaccus*) (Kramski et al.,

2010). Marmosets are lethally infected by CPXV challenges as low as 5×10^2 PFU via the intranasal route, and they develop a progression of signs reminiscent of smallpox. Although the marmoset is more distantly related to humans than are macaques, and immunological reagents are not yet readily available, this species holds significant promise for future model development.

An animal model in which VARV produces a disease similar to human smallpox is required to convincingly demonstrate the protective efficacy of vaccines and antiviral drugs for smallpox (USFDA, 2002, 2008, 2009). Because of the species specificity of VARV, it was not surprising that attempts to infect and produce disease with VARV in rodents and rabbits were unsuccessful (Marennikova, 1979). Indeed, even in primates, early experiments with VARV resulted in mild but self-limited infections. Cynomolgus macaques, exposed to aerosols containing 2×10^8 pock-forming units, developed a rash after a 6-day incubation period; virus replicated in the lungs, and secondary sites of replication were established in lymph nodes before viraemia occurred (Hahon, 1961). In the same study (Westwood et al., 1966), 109 rhesus monkeys were exposed; all developed fever by day 5 and rash between days 7 and 11, but only two died. Bonnet macaques (*Macaca radiata*) were also resistant to disease following infection (Rao et al., 1968); none of 14 died. However, the same authors demonstrated that cortisone treatment rendered monkeys susceptible; 14 of 16 died, as did one untreated but pregnant monkey. In human populations, pregnant women suffered the highest mortality following smallpox infections (Rao et al., 1963).

The historical record thus suggested that there were no suitable models for the pathogenesis of VARV in humans (US Institute of Medicine, 1999). However, infection of macaques was known to produce skin lesions and evidence of systemic infection, and a primate model was used to license MVA in Germany in the 1960s (Hochstein-Mintzel et al., 1975). It was therefore reasonable to test other VARV strains in higher doses by a variety of routes to seek a model for lethal smallpox. Aerosol exposure of cynomolgus monkeys to either the Yamada or Lee VARV strains ($10^{8.5}$ PFU) resulted in infection but no serious disease (LeDuc & Jahrling, 2001); however, when monkeys were exposed to either Harper or India 7124 VARV strains by the IV route, acute lethality resulted (Jahrling et al., 2004). Doses lower than 10^9 PFU caused lower lethality, and quantifiable parameters of disease severity diminished with declining dose.

In monkeys dying after VARV infection, the end-stage lesions resembled terminal human smallpox. Our understanding of the pathophysiology of human smallpox is imprecise, since the disease was eradicated before the development of modern tools of virology and immunology. However, the primate models may inspire re-investigation of archived specimens using modern techniques such as immunohistochemistry and cDNA microarrays, which were used in the primate model studies (Jahrling et al., 2004; Rubins et al., 2004). A recent review of all pathology reports published in English in the past 200 years (Martin, 2002) suggested that, in general, otherwise healthy patients who died of smallpox usually succumbed to renal failure, shock secondary to volume depletion, and difficulty with oxygenation and ventilation as a result of viral pneumonia and airway compromise, respectively. Degeneration of hepatocytes might have compromised health to some degree, but liver failure was not usually the cause of death.

End-stage lesions in monkeys inoculated with VARV closely resembled this human pathology (Jahrling et al., 2004). After experimental infection, a number of parameters

could be evaluated at intermediate time points before death. Monkeys inoculated by the IV route had a demonstrable artificial viraemia immediately after inoculation. Following an eclipse phase of several days, virus in the blood was associated only with monocytic cells. Animals that died had profound leukocytosis, thrombocytopenia and elevated serum creatinine levels. High viral burdens in target tissues were associated with organ dysfunction and multisystem failure. The distribution of viral antigens (using immunohistochemistry) correlated with the presence of replicating viral particles (using electron microscopy) and with pathology in the lymphoid tissues, skin, oral mucosa, gastrointestinal tract, reproductive system and liver. Histologic evidence of bleeding tendency was corroborated by elevations in D-dimers. Apoptosis of T cells occurred in lymphoid tissue, probably resulting from viral replication in macrophages and the resultant cytokine storm. “Toxaemia”, described by clinicians as the terminal event in human smallpox, probably results from overstimulation of the innate immune response, including interleukin-6 and IFN-γ, as much as from direct viral damage to target tissues.

Peripheral blood samples from the monkeys were analysed using cDNA microarrays designed for the study of human gene expression patterns (Rubins et al., 2004). VARV elicited striking and temporally coordinated patterns of gene expression (features that represent an IFN response), cell proliferation and immunoglobulin expression, correlated with viral dose and modulation of the host immune response. Surprisingly, a tumour necrosis factor-α – nuclear factor kappa B (NF-κB) response was virtually absent, suggesting that VARV gene products may ablate this response. The interaction of VARV with the human immune system can only be approximated in the monkey models, but it is less tenuous to extrapolate from primates to humans than from rodents to humans. Whether MPXV in monkeys is a better model for human smallpox than VARV in monkeys is a focus of intense investigation. Both primate models may provide insight into development of diagnostic, prophylactic and therapeutic strategies.

5.3 Conclusion

It is generally acknowledged that the primate models for either MPXV or VARV replicate some, but not all, features of human disease. IV infection with these viruses leads to a sequence of disease manifestations that is similar to the disease in humans (Breman & Henderson, 2002), although it is accelerated due to the elimination of a prodromal period. As in humans following secondary viraemia, onset of fever is followed by development of macules, papules, vesicles, pustules and eventually crusts, if the patient does not succumb to the disease. The experimental disease course can be lengthened by decreasing the IV inoculum dose; a 10-fold reduction in dose reduces lethality from 100% to approximately 33%, and increases the mean time to death. Although 33% mortality for VARV more closely resembles the human condition, use of this model for efficacy determinations must rely on surrogate end-point determinations rather than reduction in mortality. Currently, surrogate end-points include reductions in viraemia and lesion count, which are crude measures of disease severity. More time is required to develop a panel of surrogate end-points that more accurately reflect the pathophysiology of disease and its reversal. These might include panels of biomarkers and genome-wide patterns of protein expression, in conjunction with medical imaging – including magnetic resonance, positron emission tomography, single photon emission computed tomography and computed tomography.

Further investment in refined primate models should begin after the data from previous experiments have been thoroughly evaluated. These experiments include those done with lower doses of virus administered by alternative routes, including aerosol, intrabronchial, intratracheal and droplet exposures.

It is likely that no single combination of conditions will result in a model that will simultaneously satisfy all of the criteria of the ideal model smallpox infection; different models may be required to assess different indications. The value of pathophysiologic data from studies using telemetry and medical imaging should be considered. If further investment in specific models is indicated, special attention should be paid to elucidating biomarker patterns that could be used in a clinical setting as triggers for early intervention, thus increasing the likelihood of successful intervention.

Abbreviations

CPXV	cowpox virus
DNA	deoxyribonucleic acid
ECTV	ectromelia virus
IFN	interferon
IP	intraperitoneal
IV	intravenous
MPXV	monkeypox virus
MVA	modified vaccinia virus Ankara
OPV	orthopoxvirus
PCR	polymerase chain reaction
PFU	plaque-forming unit
RPXV	rabbitpox virus
VACV	vaccinia virus
VARV	variola virus
WHO	World Health Organization
WR	Western Reserve

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6 Antiviral drug development for the treatment of smallpox – status of small-molecule therapeutics

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Executive summary

Public health importance

Widespread vaccination against smallpox is extremely unlikely to occur before the first occurrence of a smallpox outbreak because of the serious and occasionally fatal events associated with current smallpox vaccines. Therefore, if smallpox re-emerges, it may be necessary to treat a large number of cases with an antiviral drug before mass vaccination campaigns have time to provide adequate protective immunity.

Previous smallpox control measures have had to rely exclusively on vaccination and supportive care of infected individuals, who may be facing a 30% chance of dying from the infection. However, experience with control of the current H1N1 influenza epidemic has shown that both vaccine and antiviral drugs can be important as part of the public health response, both to control the outbreak and to reduce mortality in those infected.

The project described in this chapter was undertaken to obtain two approved oral antiviral drugs, with different mechanisms of action, for treating clinical cases of smallpox. These drugs need to have been approved by drug regulatory agencies if they are to be used during an outbreak. Regulatory approval also provides convincing evidence of the efficacy of the drugs, which will be needed by public health officials who are formulating control strategies. Because smallpox, caused by variola virus, was eradicated by mass vaccination, the effectiveness of these drugs can only be demonstrated using variola virus–infected animal models in non-human primates.

Progress to date

Development of any antiviral therapeutic is a long and difficult process, which has been unsuccessful for many viral infections, including the common cold. For smallpox, considerable progress has been made in initial drug discovery, and a number of potential candidates need to be evaluated in animal models.

Three compounds – cidofovir, ST-246 and CMX001 – that inhibit variola virus replication, in cell culture and in multiple animal models (surrogate orthopoxvirus models) have gained investigational new drug (IND) status from the United States Food and Drug Administration (USFDA) for treatment of orthopoxvirus infections. Initial human studies are in progress. Two of these compounds (cidofovir and ST-246) have demonstrated activity in a lethal primate model of variola virus, and the third is a prodrug of cidofovir that can be given orally. Development of ST-246 and CMX001 is in progress, and clinical trials are ongoing.

Additional work requiring live variola virus to obtain an approved antiviral drug for the treatment of smallpox

Although results to date are promising, extensive industry experience with drug development suggests that fewer than 35% of compounds entering expanded safety trials (USFDA phase II) will obtain approval. The process of moving from the IND stage to the new drug application stages takes an average of five to seven years.

Since variola virus has been eradicated from the human population, traditional clinical efficacy trials are not feasible. In addition, it is not possible to conduct ethical clinical trials in humans, so demonstration of efficacy must use the USFDA “animal rule” (US 21CRF310.610). Given the uncertainties in the animal rule, and the fact that no antiviral drugs are currently approved for any type of smallpox indication (treatment or chemoprophylaxis), it is difficult to estimate the time lines or the data required for approval; the data are expected to include, but not be limited to, work with variola virus. The intensity of review and the level of scientific scrutiny applied to animal model studies proposed to support indications under the animal rule would be the same as for human clinical trials to support approval of products for other types of indications using other approval pathways.

Approval in countries other than the United States is associated with at least as much uncertainty.

It could be argued that work with live variola virus must remain an option until an adequate number of drugs, with different mechanisms of action, have gained regulatory approval and could be used worldwide to combat an outbreak of smallpox.

A report by the Institute of Medicine of the National Academies, entitled *Live variola virus considerations for continuing research*, concluded that “the most compelling reason for long-term retention of live variola virus stocks is their essential role in the identification and development of antiviral agents for use in anticipation of a large outbreak of smallpox”.

6.1 Introduction

Naturally occurring smallpox disease was being eradicated by mass vaccination at a time when antiviral therapy was still in its infancy. The drugs available at that time, including the thiosemicarbazones methisazone (Marboran) and the related M&B7714, were inactive in therapeutic and prophylactic field trials (Rao, McFadzean & Squires, 1965; Rao, McFadzean & Kamalakshi, 1966; Rao et al. 1966). These drugs have been withdrawn from the market. Cytosine arabinoside (ara-C) (Dennis et al., 1974) and adenosine arabinoside (ara-A) (Koplan et al., 1975) also failed to reduce mortality. The results of those studies have been reviewed (Smee & Sidwell, 2003).

Drug development for smallpox is a complicated, lengthy and costly process. Most of the progress in drug discovery to date has been accomplished by universities, government laboratories and a few small pharmaceutical companies. Development of a therapeutic compound begins with discovery of a compound that selectively inhibits viral replication in cell culture. This discovery guides organic chemists to synthesize similar chemical structures, to determine the most active compound in that class. Often, this is followed by a medicinal chemistry approach, in which the structure is systematically modified and antiviral activity is evaluated to produce the most potent compound, now referred to as a “lead compound”. Most published work has been done in these initial steps.

Often the lead compound does not have all the properties of a desirable drug, such as low toxicity, oral dosing, resistance to metabolic inactivation, adequate solubility and many other properties that contribute to a successful treatment. During development, the medicinal chemist will try to modify the structure to improve desirable properties such as solubility, while removing undesirable aspects such as toxicity, often having to sacrifice potency in the trade-off. Complex multidisciplinary studies in animals, conducted in compliance with good laboratory practice (GLP), are then required to understand the compound’s metabolism, pharmacokinetics, distribution in tissues and toxicity. These properties must be evaluated in multiple species, using a series of fairly standard tests, as well as in customized studies to determine whether the compound will be safe for initial administration in humans.

At this time, an extensive series of evaluations, referred to as the “microbiology section”, must be conducted in cell culture and animal models. These evaluations provide information on the compound’s ability to inhibit viral replication, and preliminary information on the ability of the compound to reduce the morbidity and mortality of the disease. Together with information from pharmacokinetic studies in the same animal species, the studies suggest the minimum concentration required for drug activity, and, in conjunction with investigational new drug (IND) phase I human studies, provide the initial estimate for the corresponding target dose in humans.

In the United States, any administration of a potential drug to humans for clinical evaluation requires the submission of an IND application, which includes a formal submission of the studies described above, plus information on the synthetic methodology and quality control procedures. Completion of the studies required to obtain an IND takes several years and must occur before the compound is evaluated in humans. Fewer than 10–25% of compounds receiving an IND ultimately obtain approval.

Phase I studies in humans begin with a small number of subjects and a concentration of the compound chosen to be far (at least 10-fold) below the level predicted to cause any toxicity. The concentration is slowly increased while the drug pharmacokinetics and distribution are determined, and an adequate drug level, based on preclinical studies, is obtained. If the drug candidate is safe at levels predicted to be therapeutic, an expanded safety study is conducted in increasing numbers of subjects. These studies ultimately involve special populations that are candidates for treatment, including those with complicating medical conditions. For smallpox, where it would not be desirable to exclude any population, this includes paediatric, adolescent, adult and geriatric populations, as well as people with conditions that can affect drug levels, such as renal or liver disease. Ultimately, safety will need to be evaluated in hundreds of subjects (evaluation in 600 subjects will detect an adverse event at the 1% level).

Historically, the efficacy data that the United States Food and Drug Administration (USFDA) has required for drug approval come from "well-controlled", pivotal human clinical trials. This requirement cannot be met with drugs for a highly pathogenic disease like smallpox. To address this problem, the USFDA has published what is commonly referred to as the "animal rule" (USFDA, 2009). The animal rule allows demonstration of efficacy using an animal model (or models) that adequately reproduces the critical aspects of disease, and in which a similar reduction in the magnitude of the disease in humans would be expected to reduce morbidity and/or mortality. However, this introduces several additional problems in drug development. Establishing the equivalent target drug dose for humans and the species used for efficacy testing has become more critical, as this will determine the human dose. Extrapolating the therapeutic window between humans and the current variola virus (VARV) primate models is also difficult, and existing models may underestimate the latest time that treatment can be initiated, due to the severity of the model.

For smallpox, the goal for an effective therapeutic treatment is a reduction in mortality when treatment is begun after onset of lesions (the only practical diagnosis during a large outbreak). This eliminates drugs that only reduce morbidity, and, given current models for VARV infection, provides a more severe requirement for the drug.

Apart from limited studies at the two World Health Organization (WHO) collaborating centres (Centers for Disease Control and Prevention in the United States, and the State Research Center of Virology and Biotechnology VECTOR [SRC VB VECTOR] in the Russian Federation), the discovery process for VARV has been conducted with surrogate viruses, due to limited access to VARV and the difficulties in conducting such research in biosafety level 4 (BSL-4) facilities. Most work has used the closely related surrogate viruses vaccinia virus (VACV) and cowpox virus (CPXV), with some additional screening with ectromelia virus (ECTV) and rabbitpox virus (RPXV). These viruses are of lower biohazard, and ECTV and RPXV can be handled under BSL-2 conditions. Highly potent compounds are then evaluated against monkeypox virus (MPXV), and a much smaller number are evaluated against VARV.

Many laboratories have contributed to efforts to discover antiviral compounds that are active against orthopoxviruses (OPVs). In 2009, a PubMed search of the terms "orthopoxvirus (OPV)" and "antiviral" found 1041 publications. Most of these involved only preliminary drug discovery efforts – many using VACV as their primary in vitro

screen – as suggested (De Clercq, 2001). Other groups routinely include CPXV; a study by the United States Army Medical Research Institute for Infectious Diseases (USAMRIID) involving more than 500 compounds determined that CPXV, among the viruses that can be handled in BSL-2 facilities, was the best predictor of VARV activity (Huggins, unpublished observation, 2003). Many of the published results have been reviewed by multiple authors (Goebel et al., 1982; Bray et al., 2000; De Clercq, 2001; Baker, Bray & Huggins, 2003; Keith et al., 2003; Kern, 2003; Smee & Sidwell, 2003; De Clercq et al., 2005; Smee, 2008), and readers are directed to these reviews for details on specific compounds. These authors looked at the activity of a number of classes of compounds, often grouping them by their proposed mechanism of action.

6.2 Drug discovery

OPVs are large, linear, double-stranded DNA (deoxyribonucleic acid) viruses that replicate exclusively in the cytoplasm, using a number of viral-encoded enzymes to carry out DNA replication. The complex replication mechanism of OPVs provides a number of targets for drug intervention. The OPV genome has a highly conserved central portion that encodes the machinery required for viral replication; the two ends, which are more variable, encode proteins that affect virulence and host range. The genome of VARV, the virus that causes smallpox, encodes a large number of proteins that interfere with the host's ability to recognize infection and to induce the mechanisms that normally allow humans to fight off infection. The machinery required for viral replication has been the most frequent target for antiviral drug design, as it is highly conserved among OPVs, allowing surrogate viruses such as VACV and CPXV to be used for initial drug discovery. Furthermore, the viral DNA polymerase shares significant sequence similarity at the active site with other DNA viruses, including herpesviruses.

6.2.1 Thiosemicarbazones

Thiosemicarbazones were the first class of drug to show activity against OPVs (Bauer, 1955); the use of methisazone to treat smallpox is discussed in section 6.1. Methisazone showed moderate antiviral activity against VARV in assays based on 50% inhibition (Baker, Bray & Huggins, 2003), but was able to inhibit 80% of replication only at the highest tolerated concentration (Huggins, unpublished observation, 2003). A large number of related compounds have been evaluated in vitro, and some in mouse models, showing moderate activity, but none have gone on to advanced evaluation in primates.

6.2.2 Evaluation of approved and investigational new drugs

Current USFDA-approved drugs were systematically evaluated in vitro against VACV and CPXV (Kern, 2003). They included drugs that are active against herpesviruses, hepatitis B virus (DNA viruses) and human immunodeficiency virus (an RNA [ribonucleic acid] virus); nucleoside reverse transcriptase inhibitors, non–nucleoside reverse transcriptase inhibitors and protease inhibitors; and several compounds with IND status. Many of the active compounds were also evaluated against VARV and MPXV (Goebel et al., 1982; Baker, Bray & Huggins, 2003). Kern et al. (2002) identified several compounds with relevant activity, including the previously known cidofovir and adefovir dipivoxil (bis-POM PMEA); however, Baker, Bray & Huggins (2003) found the latter compound less active. Two topical drugs, idoxuridine and trifluridine, were active, but could not be given orally due to toxicity. A compound library consisting of most approved drugs was

evaluated without identifying additional candidates (Huggins, unpublished results from USAMRIID screening programme, 2005).

6.2.3 Acyclic nucleoside phosphonates

The acyclic nucleoside DNA polymerase inhibitors – such as acyclovir, penciclovir and ganciclovir, and their oral prodrugs – were first developed for herpesviruses. They require phosphorylation by a viral-encoded thymidine kinase or protein kinase, and further phosphorylation by cellular kinases to produce the active triphosphate antiviral compound. Human cytomegalovirus (HCMV) thymidine kinase was not capable of phosphorylating these compounds, so a class of acyclic nucleoside phosphonates was developed. These were the equivalent of the nucleoside monophosphate, but employed chemically resistant phosphonates that are not recognized by phosphatases that would normally remove a monophosphate while in circulation. The key was substituting a stable P–C phosphonate bond that is not cleaved by cellular hydrolases for the labile P–O–C bond (De Clercq, 2003; Helliot et al., 2003; Keith et al., 2003). As for HCMV, the OPV thymidine kinase will not phosphorylate acyclic nucleosides, but cidofovir (HPMPC) and its cyclic analogue cyclic-HPMPC were both active in vitro against all OPVs tested, including VARV (Baker, Bray & Huggins, 2003). Cidofovir was the first compound to show protection in the VARV primate infection model, and has demonstrated protection in multiple small-animal models (see Table 3 in Smee, 2008 for a listing). Intravenous (IV) cidofovir (Vistide) is approved for the treatment of HCMV retinitis at a once-weekly dose of 5 mg/kg; its current status is reviewed in sections 6.5 and 6.9.

Uptake problems of cidofovir, which is taken up by pinocytosis and requires bolus dosing that can result in nephrotoxicity, were overcome by lipid derivatives of cidofovir synthesized by Hostetler and colleagues (Painter & Hostetler 2004). Lipid analogues use the chylomicron pathway for effective uptake and are orally bioavailable (Kern et al., 2002; Keith et al., 2004; Painter & Hostetler 2004; Beadle et al., 2006; Lebeau et al., 2006). A large series of lipid analogues has been evaluated, and hexadecyloxypropyl-cidofovir, later renamed CMX001, was selected for advanced evaluation (see Table 4 in Smee, 2008 for a partial listing of published evaluations). CMX001 is reviewed separately in sections 6.6 and 6.9.

6.2.4 Inosine monophosphate dehydrogenase inhibitors

Inosine monophosphate dehydrogenase inhibitors include ribavirin, the first broad-spectrum nucleoside analogue (Sidwell et al., 1972). Ribavirin, along with several other members of this class of compounds, is active in vitro against VACV in mice and RPXV in rabbits. However, ribavirin did not provide any protection in the MPXV primate infection model (Huggins, unpublished observations, 2001) when administered prophylactically at a dose, route and schedule that protected primates against infection with Junin virus (McKee et al., 1988) and Lassa virus (Jahrling et al., 1980; Stephen & Jahrling 1979). Newer analogues – FICAR, EICAR, tiazofurin (active against VARV in vitro) and selanazole – showed activity against CPXV infection of mice (Huggins, unpublished observation, 2003), but have not been evaluated in primate models (where ribavirin failed) and are not considered likely to be effective.

6.2.5 S-adenosylhomocysteine hydrolase inhibitors

S-adenosylhomocysteine hydrolase inhibitors are believed to inhibit OPVs by inhibiting a specific step in cap methylation (mRNA-capping enzyme) that is required for viral replication (see Figure 4 [De Clercq, 2001] for structures). Two members of this class, 3-deazaneplanocin A and carbocyclic 3-deazaneplanocin A, were among the most active inhibitors of VARV (Baker, Bray & Huggins, 2003), and showed activity against intranasal CPXV infection (Baker, Bray & Huggins, 2003). Although moderate activity was seen with both compounds in this infection model (Huggins, unpublished results, 2003), they have not been evaluated further.

6.2.6 Orotidine-5'-monophosphate decarboxylase inhibitors

Pyrazofurin, the prototype for the orotidine-5'-monophosphate decarboxylase inhibitors, is one of the most active antiviral compounds in vitro, but is toxic in animals. Extensive medicinal chemistry efforts to separate the antiviral portion of the molecule from the toxic portion have been unsuccessful. As a result, this drug should be considered an anticancer drug, but with significant toxicity (Huggins, unpublished observation from the USAMRIID screening programme, 2001).

6.2.7 Thymidylate synthase inhibitors

Although VACV does not encode a thymidylate synthase, thymidylate synthase inhibitors – 5-substituted 2'-deoxyuridine compounds (see Figure 9 in [De Clercq, 2001] for structures, and Table 1 in [De Clercq, 2001] for in vitro activity) – have shown activity against VACV infection of mice by tail scratch. Rapid catabolism to inactive metabolites has limited the potential of 5-iododeoxyuridine and related compounds. However, recently, the 4'-thio analogue (SRI-21950) solved metabolic instability and provided excellent protection against infection of mice with VACV or CPXV (Kern et al., 2009).

6.2.8 Compounds that interfere with virus assembly

Compounds that block specific steps in OPV assembly include rifampin and N_1-isonicotinoyl-N_2-3-methyl-4-chlorobebzoylhydrazine (IMCBH). Rifampin interacts with the 65-kilodalton polypeptide encoded by the VACV *D13L* gene (Sodeik et al., 1994), and IMCBH targets a 37-kilodalton protein encoded by the VACV *F13L* gene, which is a component of the outer envelope of extracellular enveloped virus (EEV) or extracellular virus (Schmutz et al., 1991). Rifampin acts by blocking the formation of the first infectious form of virus, called intracellular mature virus (IMV) or mature virus, so no infectious progeny are formed. In contrast, IMCBH acts by preventing the wrapping of IMV particles by a double layer of cell membrane derived from either the trans-Golgi network or early endosomes, which has been modified by insertion of several virus proteins. Although blocking virus morphogenesis at this stage does not prevent the formation of infectious IMV particles, it prevents the transport of virus out of the cell and greatly diminishes spread, which is mediated by EEV or extracellular virus.

ViroPharma and USAMRIID used a very simple high-throughput screening assay to screen one third of the million compounds from their compound library, identifying several classes of active agents. Active compounds were evaluated against MPXV. Ultimately, an extensive medicinal chemistry effort (Bailey et al., 2007) led to a limited number of candidates, which were then screened against VARV (Yang et al., 2005). ST-246 was

selected for advanced development. A partial listing of the animal models in which ST-246 has shown protection is shown in the review by Smee (see Table 4 in Smee, 2008). Further progress in the development of this compound is described in sections 6.7 and 6.9.

6.2.9 Abl-family kinases

Gleevec, approved to treat chronic myelogenous leukaemia in humans, blocks the egress of OPVs from cells. After infection of mice with a dose of VACV that induced 70% mortality, it provided protection and a 100 000-fold reduction in the VACV titre in ovaries. While this is not a severe test of protection, it suggests that Gleevec and related compounds should be evaluated further (McFadden, 2005; Reeves et al., 2005).

6.2.10 Immunobiological preparations

Vaccinia immune immunoglobulin from humans is used for prophylaxis and treatment of a number of post-vaccination complications. However, because it has all the drawbacks of a donor blood-based preparation, use of specific human anti-OPV recombinant antibodies would be preferable, especially fully human recombinant (or monoclonal) antibodies. In order to produce these, variable domains of human antibodies possessing the target activity are combined with constant domains of human immunoglobulins of the necessary isotype. The key stage in developing fully human recombinant antibodies is selecting variable domains responsible for the antibody specificity, affinity and biological properties. One way to produce them is to select variable domains from combinatory phage libraries of mini-antibodies using the VARV Ind3a and Butler strains. In follow-up studies, 34 antibodies constructed at VECTOR were tested for their ability to neutralize the infectivity of VARV strains Ind3a and Butler, and five scFv antibodies capable of neutralizing VARV were found (Tikunova, unpublished data, 2006). Fully human antibodies that were constructed on the basis of variable domains of four scFv antibodies were confirmed to bind VARV and other OPVs, and to neutralize VARV in cell culture. These antibodies will require evaluation in animal models of VARV infection.

6.2.11 Miscellaneous compounds

A series of analogues of adenosine-N1-oxide with activity against VACV – both in vitro and following infection of mice via tail scratch (Kwong et al., 1998) – were evaluated against VARV and VACV in vitro (Huggins, unpublished data, 2002). The correlation between potency against VACV and potency against VARV was low. If only activity against VACV had been used to select a compound for further development, a compound would have been selected that is 40-fold less active than the one that is most active against VARV. This is a clear example of the risks in relying too heavily on surrogate viruses, unless it is known that the targets are identical.

6.2.12 Compounds whose structure will not be revealed until late in development

The initial steps in identifying compounds with activity against OPVs involved a number of government-sponsored laboratories (National Institutes of Allergy and Infectious Diseases, USAMRIID and VECTOR) screening large numbers of compounds against surrogate OPVs, and both WHO collaborating centres have screened several thousand

compounds against VARV using an in vitro cell culture–based assay. The results of screening assays were reported to the WHO Advisory Committee on Variola Virus Research during the yearly meeting (WHO, 2001, 2002). Most compounds did not show significant VARV activity and were not evaluated further. Many of the compounds initially evaluated against VARV were identified by a supplier identification number rather than being described by chemical class. The structures of all compounds evaluated in the VARV animal models have been revealed, including all three compounds that are in clinical development. Although patent protection varies by country, in general it lasts for only a fixed number of years, and in several countries the clock begins running at the first public disclosure of the compound's structure. This can lead to undesired consequences, including difficulty in developing a compound that does not have a clear patent. This consideration sometimes leads to an industry practice of not disclosing structures of compounds under development until just before initial human trials begin(Huggins, unpublished results; US Institute of Medicine, 2009).

6.2.13 Molecular biology–based approach to inhibiting viral replication, such as RNA interference

RNA interference is a mechanism that cells normally use to regulate gene expression, including suppression of foreign RNA (Fire et al., 1998). Practical use of this mechanism to inhibit viral replication had to await the development of delivery systems that would allow RNA interference and small interfering RNA to survive inactivation in the circulation during delivery to target cells (Nguyen et al., 2008). Recently, Alkhalil et al. (2009) described the inhibition of MPXV by RNA interference. This new field is expanding rapidly, and may well provide the next generation of antivirals.

6.3 Variola and monkeypox primate animal models

Development of a primate model for VARV infection has proven to be difficult. Early work in the 1960s did not produce an acceptable model (Hahon & Wilson, 1960; Hahon, 1961; Hahon & McGavran, 1961; Lancaster et al., 1966; Westwood et al., 1966), probably because VARV naturally only infects humans. There is no evidence of a naturally occurring infection of any non-human primate, and experimental models in non-human primates could only be developed by IV inoculation with 100 000 times the estimated human dose.

To allow evaluation of smallpox in animals, two models of IV infection of cynomolgus monkeys with VARV were developed. In humans, the extent (number of lesions) of the characteristic smallpox and monkeypox rash correlates with disease severity. The WHO scoring system categorizes the severity of disease based on the number of pox lesions – the standardized category "grave" is generally associated with high mortality, while the "mild" or "moderate" disease categories are generally associated with non-lethal disease (Jahrling & Huggins, 2005). IV infection with more than 10^7 plaque-forming units (PFU) of MPXV produced a lethal model that faithfully reproduced the rash lesional disease characteristics of smallpox and monkeypox. Similar studies with 10^8 PFU (2×10^9 genomes) of the Harper strain of VARV produced a similar lesional disease, with more than 1000 lesions (WHO category grave) and 33% mortality (days 11 and 13) in cynomolgus monkeys – the same mortality reported for human smallpox. Increasing the VARV challenge dose 10-fold (to 1×10^9 PFU or 2×10^{10} genomes) resulted in a 100% acutely lethal disease (mean time to death of 4 days) that more closely mimicked

haemorrhagic smallpox. Viral titres in organs at the time of death were 1000 to 10 000 times greater than in monkeys infected with 10 times less virus (Jahrling et al., 2004; Rubins et al., 2004). Mortality in both models depends on the infecting viral dose, which correlates with the total number of lesions. The authors of this chapter postulate that IV infection produces an artificial secondary viraemia, which bypasses the incubation period, resulting in rapid development of high levels of virus and rapid onset of disease.

The public health concern is that realistic diagnostic criteria for treating a large number of cases should be used to define therapeutic intervention. This results in selection of the onset of rash, illness and lesions as the time for initiation of treatment, based on clinical symptoms. However, by this stage, all organs have a substantial viral burden of more than 10^6 genomes per gram of tissue when treatment is initiated, on day 4 after infection.

A trial showing pivotal efficacy must be designed to provide stand-alone data (not dependent on other studies) that emulate, as far as possible, a human phase III clinical study, because medical reviewers will use the trial in place of human clinical trials to assess drug efficacy. In a report by the United States Institute of Medicine entitled *Live variola virus considerations for continuing research*, Table 4-1 characterized the non-human primate model as "most useful in suggesting likely benefit from candidate therapeutics and vaccines against variola in the human population" (US Institute of Medicine, 2009). To seek a drug label indication for reduction of smallpox mortality, the MPXV model in primates must be one of the pivotal studies. The only animal model for VARV developed to date that resembles classical smallpox is 33% lethal, and the only laboratory where VARV research can be conducted cannot accommodate enough primates to provide a statistically significant result (study design criteria to provide increased confidence in the study results due to the sample size are 120 non-human primates required for $P = 0.05$, 2-tailed with 79% power). Therefore, to achieve statistical significance, proof of reduction of mortality must use the model of the closely related MPXV infection of primates that induces greater than 90% mortality.

6.4 Human data to validate animal models

Smallpox was eradicated at a time when most of the modern methodology now used to characterize disease pathogenesis was in its infancy. As a result, little human data exist to compare with the results of pathogenesis studies conducted using VARV and MPXV infection of cynomolgus monkeys. The need for this information led the USFDA to encourage USAMRIID to conduct a natural history study of human monkeypox, as the closest currently occurring relative of smallpox, to validate an animal model that would meet the animal efficacy rule requirement. This study is currently under way at L'Hôpital Général de Référence de Kolé in Kolé town, Sankuru District, the Democratic Republic of Congo. It is being run in conjunction with l'Institut National de Récherche Biomédicale in Kinshasa, the Democratic Republic of the Congo, and USAMRIID (Huggins, personal communication, 2010).

6.5 Intravenous cidofovir

IV cidofovir (Vistide) is approved by the USFDA for treatment of HCMV retinitis in acquired immunodeficiency syndrome (AIDS) patients. Cidofovir, a small molecule

nucleoside analogue that selectively inhibits the viral DNA polymerase, was uniformly active against 35 VARV isolates selected to represent as large a geographic area and time span as possible (LeDuc et al., 2002). It reduced the replication of VARV 1000- to 100 000-fold in cell culture (Baker, Bray & Huggins, 2003). The efficacy of cidofovir for treating CPXV infection of BALB/c mice was investigated to evaluate new therapies for virulent OPV infections in a small-animal model. One intramuscular inoculation of 100 mg/kg of cidofovir on day 0, 2 or 4 after infection, with respect to intraperitoneal, aerosol or intranasal infection, respectively, resulted in 90–100% survival in the otherwise uniformly lethal models. Treatment on day 0 reduced peak pulmonary viral titres 10- to 100-fold, reduced the severity of viral pneumonitis and prevented pulmonary haemorrhage (Bray et al., 2000).

Two primate models of post-exposure prophylaxis were used to demonstrate drug efficacy. The first was a lethal lesion model with MPXV, in which evaluation of drug efficacy was based on reduction in mortality, lesion count and viral load. The second was a lesion VARV model, in which evaluation of drug efficacy was based on reduction in lesion count and viral load. In both models, treatment was initiated 24 hours after infection, when replication reached more than 10^4 genomes/g of tissue in all organs. To determine whether a lesion model could be treated successfully, the lethal intravenous MPXV model, which corresponds more closely with the VARV model following infection with 10^8 PFU, was used to show that cidofovir prophylaxis completely protected the animal. Cidofovir-treated monkeys showed no signs of illness, and viral replication in blood was controlled; in contrast, the placebo-treated animal had more than 850 lesions and levels of virus in blood greater than 10^7 genomes/ml, and died on day 12. Studies with MPXV demonstrated that doubling the cidofovir dose (four times the approved human dose) resulted in better control of viral replication; however, this higher dose is not approved for treatment of HCMV retinitis in human AIDS patients. Cidofovir is associated with potential significant nephrotoxicity – requiring IV prehydration, administration of probenecid and post-IV hydration – which would be a serious drain on medical facilities during an outbreak. Controversy exists around the possibility that a higher dose would be tolerated, but no patient population has been identified that would benefit sufficiently from this higher dose to justify its evaluation in humans.

Cidofovir was then evaluated against VARV in the post-exposure prophylaxis model. Groups of three cynomolgus monkeys were treated beginning on days 0, 1 or 2 and compared with placebos. One of three (33%) placebo-treated monkeys died, and all three were critically ill. None of the cidofovir-treated monkeys died or became seriously ill. Lesion counts and viral load were reduced in all cidofovir-treated groups compared with the placebo group ($P < 0.01$). The model of haemorrhagic smallpox was used to demonstrate successful prophylaxis with cidofovir, but the overwhelming nature of the infection makes the haemorrhagic smallpox model inappropriate for determining treatment efficacy for typical smallpox.

These results demonstrated that cidofovir given before the onset of rash illness, but not afterwards (data not shown), can prevent mortality. However, optimum results required a dose of 20 mg/kg. Although the exact dose equivalency between human and cynomolgus monkeys has not been fully resolved, this exceeds the dose of 5 mg/kg that has been approved for treating HCMV retinitis in humans. Limited safety data in humans suggest that the 10 mg/kg dose might be tolerated, but the safety studies that would

have to be conducted could not be justified, as no patient population exists in which the higher dose would provide sufficient benefit to outweigh the increased potential for nephrotoxicity. There is therefore no way to obtain the safety data required to support approval of a higher dose. Importantly, intervention after onset of lesions was not successful in these models. Both models may produce disease that is more severe than smallpox, especially as the disease course is accelerated, and it is possible that cidofovir would be an effective treatment for smallpox, but efforts to develop less severe but symptomatic models in which to demonstrate efficacy after onset of lesions have not been successful.

6.6 Oral CMX001

Cidofovir is taken up by pinocytosis, and requires IV infusion that can result in nephrotoxicity. The lipid analogue, 1-O-hexadecyloxypropyl-cidofovir (HDP-cidofovir, CMX001), is orally bioavailable, and no nephrotoxicity has been detected in preclinical toxicity studies or human trials (Kern et al., 2002; Keith et al., 2004; Painter & Hostetler, 2004; Beadle et al., 2006; Lebeau et al., 2006); CMX001 is reviewed in section 6.9. Mechanistically, the lipid moiety of CMX001 dictates the drug's pharmacokinetic properties in target organs, while the antiviral activity is contained within the nucleotide residue. Compared with cidofovir, which is taken up into cells by inefficient processes, the conjugate is designed to act like lysophosphatidylcholine, using natural lipid uptake pathways to achieve high intracellular concentrations. Once inside target cells, the lipid side chain of CMX001 is cleaved, presumably by phospholipase C, to yield free cidofovir. Conversion of cidofovir to the active antiviral agent, cidofovir diphosphate, occurs via a two-step phosphorylation process catalysed by intracellular anabolic kinases. Cidofovir diphosphate exerts its antiviral effects intracellularly, by acting as a potent alternative substrate inhibitor of viral DNA synthesis.

The antiviral activity of CMX001 has been characterized against OPVs in vitro and in vivo – in mice, rabbits and non-human primates. The in vitro potency of CMX001 against VARV is 0.1 µM, and ranges from 0.5 to 0.9 µM against CPXV, VACV, ECTV and RPXV (Hostetler, 2009). In mice, CMX001 is effective in preventing mortality after intranasal infection with a lethal inoculum of ECTV, CPXV, VACV or MPXV, when administered several days after infection. Effective doses are in the range of 1–20 mg/kg once per day for 5 days. Alternatively, a single dose of 20–100 mg/kg is effective in some cases. In a rabbit model, CMX001 is also effective in preventing mortality after a lethal infection with RPXV. Effective doses ranged from 1 mg/kg twice daily for 5 days, to 20 mg/kg once daily for 5 days. A single dose of 20 mg/kg is also effective in some cases. In a recent randomized, blinded, placebo-controlled study of RPXV-infected rabbits, where treatment was initiated after the onset of lesions, three doses of 20 mg/kg administered every other day (60 mg/kg total dose) provided statistically significant protection from mortality after intradermal inoculation of rabbits with a lethal dose of RPXV (11/12 survivors in the CMX001 group versus 2/12 in the placebo group).

Because of differences in metabolism and exposure to CMX001 in non-human primates, studies of CMX001 in cynomolgus monkeys are not relevant to humans. However, cidofovir has been shown to be efficacious in monkey models of infection, and both cidofovir and CMX001 deliver the same active antiviral compound, cidofovir diphosphate. For example, monkeys were protected from mortality after a lethal

intravenous inoculation of MPXV when cidofovir was administered at 20 mg/kg on days 1, 6 and 11 after infection; there were 7/8 survivors in the cidofovir group versus 1/8 survivors in the placebo group (Huggins, unpublished results, 2004).

Overall, the composite animal studies for CMX001 and cidofovir show that these compounds are effective against various OPV infections in vivo in scenarios involving pre-exposure treatment and post-exposure symptomatic disease treatment (i.e. after development of lesions). Furthermore, IV cidofovir can be used to model an efficacious exposure to cidofovir diphosphate in non-human primates where, due to metabolic differences, the direct evaluation of CMX001 is not possible. Systemic exposure to CMX001 and/or cidofovir and peripheral blood mononuclear cell (PBMC) levels of cidofovir diphosphate at the efficacious dose and regimen will be determined. The proposed studies will be conducted in RPXV-infected rabbits treated with orally administered CMX001, and in MPXV- and VARV-infected cynomolgus monkeys treated intravenously with cidofovir.

Pharmacokinetic data for CMX001 and/or cidofovir, as well as intracellular levels of cidofovir diphosphate in PBMCs of healthy and infected rabbits and monkeys, will be used to scale the efficacious dose to humans. In humans, the cidofovir diphosphate levels in PBMCs will be determined after varying doses of CMX001. For treatment of smallpox in humans, the efficacious dose and dosing interval of CMX001 will maintain a concentration of cidofovir diphosphate in PBMCs equal to or greater than the efficacious concentration (as determined in the animal models), for a duration corresponding to the normal course of smallpox.

CMX001 is well absorbed in humans, leading to high plasma concentrations of drug. A human patient with progressive VACV infection was treated successfully with a regimen that included CMX001; details of this case can be found on the *Morbidity and Mortality Weekly Review* website.[13] CMX001 is currently in phase II human clinical development for BK virus and HCMV; to date, it has been administered to more than 80 healthy volunteers and patients in three clinical trials, with no drug-related serious adverse events.

6.7 Oral ST-246

ST-246 (tecovirimat) is a small molecule compound that is potent, selective and active against multiple OPVs, including MPXV, camelpox virus, CPXV, ECTV and VARV (Yang et al., 2005; Bailey et al., 2007). The drug targets a gene (CPXV *V061* gene, VACV *F13L* gene, or VARV Bangladesh gene *C17L* of VARV-ORF-040) that encodes a major envelope protein – p37, found on the outer membrane of EEV. This protein is required for production of extracellular virus. In the presence of ST-246, plaque formation and virus-induced cytopathic effects were inhibited. In addition, formation of extracellular virus was reduced 158-fold, while production of IMV was reduced 11-fold in virus yield assays that used a low multiplicity of infection. ST-246 did not cause a defect in production of IMV, but failure to form EEV reduced the spread of virus to uninfected cells. IMV particle

[13] http://www.cdc.gov/mmwr/

formation and morphology are not affected by ST-246 treatment, as visualized by transmission electron microscopy. In vivo, oral administration of ST-246 protected BALB/c mice from lethal infection after intranasal inoculation with $10 \times LD_{50}$ of VACV (International Health Department strain J). Drug-treated mice that survived infection acquired protective immunity, and were resistant to subsequent challenge with a lethal dose ($10 \times LD_{50}$) of VACV (Yang et al., 2005). ST-246 administered at 50 mg/kg twice daily protected ANC/R mice from lethal infection after intranasal inoculation with 40 000 × LD_{50} of ECTV. Infectious viral titres in liver, spleen and lung at day 8 after infection were below the limits of detection (<10 PFU/ml) in animals treated with ST-246. In contrast, mean viral titres in liver, spleen and lung tissue from placebo-treated mice were 6.2×10^7, 5.2×10^7, and 1.8×10^5 PFU/ml, respectively. Oral administration of ST-246 inhibited VACV-induced tail lesion in NMRI mice inoculated via the tail vein. Oral administration of ST-246 also protected against a lethal challenge of MPXV up to 3 days after infection in the 13-line ground squirrel model (Sbrana et al., 2007). Taken together, these results validate protein p37 as an antiviral target, and demonstrate that an inhibitor of EEV formation can protect mice from OPV-induced disease. SIGA Technologies has been granted an IND and Fast Track status for ST-246, based on its very safe preclinical safety drug profile. (Fast Track status allows data from clinical trials to be submitted to the USFDA as they become available, rather than at the end of the studies.)

Oral ST-246 was evaluated for post-exposure prophylaxis activity against VARV infection of cynomolgus monkeys, which closely resembles human smallpox. The placebo group developed typical disease, with more than 1250 pox lesions and 33% mortality. Oral gavage with ST-246 began 24 hours after infection in the treatment group, when bone marrow, spleen, some lymph nodes and liver had more than 10^8 genomes/g and all tissues had 10^4–10^6 genomes/g. The treatment eliminated disease, as judged by a complete lack of lesion formation (the best predictor of smallpox disease severity in humans) and the lack of any significant clinical or laboratory findings. Viral titres in blood did not increase over pretreatment levels (10^6 genomes/ml), and virus was cleared in 6 days, compared with 16 days for placebo animals (based on historical data) (Huggins et al., 2009).

ST-246 was next evaluated using the MPXV infection of cynomolgus monkeys that closely resembles human smallpox. The placebo-treated group demonstrated typical disease, with more than 1500 pox lesions and 100% mortality. Oral gavage treatment with ST-246 began 24 hours after infection, when bone marrow, spleen, some lymph nodes and liver had more than 10^7 genomes/g and all tissues had 10^5–10^6 genomes/g. Treatment eliminated disease, as judged by a complete lack of lesion formation and the lack of any significant clinical or laboratory findings. Viral titres in blood did not increase over pretreatment levels, and virus was cleared in 4 days, compared with 16 days for placebo animals or animals treated with IV cidofovir (based on historical data). In a separate experiment, oral gavage treatment with ST-246 began 3 days after infection, when bone marrow, spleen, some lymph nodes and liver had more than 10^8 genomes/g and all tissues had more than 10^6 genomes/g. Again, treatment eliminated disease, as judged by a complete lack of lesion formation in two out of three monkeys and less than 5% of control lesions in the other monkey (these lesions did not progress), as well as the lack of any significant clinical or laboratory findings. Viral titres in blood did not increase over pretreatment levels, and virus was cleared in 6 days, compared with 16 days for the

placebo (Jordan et al., 2009). ST-246, at the monkey dose of 10 mg/kg (which is equivalent to the proposed human dose), can treat monkeypox successfully after the onset of lesions. Initial studies were conducted at 300 mg/kg, with even more dramatic results.

ST-246 was selected from related analogues after an extensive effort to optimize the compound's potency and stability in S9 liver metabolism assays. The compound showed moderate protein binding (around 80% in humans, around 88% in mice and monkeys, from 0.03 µM to 50 µM). Exposure was limited by absorption, which reduced bioavailability to approximately 30%. Given these limitations, the potency of the compound is extremely important in ensuring efficacy. Protein binding, absorption and excretion of small-molecule compounds vary between species, and this can have a dramatic impact on efficacy in animal models, thereby limiting their predictive value for humans. The unanticipated problems that arise from these differences are concrete examples of why activity in a small-rodent model may not lead to a usable clinical product.

6.8 Effect of antiviral administration on vaccine protection

6.8.1 Cidofovir and Dryvax

The effect of co-administration of cidofovir and Dryvax has been examined in mice and monkeys. In cynomolgus macaques, co-administration of a single dose of cidofovir (20 mg/kg) and Dryvax reduced VACV viral loads and Dryvax adverse events compared with vaccine alone; however, cidofovir also reduced immunity, as measured by humoral or cellular responses, and decreased protection from an MPXV challenge (survival/total was 2/6 for placebo, 6/6 for Dryvax and 5/6 for cidofovir plus Dryvax) (Wei, 2009). When Dryvax and cidofovir (12.5 mg/kg) were co-administered to A/NCR mice, lesion sites were smaller and healed faster than when vaccine alone was used. As with the monkey study, antibody responses were reduced; however, in contrast to the monkey study, there was no discernible reduction in protection from heterologous OPV challenge (ECTV). These data are consistent in showing a reduction in antibody titres when cidofovir is co-administered with Dryvax, as expected when a drug that inhibits viral replication at an early stage in the life-cycle is combined with a vaccine that requires multiple rounds of replication with the typical inoculums. Studies are planned to evaluate CMX001 and vaccination with the modified vaccinia Ankara (IMVAMUNE) vaccine that is given at high dose, since replication does not occur in human cells.

6.8.2 ST-246 (tecovirimat), and Dryvax and ACAM2000

Studies have shown that Dryvax and ACAM2000 vaccine efficacy is not compromised by ST-246 treatment given at the time of vaccination. Normal immunocompetent mice were vaccinated with Dryvax (Grosenbach et al., 2008) or ACAM2000 (Berhanu et al., 2010), using the standard human dose and route, and treated with ST-246 immediately after vaccination. The severity of vaccine lesions and time to resolution were reduced by ST-246 treatment. Furthermore, virus shedding from the lesion site was reduced (Berhanu et al., 2009). Humoral immune responses may have been slightly reduced by ST-246 treatment, but cellular immune responses appeared to be slightly increased. Animals that were vaccinated and treated with ST-246 were equally protected from a subsequent lethal challenge in both short- and long-term experiments, clearly demonstrating that ST-

246 does not adversely affect vaccine efficacy. In a series of follow-up experiments, numerous murine models for immunodeficiency were vaccinated with ACAM2000, using the standard human dose and route, and then treated with ST-246 (Berhanu et al., 2010). ST-246 was effective in all models except those completely deficient for cellular immune responses (combined $CD4^+$ and $CD8^+$ deficiency). ST-246 reduced vaccine reactogenicity and the amount of time necessary for resolution of the vaccine lesion. Virus shedding from the lesion site was also reduced by ST-246 treatment. In models with partial immunodeficiencies, animals were safely vaccinated and able to resist subsequent lethal challenge in short- and long-term experiments. This demonstrated that, even in a partially immunodeficient setting, ST-246 improves vaccine safety while allowing the induction of robust immune responses that are capable of resisting lethal challenge.

6.9 Drugs under clinical development

The United States Institute of Medicine report *Live variola virus considerations for continuing research* concluded that "… the most compelling reason for long-term retention of live variola virus stocks is their essential role in the identification and development of antiviral agents for use in anticipation of a large outbreak of smallpox" (US Institute of Medicine, 2009).

Although numerous compounds have been identified that inhibit OPV replication in multiple in vitro test systems (including limited evaluation against VARV), a smaller number of these have been evaluated in small-rodent models, usually in mice. The OPV animal models, mostly murine, that have been used to evaluate compounds were recently reviewed by Smee (Smee, 2008). Useful information can be obtained from these models, but they are only the first step in the long, complicated, multiyear process required to obtain approval for a drug.

Most compounds that show protection in initial animal models will encounter some problem that keeps them from becoming a successful drug, such as excessive toxicity, metabolic inactivation, failure to produce adequate levels in target tissues, economic unfeasibility of production and/or numerous other possible problems.

To date, three compounds have undergone adequate development to allow their evaluation in humans under an IND application: cidofovir, a drug approved for HCMV retinitis; CMX001, a lipid prodrug of cidofovir; and ST-246. All three show activity in a spectrum of small-rodent models with surrogate OPVs, and both cidofovir and ST-246 have shown protection in the primate models of VARV infection. None, however, have been approved for treatment of smallpox, as the required evaluations have not been completed.

Because clinical trials using smallpox are not ethical, the USFDA had to develop a new approach for drug approval that would allow drug efficacy to be evaluated in animal models; this approach is often referred to as the "animal rule". Under this rule, animal model studies are subject to the same intensity of review and the same level of scientific scrutiny as human clinical trials proposed to support approval of products for other types of indications using other approval pathways.

The USFDA Center for Drug Evaluation and Research has not seen any compelling arguments that a smallpox indication could be justified without VARV data. This means that the USFDA might not license a drug against smallpox without receiving data on the efficacy of the drug against VARV in animal models. Because of the unique history and virulence of VARV, pathways to approval for smallpox indications are not clear or uniform even *with* a VARV challenge model. The equivalent regulatory agencies in countries other than the United States have given even less formal guidance. Some sponsors may choose to pursue development of their product for another disease or condition, while having IND status or the equivalent for that product's use against smallpox. Using an IND product during an emergency, however, imposes additional complications, including logistical ones. To partially address these problems, the USFDA has approved an Emergency Use Authorization process, but how it would be used is still being developed.

The USFDA has also published draft guidance (USFDA, 2007) on the use of the animal rule. However, mainly because of a lack of detailed knowledge of human smallpox, the USFDA has not yet determined that any animal models for smallpox are adequate under the rule. Any drug approved under this rule must still meet all other licensure requirements, including demonstrating safety in humans. Unless the drug can also be used to treat diseases other than those caused by OPVs, it must be tested in healthy volunteers. This can only happen with drugs that have insignificant side-effects, as the experimental subjects would receive no benefit. This requirement significantly limits the candidate drugs that can be developed. Although some toxicity could be tolerated when treating a smallpox patient with a 30% mortality risk, that consideration is not relevant to safety testing in healthy subjects.

6.10 Time lines for development of smallpox therapeutics

All smallpox antiviral therapeutics must use the animal rule for efficacy evaluation, but requiring efficacy evaluation only in animals is clearly not a shortcut to drug approval. In fact, it makes progress towards approval more complicated, because the underpinning regulatory science is still evolving. Because only two drugs have been evaluated to date under the animal rule, it is too early to estimate the types and number of studies that will be required to establish efficacy for smallpox therapeutics. Many scientific challenges must still be worked through in order to answer these questions, and this introduces uncertainty about the time that will be required to obtain approval for a smallpox drug.

Considerable uncertainty still exists about the combination of in vitro and animal model data that will suffice to demonstrate efficacy. Drug regulatory agencies offer guidance about the types of studies that could be useful in evaluating a drug candidate, but it is difficult for them to provide precise guidance; instead, they ask the drug sponsor to provide a clinical development plan for their review. In some areas, where multiple products have been approved to treat a specific disease, a road map to a successful application has become clearer. However, there is no experience beyond public USFDA guidance to industry, USFDA workshops, and private drug-specific meetings between the USFDA and a drug sponsor (which are not typically made public) that might allow us to predict a time line. Even less guidance is available from most other equivalent bodies. Each country will need to approve use of a drug within its borders. Ultimately, it is the responsibility of the sponsor to prepare a clinical development plan, which guides a

submission package that must adequately demonstrate both safety and efficacy for the product in question. To assist in this process, the USFDA has issued draft *Guidance to industry* for smallpox therapeutics. The USFDA will convene a "USFDA Advisory Panel" of technical experts to assist in determining what combination of animal models will be needed to replace human clinical trials for studies intended to demonstrate efficacy in a VARV challenge model. These trials must be "well-controlled"; for animal studies, this means that they must be in compliance with GLP guidelines. Complying with GLP in a BSL-3 environment for MPXV has proven difficult but possible (Huggins, unpublished data, 2008), but it may not be practical to comply with all GLP regulations when conducting drug studies with VARV under BSL-4 conditions, because of the restrictions imposed by working in a protective "space suit". Because of the large number of unknowns, unanticipated additional studies with live VARV may be required at any time until final drug approval. Current USFDA regulations require materials from critical studies to be retained for two years after drug approval, to allow for re-evaluation if issues emerge during clinical use. How this would apply to a smallpox drug is not known.

Industry experience suggests that drugs entering phase I studies have about a 10–25% chance of gaining approval, and this process typically takes five to eight additional years. Given these constraints, it is remarkable that three drugs have achieved IND status for the treatment of smallpox, and that two companies are working with the USFDA to develop a road map for drug approval. Realistically, approval is likely to take at least 5–10 years, even if all studies are successful. However, it is critical that adequate studies be performed, not just to obtain regulatory approval to sell a drug, but also so that public health officials have sufficient information to allocate scarce health-care resources for maximum impact in both halting an epidemic and minimizing the mortality and morbidity of clinical cases. Obtaining this information may require additional studies, especially to translate the treatment benefit in animal models to the likely benefit in humans, and to assess the likely impact on the requirement for medical resources.

6.11 Concluding discussion

Antiviral therapeutics do not exist, even as IND products, for many viral diseases – including such things as the common cold, where there is enormous potential for profit for a successful drug product. This is clear evidence that the process of developing antiviral drugs is difficult.

It is remarkable that three drug candidates have obtained IND status for treatment of disease caused by OPVs, and that all three have been used to treat adverse events associated with VACV vaccinations. None have been used to treat human MPXV, and additional safety information is still needed before it would be safe to investigate their use in remote areas where monkeypox is currently transmitting.

There is a much larger number of compounds that show promise but are not as far along in development. Hundreds of compounds are active in cell culture–based assays, and more than a dozen are in initial evaluation in small-rodent models. It could be argued that it is critical that work continues on these compounds, along with discovery efforts for newer approaches to inhibiting viral replication. Experience from industry suggests that we cannot yet predict whether any of the three current IND products will obtain regulatory approval, and that we should therefore continue and expand our efforts to

develop additional drug candidates for the treatment of smallpox. This would have to include drug discovery efforts with surrogate OPVs, evaluation of promising compounds against VARV, and then – much later in the development process, after the primate equivalent of the proposed human dose has been established – evaluation of efficacy in primate model(s) of VARV infection, initiated when clinical diagnosis and treatment are feasible.

It is not currently possible to predict how much longer work with live VARV will be required. However, it would certainly be required until at least two drugs are approved for treatment of clinical smallpox.

The USFDA has repeatedly stated that it does not see a path to drug approval that does not require efficacy data in a VARV challenge model. Because it will not be possible to conduct clinical trials against smallpox in humans, demonstration of efficacy must use the USFDA animal rule (US 21CRF310.610) or other processes mandated by other countries' drug regulatory agencies for approval in their country. Given the uncertainties in the practicalities of the animal rule, due to its infrequent use to date, and the fact that there is no drug currently approved for smallpox, it is difficult to provide firm estimates on the time line or the data required for USFDA approval. Approval in other countries is at least as uncertain.

Abbreviations

AIDS	acquired immunodeficiency syndrome
BSL	biosafety level
CMX001	HDP-cidofovir, 1-O-hexadecyloxypropyl-cidofovir
CPXV	cowpox virus
DNA	deoxyribonucleic acid
ECTV	ectromelia virus
EEV	extracellular enveloped virus
GLP	good laboratory practice
HCMV	human cytomegalovirus
HPMPC	cidofovir
IMCBH	N_1-isonicotinoyl-N_2-3-methyl-4-chlorobebzoylhydrazine
IMV	intracellular mature virus
IND	investigational new drug
IV	intravenous
MPXV	monkeypox virus
OPV	orthopoxvirus
PBMC	peripheral blood mononuclear cell
PFU	plaque-forming unit
RNA	ribonucleic acid
RPXV	rabbitpox virus
USAMRIID	United States Army Medical Research Institute for Infectious Diseases
USFDA	United States Food and Drug Administration
VACV	vaccinia virus
VARV	variola virus

VECTOR	Russian State Research Center of Virology and Biotechnology
WHO	World Health Organization

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