Global transmission of influenza viruses from humans to swine

Martha I. Nelson,¹ Marie R. Gramer,² Amy L. Vincent³ and Edward C. Holmes¹,⁴

To determine the extent to which influenza viruses jump between human and swine hosts, we undertook a large-scale phylogenetic analysis of pandemic A/H1N1/09 (H1N1pdm09) influenza virus genome sequence data. From this, we identified at least 49 human-to-swine transmission events that occurred globally during 2009–2011, thereby highlighting the ability of the H1N1pdm09 virus to transmit repeatedly from humans to swine, even following adaptive evolution in humans. Similarly, we identified at least 23 separate introductions of human seasonal (non-pandemic) H1 and H3 influenza viruses into swine globally since 1990. Overall, these results reveal the frequency with which swine are exposed to human influenza viruses, indicate that humans make a substantial contribution to the genetic diversity of influenza viruses in swine, and emphasize the need to improve biosecurity measures at the human–swine interface, including influenza vaccination of swine workers.

INTRODUCTION

The 2009 pandemic H1N1 virus (H1N1pdm09) represents the best-documented emergence of a swine pathogen in humans, particularly as the virus was associated with widespread global morbidity, mortality and years of life lost in humans (Viboud et al., 2010). The H1N1pdm09 virus was generated by a reassortment event between Eurasian swine H1N1 influenza viruses and North American triple-reassortant H1 viruses, with the former contributing the N1 and M segments and the latter donating the PB2, PB1, PA, H1, NP and NS segments (Garten et al., 2009). Although similar influenza virus reassortants containing segments of both Eurasian swine virus and triple-reassortant swine virus origins have been detected in Asia (Lam et al., 2011; Smith et al., 2009), no ‘smoking gun’ viruses that are closely related progenitors have been detected in swine in any locality.

Following the identification of H1N1pdm09 in humans in April 2009, the virus was transmitted rapidly from humans back into swine. The first isolation of a H1N1pdm09 virus in swine was from a pig farm in Alberta, Canada, in May 2009 (Howden et al., 2009), and the H1N1pdm09 virus was subsequently isolated from outbreaks in swine in all other major continents: Africa (Njabo et al., 2012), Asia (Kim et al., 2011; Sreta et al., 2010), Australia (Holyoake et al., 2011), Europe (Hofshagen et al., 2009; Howard et al., 2011) and South America (Pereda et al., 2010). Upon introduction into the swine population, H1N1pdm09 viruses co-circulated with endemic swine influenza viruses and exchanged genome segments via reassortment (Ducatez et al., 2011; Lam et al., 2011; Vijaykrisna et al., 2010). In 2011, 12 reassortant H3N2 (H3N2v) swine influenza viruses with matrix (M) segments of pandemic H1N1pdm09 origin were isolated from humans in the USA (CDC, 2012).

Seasonal human influenza viruses have also been isolated periodically from swine, with a few major lineages becoming endemic in swine: most notably the human-origin H3N2 viruses that proliferated in European swine in the 1970s (Ottis et al., 1982), the H1α lineage of human origin that emerged in North American swine in 2003 (Karasin et al., 2006), and the human H3, N2 and PB1 segments that were associated with the triple-reassortant viruses that were identified in 1998 in North American swine (Zhou et al., 1999). In addition, singletons and smaller clusters of swine influenza viruses that contain one or more segments of human seasonal influenza virus origin have been identified in several countries, including Argentina (Cappuccio et al., 2011; Pereda et al., 2011) and Italy (Moreno et al., 2012).
Table 1. Country and state/province of virus collection, month and year of collection, number of swine isolates, and bootstrap support $\geq 70\%$ for 49 introductions of H1N1pdm09 influenza virus from humans into swine during 2009–2011

<table>
<thead>
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<th>Introduction no.</th>
<th>Country of collection*</th>
<th>State or province of collection (if available)*</th>
<th>Month/year of collection</th>
<th>No. of isolates (swine)</th>
<th>Bootstrap support (%) for viral introductions†</th>
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The increased global surveillance of influenza in pigs provides the first opportunity to estimate the extent of human-to-swine transmission of the H1N1pdm09 virus. The main aim of our study was therefore to estimate, using a simple phylogenetic approach, the total number of introductions of the H1N1pdm09 virus from humans into swine using a dataset of H1N1pdm09 viruses collected globally in swine during 2009–2011. We then compared this estimate with the equivalent transmission frequency of human seasonal (non-pandemic) H1 and H3 into swine since 1990.

RESULTS

Frequent transmission of H1N1pdm09 virus from humans into swine

Across the haemagglutinin (HA), neuraminidase (NA) and concatenated internal gene phylogenies, we identified a total of 49 discrete introductions of H1N1pdm09 influenza virus from humans into swine during 2009–2011 [Table 1; Figs 1 and S1–S3 (available in JGV Online)]. These introductions were observed in 12 countries and semi-autonomous regions: Australia, Canada, China, Costa Rica, Cuba, Hong Kong (SAR), Italy, Singapore, South Korea, Taiwan, Thailand and the USA. The majority of viral introductions into swine were identified in the USA and Canada (19 and eight introductions, respectively; Table 1), reflecting the higher number of H1N1pdm09 influenza virus sequences available from North American swine. Given our strict criteria for defining viral introductions (bootstrap values ≥70 %) and limited global sampling, these numbers are likely to underestimate the extent of global spillover from humans to swine of the H1N1pdm09 virus significantly. Our conservative methods are particularly prone to miss introductions involving single isolates. For example, single H1N1pdm09 isolates from Mexico, Japan, England, Cameroon, Brazil and Colombia (A/sw/4/Mexico/2009, A/sw/Osaka/1/2009, A/sw/England/73690/2010, A/sw/Cameroon/11rs149-198/2010, A/sw/Brazil/12A/2010, A/sw/Colombia/1403/2010) are likely to represent additional introductions. These additional probable introductions are identified by on the detailed HA, NA and concatenated phylogenies presented in Figs S1–S3.

Frequency of human-to-swine transmission of seasonal influenza viruses

Across the H1, H3, N1 and N2 phylogenies, at least 23 separate introductions of seasonal influenza virus from humans to swine were observed during the period 1990–2011 (Figs S4–S7). The vast majority of these introductions were identified after 1996, when surveillance of influenza virus in swine increased. Six human-to-swine transmission events involved the human seasonal H1 segment, eight involved the human H3 segment, six involved the human N1 segment and 16 involved the human N2 segment (Table 2). The higher number of introductions of the human N2 segment is...
probably related to several factors, including (a) the overall higher number of H3N2 virus introductions (n=12) due to the dominance of the H3N2 subtype in humans during the period of this study, (b) the frequency of introductions (n=3) of the human H1N2 variant that circulated globally in humans from 2001 to 2003, and (c) the frequency of reassortment involving N2 segments of human origin and HA segments of swine origin. For example, the 20 H1N2 reassortant viruses from Italy that are associated with introduction #11 have N2 sequences that are related most closely to the N2 of human seasonal H3N2 isolates collected in 1997–1998, but H1 sequences related to European swine influenza viruses.

Twelve of the introductions of seasonal influenza virus were first identified in Asia, six were first identified in the USA, three in Canada and two in Europe (Table 2). As with the H1N1pdm09 viruses, the methods that we used to define a human-to-swine transmission event are highly conservative, and numerous probable introductions were excluded from our final estimate, particularly those involving singletons. Six singleton swine isolates, including three from Argentina, which were identified to be of human origin on both the HA and NA trees but were not supported by high bootstrap values, are highlighted (letters a–g, Table 2; Figs S4–S7). Additionally, eight swine H3N2 isolates collected in Hong Kong during 2000–2002 are interspersed with human isolates collected from that same time period on both the H3 and N2 phylogenies, suggesting additional introductions of human H3N2 viruses into swine in Hong Kong, although again lacking the phylogenetic resolution required to be defined as discrete introductions.

Estimated time periods of human-to-swine transmission

The long branch lengths associated with several introductions of human-origin influenza viruses into swine indicate
Table 2. Country and year of collection, viral subtype, year of collection of the most phylogenetically related human influenza viruses, number of swine isolates, and bootstrap support \( \geq 70 \% \) for 23 introductions of seasonal influenza viruses (H1, H3, N1 and N2 segments) from humans into swine during 1990–2011

<table>
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<th>Introduction no.</th>
<th>Country and year of collection</th>
<th>Subtype</th>
<th>Related human viruses (year)</th>
<th>No. of isolates (swine)</th>
<th>Bootstrap support (%) for viral introductions</th>
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<td>H3N2</td>
<td>1997</td>
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<td>H3N2</td>
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<td>2002–2003</td>
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</table>

*Bootstrap support was \( \geq 70 \% \), but only for a subset of the isolates in a given cluster.

†In cases of reassortment, the number of isolates on HA phylogeny is provided.

§In cases of reassortment, bootstrap values for both NA clades that are associated with an introduction on the HA tree are provided.

$\$The HA associated with this introduction was identified to be of human seasonal influenza virus origin on the HA phylogeny (or the HA1 phylogeny; introduction no. 3), but was not associated with a significant bootstrap value.

||These H1N2 viruses are not related to the seasonal H1N2 viruses that circulated globally in humans in 2001–2003, but rather are human–swine reassortants.
that numerous viral introductions circulated in swine for many years before being detected. Given the high intensity of sampling of influenza virus in humans, the duration of unsampled circulation in swine can be estimated simply and directly by the differences in time between when the isolates were collected in swine and when the most closely related human isolates were collected. For example, the eight H3N2 viruses associated with introduction #13 were collected in swine in Thailand in 2004–2009, but are related most closely to A/Wuhan/359/1995(H3N2)-like viruses from 1995–1996 on the H3 and N2 phylogenies (Figs S4 and S6, respectively), representing 8 years of unsampled circulation in swine. The time difference between the 2008 H3N2 and 2010 H1N2 isolates collected in swine in Argentina and the most closely related human seasonal isolates is approximately 6–8 years. Likewise, the isolate A/sw/Argentina/CIP051-StaFeN2/2010(H1N2) is related most closely to the reassortant H1N2 viruses that circulated in humans during 2002–2003 (Figs S5 and S6), suggesting that this virus has circulated undetected in swine in Argentina or elsewhere since at least 2003, when the H1N2 viruses disappeared globally in humans. Incorporating more viral sequence data from swine by also including partial HA (HA1) sequences filled in some of these gaps in surveillance (H1–HA1 and H3–HA1 trees available from the authors upon request). For example, the USA 2007–2011 (δ-1) introduction (#18) is detected 2 years earlier in 2005 when HA1 data are included. Finally, 11 years separate the three H3N2 swine isolates collected in South Korea in 2007 (introduction #17) from the most phylogenetically related human seasonal H3N2 viruses collected in 1995–1996. However, the relatively short branch lengths associated with these 2007 South Korean isolates raises the question of whether the evolutionary rate was particularly low on this branch, or whether these sequences could be erroneous. The short branch length that separates A/sw/Fujian/0325/2008(H1N1) (introduction #19) from the most closely related human seasonal H1N1 viruses collected in 2000–2001, relative to the approximately 7–8-year difference in collection date, also raises the question of possible sequencing error.

Spatial patterns of human-origin influenza viruses in swine

The number of swine H1N1pdm09 isolates associated with each introduction (ranging from one to ten isolates; Table 1) represents the extent of onward swine-to-swine transmission that can be inferred from our phylogenetic analysis. Although the vast majority of introductions of human H1N1pdm09 viruses into swine exhibit local onward transmission in pigs, reflected in introductions that are associated with more than one swine isolate, we infer that global viral movement has not yet been important in the dissemination of the H1N1pdm09 virus in swine. Specifically, each cluster of swine H1N1pdm09 influenza viruses that are associated with a human-to-swine transmission event is highly spatially structured, with no swine introduction including viruses collected in more than one country. However, within-country spread of H1N1pdm09 viruses has occurred in the USA, as six viral US introductions include multiple states, representing viral dissemination within the Midwestern USA (Table 1). Limited spread within Canada between Alberta and Manitoba was also detected (introduction #2; Table 1). Within-country spread was not observed in other countries where information on the province or region of viral collection was also available, including China and Australia.

Similarly, 18 of the 23 introductions of human seasonal H1 and H3 influenza viruses into swine involve a single country (Table 2). Six of these single-country introductions involve singleton swine influenza viruses, with no evidence of onward transmission. In contrast, four viral introductions include isolates collected in different countries, indicating viral migration within North America and into and within Asia. Aside from the major introduction of triple-reassortant H3N2 viruses that spread from North America to Asia (introduction #4) and from the USA to Canada (introduction #10), possible limited viral migration occurred between Taiwan and Indonesia (introduction #9) and the USA and South Korea (introduction #8).

DISCUSSION

Despite evolving the capability to transmit efficiently in humans, our results indicate that the H1N1pdm09 influenza virus has retained its ability to transmit back into swine and to co-circulate with other swine influenza viruses, increasing genetic diversity and providing the opportunity for reassortment. The large number of H1N1pdm09 transmission events observed in this study, even using conservative methods, highlights the frequency of human–swine contact rates globally and the continual exposure of swine to human influenza viruses. Indeed, this transmission rate is considerably higher than would be inferred from the relatively lower number (n=23) of human-to-swine transmission events of seasonal H1 and H3 influenza viruses that were identified during the past two decades. Importantly, detection of seasonal human influenza viruses in swine did not increase during 2009–2011, despite widespread global circulation of seasonal H3N2 viruses in humans during 2010–2011. Hence, the different transmission frequencies observed are not explained by the increase in swine influenza surveillance since 2009 and are unlikely to be an artefact of sampling. Furthermore, the low phylogenetic resolution of the H1N1pdm09 phylogeny, due to the virus’s recent emergence and relatively low genetic diversity, impeded the identification of what are likely to be many more introductions of this virus into swine than could be defined here.

 Although it is tempting to compare the frequencies of human-to-swine transmission with the 27 swine-origin influenza viruses that were identified in humans in the USA over a time period similar to that of our study (1990–2010) (Shu et al., 2012), such a comparison is biased greatly by the substantially higher levels of global surveillance of
influenza in humans than consistently occurs in swine. However, the intensity of influenza surveillance in humans does facilitate estimation of the time of emergence of the 23 introductions of human seasonal influenza viruses into swine. For example, the introductions of H1N1pdm09 viruses were generally detected in swine within 1 year, whereas several of the human seasonal influenza viruses that were introduced into swine were not detected for 5–10 years. Whilst the time to detection in swine may relate to intrinsic viral characteristics (e.g. the virus must first adapt, then replicate competently in the new host, then spread to many hosts before being detected), it is also possible that the difference in time between when a human-to-swine transmission event is detected in swine and the year of collection of the most closely related human influenza viruses provides an indication of intensity of swine surveillance in a given region. The smallest gaps in detection are in Canada, the USA and Hong Kong, where ongoing viral surveillance is conducted in swine, while larger time gaps occurred in countries such as South Korea, Italy, Thailand and Argentina, where sampling appears to be more sporadic, based on publicly available sequence data.

The global frequency of introductions of H1N1pdm09 viruses into swine also provides an opportunity to track, in detail, the onward transmission and spatial dissemination of these new viral clades and how they relate to swine movements (Nelson et al., 2011). It will be particularly important to determine which of these H1N1pdm09 lineages sustains transmission in swine over the long term, to observe their population dynamics in a new swine host and to identify which lineages spread globally. The multiple introductions of human influenza viruses into swine provide a natural experiment for observing adaptive evolution of influenza from human to swine hosts, in identifying instances of parallel adaptive evolution, and in the comparison of rates of evolutionary change and selection pressures along the branches that define human-to-swine transmission events. Clearly, the ability to trace such dynamics relies upon the continued surveillance of influenza in swine globally, even after the initial interest in the 2009 H1N1 pandemic has subsided.

**METHODS**

**Sequence data used in this study.** To estimate the number of human-to-swine transmission events of the H1N1pdm09 virus, we compiled gene sequence data from 263 H1N1pdm09 influenza viruses that were collected in swine during 2009–2011 in 18 countries: Argentina, Australia, Brazil, Cameroon, Canada, China, Colombia, Costa Rica, Cuba, Hong Kong, Japan, Mexico, Singapore, South Korea, Taiwan, Thailand, the UK and the USA (Table S1). Due to the frequency of reassortment between H1N1pdm09 viruses and other swine influenza viruses involving the HA and NA segments, concatenated internal gene segments (PB2, PB1, PA, NP, M and NS) and the HA and NA segments were studied separately. Separate phylogenies were also inferred for each of the internal gene segments to identify and remove any viruses with reassorted internal genes. As background data, 1008 complete genome sequences from H1N1pdm09 influenza viruses that were collected in humans during 2009–2011 were downloaded from the Influenza Virus Resource on GenBank; due to the size of the original dataset \((n=2718\) genomes), 50 genome sequences were selected randomly from highly sampled countries (i.e. those with >50 genome sequences) (Bao et al., 2008).

To identify swine influenza viruses related to human seasonal H1 and H3 influenza viruses, four phylogenetic trees were inferred using all full-length H1 \((n=991\), excluding pandemic viruses), H3 \((n=326)\), N1 \((n=858)\) and N2 \((n=559)\) sequences collected from swine since 1990 that are available in GenBank (accession numbers are available from the authors). Due to the frequency of reassortment and low availability of whole-genome sequence data for these swine viral isolates, alignments of the concatenated internal genome segments were not included for this part of the study. These data came from Argentina, Belgium, Canada, China, Denmark, France, Germany, Hong Kong, Hungary, Italy, Japan, Spain, South Korea, Sweden, Taiwan, Thailand, the UK and the USA. From these phylogenies, 152 human-origin swine H1 sequences, 221 human-origin swine H3 sequences, 18 human-origin swine N1 sequences and 430 human-origin swine N2 sequences were identified globally and used in the final analysis. This analysis also included, as background data, 1000 randomly selected human seasonal H1, H3, N1 and N2 segments collected from 1990 to 2011. A large clade of European sequences (e.g. A/sw/Scotland/410440/1994) that were of human seasonal H1 virus origin were not included because they clearly were introduced from humans into swine at least a decade prior to 1990, the beginning of our study period.

To identify any additional human-to-swine transmission events that could not be detected from trees inferred with full-length HA sequence data, two additional phylogenies were inferred using all partial HA1 sequence data \((918\) nt) from the swine H1 \((n=1200\) sequences) and H3 \((n=1290\) sequences) subtypes available since 1990, including HA1 sequence data from the same 1000 human influenza viruses that were selected as background in the full-length HA phylogenies. Using HA1 data, we identified 50 additional swine H1 sequences of human origin and 70 additional swine H3 sequences of human origin. However, all but two isolates [A/sw/Obihiro/3/1993(H3N2) and A/sw/Obihiro/1/1994(H3N2)] were associated with introductions that are already identified in the analysis of full-length HA and NA sequences.

**Phylogenetic analysis of H1N1pdm09.** Alignments were constructed manually using the Se-Al program (Rambaut, 2002) for three sets of swine H1N1pdm09 virus sequence data, with the 1008 human H1N1pdm09 isolates included as background: (i) the HA segment \([n=232\) swine H1N1pdm09 isolates, 199 of which were downloaded from GenBank, and 53 provided by the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL)] from samples collected from their routine veterinary diagnostic laboratory submission and/or tested via the US Department of Agriculture (USDA) National Animal Health Laboratory Network (NAHLN) Swine Influenza Surveillance System; (ii) the NA segment \([n=229\) swine H1N1pdm09 isolates, 197 from GenBank and 32 from UMVDL]; and (iii) concatenated internal gene segments \([n=100\) swine H1N1pdm09 isolates, all downloaded from GenBank, with all reassortants identified from individual gene trees and excluded). For each alignment, a maximum-likelihood (ML) tree was inferred using the PhyML v3.0 program (Guindon et al., 2010), employing SPR (subtree pruning and regrafting) branch-swapping and a general time-reversible model (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites. Statistical support for individual nodes was estimated using 1000 replicate neighbour-joining trees inferred using RAUP* and assuming the GTR+gamma model of nucleotide substitution (Swofford, 2003). Well-supported nodes (\( >70\%\) bootstrap support) defining human-to-swine viral introductions were identified visually on each phylogeny. To mitigate possible effects of sequencing
error, the several isolates that were separated by extremely long branch lengths were only categorized as discrete introductions when multiple isolates from the same country exhibited the same phylogenetic pattern.

**Phylogenetic analysis of seasonal H1 and H3.** Alignments were manually constructed using Se-Al for four sets of swine influenza virus sequences that were identified as of human seasonal (non-pandemic) influenza virus origin: (i) 152 H1 sequences, (ii) 221 H3 sequences, (iii) 18 N1 sequences and (iv) 430 N2 sequences. As background, 1000 human influenza viruses were selected randomly for each segment and downloaded from GenBank. For each alignment, an ML tree was inferred as described above to identify well-supported nodes (≥ 70% bootstrap support) defining introductions of influenza viruses from humans to swine. The estimated time periods of human-to-swine transmission were inferred directly from the phylogeny by identifying the most closely related human viruses (the sampling date of which is known). This simple phylogenetic method produced results (i.e. times to common ancestry) similar to those obtained using the Bayesian Markov chain Monte Carlo approach available in the BEAST program (Drummond & Rambaut, 2007) and performed during a previous analysis of human influenza viruses that were transmitted to swine (Nelson et al., 2011).

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Plant Viruses. Invaders of Cells and Pirates of Cellular Pathways¹

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Plant viruses, discovered over a century ago when the science of virology was born (for review, see Creager, 2002), are obligate parasites on their hosts. Through their life cycle, from virus accumulation to intracellular, local, and systemic movement, viruses utilize plant proteins, normally involved in host-specific activities, for their own purposes. Although the first identification of a host protein interacting with plant viral RNA took place more than 25 years ago (for review, see Buck, 1999; Wain et al., 2004), the true complexity of this interaction between plant viruses and their hosts to allow virus accumulation and spread is just now becoming understood.

In addition, the ability of the host to defend itself against virus replication and spread is now known to be much more complex than was thought not long ago. During the early 1990s, the first findings were published suggesting that a plant host defense system targeting viral RNA with extreme sequence specificity existed (e.g. de Haan et al., 1992; Lindbo and Dougherty, 1992). Initially, these observations were not fully understood to represent an RNA-mediated host defense system now referred to as the RNA interference (RNAi), but with time were well differentiated from the better studied host and transgene defense systems mediated through proteins (e.g. the hypersensitive reaction of Nicotiana tabacum cv Xanthi NN against tobacco mosaic virus [TMV] and coat protein-mediated resistance; Beachy, 1999; Marathe et al., 2002). In the last few years, plant molecular virologists and biologists have moved with increasing speed to document the incredibly complex interactions between virus and host factors necessary to allow or defeat virus infections in the presence of RNAi (e.g. Baulcombe, 2004). Thus, plant viruses, besides their traditional role as causative agents of numerous plant diseases, represent molecular tools to examine and dissect diverse basic cellular processes in plants, ranging from intracellular transport and nucleocytoplasmic shuttling (Lazarowitz and Beachy, 1999; Oparka, 2004) to intercellular transport (Waigmann et al., 2004) to gene silencing (Moissiard and Voinnet, 2004).

This focus issue reports new insights into how viruses may utilize host factors to accumulate and move intracellularly to position for intercellular movement (Chen et al., 2005; Ju et al., 2005; Liu et al., 2005). Also, information further illuminating the “give and take” between virus and host factors battling for control during RNAi is presented (Chellappan et al., 2005; Liu et al., 2005; Schwach et al., 2005). Update articles on virus-host interactions during virus replication and movement in this issue review recent information in these areas to provide clues for productive future research (Bovign and Oparka, 2005; Thivierge et al., 2005). In this State of the Field editorial, we introduce the research and Update articles in this issue and review recent literature on virus-host interactions not addressed in the Updates.

VIRUS ACCUMULATION

For both DNA and RNA plant viruses, the accumulation of progeny virus involves translation and replication of viral sequences (Buck, 1999; Ahlquist et al., 2003; Noueiry and Ahlquist, 2003; Hanley-Bowdoin et al., 2004; Ishikawa and Okada, 2004; Räjamäki et al., 2004, and refs. therein). These plant viruses rely on the host to provide factors to aid their accumulation. The Update article by Thivierge et al. (2005) presents a summary of recent insights into the mechanisms by which positive-sense single-stranded RNA viruses take advantage of the host cell mRNA processing and translation machinery.

Research on virus-host interactions during DNA virus accumulation has also moved forward. For example, an NAC domain protein, SINAC1, from tomato (Solanum lycopersicum) that interacts with a geminivirus replication enhancer (REN) protein was identified and suggested to participate in viral replication (Seth et al., 2005). NAC family members, which function in plant development and defense responses (e.g. Xie et al., 2000; Hegedus et al., 2003), are known to interact with other geminivirus proteins, such as RepA, but in those instances they inhibited rather than promoted viral replication (Xie et al., 1999). Furthermore, a NAC protein interaction with an RNA virus coat protein is

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necessary during a resistance response in Arabidopsis (Arabidopsis thaliana; Ren et al., 2000). That host proteins from a single family display different functions during DNA and RNA virus infection illustrates the complexity of the virus-host interaction process.

Large-scale screening for host factors that affect RNA virus accumulation has been undertaken using yeast as an alternative host distinguished by a wealth of well-characterized mutants (e.g. Kushner et al., 2003; Panavas et al., 2005). These experiments showed that host genes involved in viral accumulation may differ between viruses. For example, while brome mosaic virus and tomato bushy stunt virus each are affected in their accumulation by approximately 100 host genes, only 14 of these genes overlap between viruses. The overlapping genes encoded proteins belonging mainly to three functional groups: protein biosynthesis, protein metabolism, and transcription/DNA remodeling (Panavas et al., 2005). Interestingly, no overlap existed between tomato bushy stunt virus and brome mosaic virus for genes involved in protein targeting, membrane association, vesicle transport, or lipid metabolism (Panavas et al., 2005), suggesting that there are important differences between these viruses for host membrane targeting and intracellular transport. Although analysis in yeast allows a high-throughput analysis of yeast host factors that affect plant virus accumulation, it is important to supplement these data with information obtained in plant cells, for example, using a recently developed technology to study virus replication in a cell-free system of membrane-containing extract from uninfected vacuolated plant protoplasts (Komoda et al., 2004). The potential to utilize protoplasts from mutant plants silenced for expression of specific plant genes identified through the yeast-based selection is very exciting.

INTRACELLULAR AND INTERCELLULAR MOVEMENT

To spread between cells, viruses must first move from their replication sites to plasmodesmata at the cell periphery and then traverse these intercellular channels to enter the neighboring cell. Cell-to-cell transport of most plant viruses is mediated by specific virally encoded factors termed movement proteins (MPs), the function of which may be augmented by other viral proteins (for review, see Morozov and Solovyev, 2003; Rajamaki et al., 2004; Waigmann et al., 2004). The majority of the cell-to-cell transport machinery, however, is presumed to be provided by the host cell. One such host transport apparatus is the cytoskeleton. Although plant cytoskeletal elements were implicated in viral cell-to-cell transport a decade ago (Heinlein et al., 1995; McLean et al., 1995), the relative roles of microtubules and microfilaments in the transport process are just emerging. Recent data suggest that, for TMV, microfilaments participate in the cell-to-cell movement of the virus, whereas microtubules and microtubule-associated proteins may be involved in degradation of the viral MPs (Gillespie et al., 2002; Kragler et al., 2003). In this issue, Liu et al. (2005) demonstrate the role of microfilaments in cell-to-cell movement of TMV. Disruption of microfilaments by pharmacological agents or by virus-induced gene silencing compromised TMV spread from cell to cell, but it did not significantly affect viral accumulation within the infected cells (Liu et al., 2005). Furthermore, this study demonstrated the potential involvement of another TMV factor, the 126-kD protein, in viral transport along microfilaments; the 126-kD protein was shown to associate with viral replication complexes, modulate their size, and potentially mediate their interaction with and movement along the microfilament network (Liu et al., 2005).

Increasing evidence suggests that the cytoskeletal network does not function alone in viral transport to and through plasmodesmata. Instead, it may act together with the endomembrane transport system of the host cell. Specifically, many viral MPs may be delivered to plasmodesmata via the endoplasmic reticulum (ER), while actin/myosin filaments may regulate the flow of proteins in the ER membrane (Boevink and Oparka, 2005). Two articles in this issue address the role of ER in viral cell-to-cell transport and plasmodesmal targeting. Ju et al. (2005) show that the potato potexvirus X (PVX) triple gene block (TGB) p2, one of the proteins required for movement of this group 2 member of the TGB-containing viruses, associates with ER-derived vesicles, which in turn colocalize with actin filaments. Intriguingly, no association of the TGBp2 with Golgi vesicles was detected (Ju et al., 2005), consistent with findings in a recent report studying the movement of TGBp2-containing structures in tissue infected with potato mop-top virus, a group 1 member of the TGB-containing viruses (Haupt et al., 2005). Thus, these viruses likely use an ER-dependent pathway for plasmodesmal targeting, which is different from the Golgi-dependent targeting to plasmodesmata recently demonstrated for some cellular proteins (Sagi et al., 2005).

The association of the potexviral TGBp2 MP with microfilaments and ER resembles similar associations of the tobamoviral MP and the 126-kD protein (McLean et al., 1995; Heinlein et al., 1998; Hagiwara et al., 2003; Liu et al., 2005). This resemblance indicates physical and functional similarities between MPs and movement-associated proteins of potexviruses and tobamoviruses, suggesting that both viral groups utilize a similar method of intracellular movement, at least through a portion of this passage (Nelson, 2005).

For TMV, the role of the ER translocation and plasmodesmal targeting was explored by Chen et al. (2005) using calreticulin, a cellular protein that localizes to plasmodesmata (Baluska et al., 1999; Michalak et al., 1999; Chen et al., 2005). This study showed that the N-terminal signal peptide was critical for the ability of calreticulin to accumulate within plasmodesmata (Chen et al., 2005). Based on these observations, it is tempting to speculate that plasmodesmal targeting involves two dis-
tinct signals, a signal to enter the ER network and a putative plasmodesmata localization signal. Consistent with this idea, several types of viral MPs that “gate” plasmodesmata (e.g. Waigmann et al., 1994; Tamai and Meshi, 2001) have been shown also to associate with the ER (e.g. Heinlein et al., 1998; Haupt et al., 2005; Ju et al., 2005).

Chen et al. (2005) also showed that calreticulin interacts with TMV MP and that overexpression of calreticulin in transgenic plants redirects TMV from plasmodesmata to microtubules and compromises cell-to-cell transport of the virus. A potential, albeit indirect, functional link between viral MPs and calreticulin also may be inferred from the observations that one of the two MPs of the turnip crinkle virus (TCV) interacts with an Arabidopsis protein containing two RGD cell-attachment sequences (Lin and Heaton, 2001) that are recognized by integrins (Campbell et al., 2000), which in turn interact with calreticulin (Dedhar, 1994).

Possible roles of the calreticulin-MP interaction in regulation of plasmodesmal permeability are discussed in the Update article by Boevink and Oparka (2005) in this issue. These authors present a review of the latest trends and discoveries regarding the role of the ER/actin network in intracellular transport, recognition of adhesion sites at the cell periphery, modification of plasmodesmata by alteration of the cell wall structure, Hsp70 chaperones as potential translocation factors, and regulation of viral cell-to-cell movement (Boevink and Oparka, 2005).

Recently, a potential link between virus accumulation and cell-to-cell movement was identified when the eukaryotic translation factors eIF4E and eIF(iso)4E, which are required for accumulation of potyviruses (Duprat et al., 2002; Lellis et al., 2002; Ruffel et al., 2002; Nicaise et al., 2003), were also shown to aid in virus cell-to-cell movement (Gao et al., 2004). These observations supported earlier findings where plant mutants with altered eIF4E activity exhibit limited virus spread (Arroyo et al., 1996). It has been speculated that potyvirus intracellular movement may occur via an interaction of eIF4E with eIF4G, which then binds microtubules (Lellis et al., 2002). Regardless of the mechanism of eIF4E-mediated virus movement, it is important to realize that host proteins may function in several steps of the virus infection process, e.g. in the case of eIF4E, both in virus translation and/or replication and in viral cell-to-cell movement.

Finally, in recent years, viral MPs have been shown to interact with numerous other cellular proteins, such as pectin methylesterases (Dorokhov et al., 1999; Chen et al., 2000; Chen and Citovsky, 2003), protein kinases (Yoshioka et al., 2004), homeodomain proteins (Desvoyes et al., 2002), DnaJ-like proteins (Soellick et al., 2000; von Bargen et al., 2001), rab acceptor-related proteins (Huang et al., 2001), β-1,3-glucanase-interacting proteins (Fridborg et al., 2003), and transcriptional coactivators (Matsushita et al., 2001, 2002). To date, only protein kinases have been shown to play a role in viral intercellular movement (Citovsky et al., 1993; Kawakami et al., 1999; Waigmann et al., 2000; Trutnyeva et al., 2005), while the functions of other MP-interacting proteins in this process remain obscure, awaiting future studies.

**VIRUSES VERSUS RNAi HOST DEFENSE**

Viruses-host interactions during RNAi in plants are complex and understood only at a rudimentary level. In general, plants have multiple RNA silencing pathways with diverse biological roles (Baulcombe, 2004). These include the regulation of gene expression and importantly, for this short review, the control of virus accumulation. The analysis of the RNAi pathway controlling virus accumulation is complicated because some of the host genes involved in this process also function in regulating host gene expression. In addition, viruses themselves modify the final outcome by their expression of proteins that defeat the system, i.e. suppressors of RNA silencing. For a more complete understanding of this rapidly evolving area, there are many excellent recent reviews (Baulcombe, 2004; Ding et al., 2004a; Moissiard and Voinnet, 2004).

RNA silencing involves the recognition of a target RNA and its subsequent destruction. This occurs via a multistep enzymatic pathway including, in plants, an RNA-dependent RNA polymerase (RdRP; now referred to as RDR), an RNase-III-type dicer-like endonuclease (DCL), putative members of the RNA-induced silencing complex such as Argonaute, which likely binds RNA, and other proteins that may support RNA-induced silencing complex activity, such as DEAD box helicases (SDE3; for review, see Baulcombe, 2004; Meister and Tuschi, 2004). The majority of these proteins are members of gene families, and it is this multiplicity of family members that allows the plant to respond to widely varying needs (e.g. plant development and defense against virus invasion) and complicates our ability to understand each system.

One way to simplify this issue is to identify natural or created plant knockout mutants for each gene involved in RNA silencing and study their loss-of-function phenotype during virus challenge. Using this approach, Arabidopsis DCL2 was found to be required for protection against TCV (Xie et al., 2004). Arabidopsis SDE3 was required for protection against cucumber mosaic virus (CMV) but not tobacco rattle virus (TRV; Dalmay et al., 2001). For the RDRs, the tobacco RDR1 was required for protection against TMV and PVX, while its Arabidopsis homolog was required for protection against TMV-cg, a toamovirus very closely related to turnip vein clearing virus (TVCV; Larrey et al., 1993), and TRV (Xie et al., 2001; Yu et al., 2003). Interestingly, *Nicotiana benthamiana* is a natural mutant for RDR1, and transgenic expression of an RDR1 ortholog from *Medicago truncatula* enhanced its susceptibility to TMV, TVCV, and sunn hemp mosaic virus (a toamovirus, but only distantly related to TMV and TVCV), but not CMV or PVX (Yang et al., 2004). Similarly, RDR6 was required for protection against CMV, but not turnip mosaic virus,
TVCV, TCV, or TRV in Arabidopsis (Dalmay et al., 2000; Mourrain et al., 2000). Thus, specific RDRs likely recognize different viruses; RDR1 is required for protection against tobamoviruses and TRV, while RDR6 is required for protection against CMV.

In this issue, Schwach et al. (2005) report that RDR6 in *N. benthamiana* is required to inhibit infections by PVX, potato virus Y, and CMV, in the presence of its Y satellite RNA, but has no effect on infections by TMV, TRV, TCV, and CMV, in the absence of the Y satellite RNA. During infection with PVX, RDR6 prevented the systemic (including meristems), but not local, infection of plants (Schwach et al., 2005).rafting experiments showed that RDR6 is required for cells to respond to a systemically moving silencing signal. The results of this study suggested that RDR6 produces double-stranded RNA precursors from the silencing signal that are used to generate short-interfering RNAs (siRNAs), which in turn allow an immediate silencing response against the target virus on its arrival (Schwach et al., 2005). This information advances our understanding of the mechanism of the host RNAi-mediated resistance pathway against virus infection. For example, as Schwach et al. (2005) suggest, exclusion of virus from the meristem is mediated by RNAi, and RDR6 is involved in this process. These results also raise issues to consider for future work in this area. For example, what are the virus and satellite RNA targets telling us about the substrate structural requirements for each RDR, or is it that factors other than substrate suitability control the ability of particular RDRs to control accumulation by specific viruses? Also, why does the Arabidopsis RDR6, but not *N. benthamiana* RDR6, protect against CMV?

It was also interesting that Schwach et al. (2005) showed that RDR6 did not control cell-to-cell movement of PVX, indicating that the silencing pathway in which this enzyme functions does not target virus intracellular or intercellular movement. In this issue, Liu et al. (2005) reported that mutant TMVs expressing 126-kD protein silencing suppressors of varying strengths were also not altered in cell-to-cell movement (for TMV suppressor characterization, see Kuba et al., 2003; Ding et al., 2004b). Earlier, such an unlinking of RNA silencing suppressor activity from cell-to-cell movement was demonstrated for the P15 suppressor from peanut clump peculovirus (Dunoyer et al., 2002). It will be interesting to determine whether or not RNA silencing ever directly targets the intracellular or cell-to-cell movement forms of the viral RNA. It may be that these forms are always protected from the host silencing machinery.

Another article in this issue reports the effect of temperature on the production of siRNAs in plants challenged with various geminiviruses, demonstrating that RNA silencing increased as the temperature was raised from 25°C to 30°C (Chellappan et al., 2005). This finding extends to DNA viruses what was found for an RNA virus, Cymbidium ringspot virus, in *N. benthamiana* (Szittyia et al., 2003). Importantly, the increase in siRNA steady-state levels was most striking (3- to 6-fold) for geminiviruses not associated with a recovery phenomenon (i.e. producing fewer symptoms over time) compared with those that were associated with a recovery phenomenon. This dramatic increase in siRNAs also was correlated with the presence of one of two viral suppressors in these geminiviruses (Vanitharani et al., 2004; Chellappan et al., 2005). The critical importance of controlling temperature when studying RNAi or applying it in agriculture is also highlighted in this work (Chellappan et al., 2005).

Last, it is interesting that connections between the induction of stress in cells, which could be considered a host defense response, and virus movement may exist. For example, exposure of plants to abiotic stress, e.g. low levels of heavy metal cadmium, blocks viral systemic movement (Citovsky et al., 1998; Ghoshroy et al., 1998; Ueki and Citovsky, 2002). At the other extreme, stress may aid movement because host heat shock protein (HSP) 70 (Aoki et al., 2002) and virus- encoded HSP70-related proteins (Medina et al., 1999; Alzhanova et al., 2001; Prokhnenvsky et al., 2002) likely help viral and/or host macromolecular transport through plasmodesmata. Interestingly, the induction of host HSP70s during infection by plant RNA viruses is driven by a general mechanism that senses the level of misfolded proteins in the cell, regardless of protein origin, viral or host (Aparicio et al., 2005). The study by Ju et al. (2005) in this issue touched on the role of stress in viral movement by showing that turnover of TGBp2 was greater during virus infection than when it was expressed alone in plant cells. They also determined that, in plant cells, TGBp2: green fluorescent protein had a longer half-life than free green fluorescent protein. Based on these observations, Ju et al. speculated that cell stress, represented by increased protein turnover, could aid movement of PVX between cells by translocating TGBp2 or viral movement complex out of the ER into the cytosol and making it available not only for degradation but also for transport through plasmodesmata (Ju et al., 2005). It will be interesting to see if viruses indeed have pirated the host stress response for their own purposes.

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**LITERATURE CITED**


State of the Field


Virus evolution: how far does the double β-barrel viral lineage extend?

Mart Krupovič and Dennis H. Bamford

Abstract | During the past few years one of the most astonishing findings in the field of virology has been the realization that viruses that infect hosts from all three domains of life are often structurally similar. The recent burst of structural information points to a need to create a new way to organize the virosphere that, in addition to the current classification, would reflect relationships between virus families. Using the vertical β-barrel major capsid proteins and ATPases related to known viral genome-packaging ATPases as examples, we can now re-evaluate the classification of viruses and virus-like genetic elements from a structural standpoint.

Viruses are the most abundant biological entities on the Earth (there are $10^{30}$–$10^{32}$ virions in the biosphere; see Glossary). Nevertheless, classification of living organisms on the basis of their 16s rRNA genes left no room for viruses on the universal tree of life. However, it is now appreciated that viruses are not only ancient, but might also have played a major part in the emergence and consequent structure of modern cellular life forms. It is thus clear that comprehension of the virosphere, including evolutionary relationships between individual viruses and viral families, is crucial to fill the gaps in our understanding of the biosphere. It has been generally considered that viruses which infect evolutionary distant hosts (for example, bacteria and humans) have different origins. However, recent accumulation of structural information has led to the proposal of a more unified common-ancestral-based virus classification.

Structural virology, already in its youth when human rhinoviruses were found to be structurally related to small plant RNA viruses, offered unexpected answers to the vague questions regarding origin and evolution of viruses. The realization that the bacterial tectivirus PRD1 (Tectiviridae family) and human adenovirus (Adenoviridae family), which infect hosts from two different domains of life, might be evolutionary related was even more surprising. Not only did PRD1 and adenovirus contain linear double-stranded (ds) DNA genomes that were replicated in a protein-primed manner, but the topology of their major capsid proteins (MCPs) and the organization of these subunits in the capsid surface lattice, as well as the structure of their penton and spike proteins, were found to be similar. In the same way, parallels were drawn between other icosahedral viruses: tailed dsDNA bacteriophages (Caudovirales order) and herpesviruses (Herpesviridae family) as well as dsRNA bacteriophages (Cystoviridae family) and reoviruses (Reoviridae family). These observations gave rise to the viral lineage hypothesis, which predicts a common origin for viruses that share the same capsid architecture, but infect hosts from different domains of life. Membership of a lineage is not strictly dependent on genome-sequence similarities, mode of replication or genome organization (circular versus linear), as these traits vary between the members of the proposed lineages. Rather, it relies on common principles of virion architecture. The set of genes that encode a capsid is considered to be the hallmark of the virus, as only these determinants can define a replicon as belonging to a virus rather than some other entity; for example, a plasmid. This block of genes is therefore likely to be inherited vertically in every virus lineage. Ascribing viruses into such structure-based lineages not only bypasses the problem of the mosaic nature of viral genomes, but also limits the enormous virosphere to a comparatively small number of lineages.

Table 1 | Viruses from the PRD1–adenovirus lineage

<table>
<thead>
<tr>
<th>Family*</th>
<th>Host</th>
<th>Morphology</th>
<th>Genome topology</th>
<th>Genome length (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tectiviridae</td>
<td>Gram-negative and Gram-positive bacteria</td>
<td>Icosahedral</td>
<td>Linear</td>
<td>~15</td>
</tr>
<tr>
<td>Corticoviridae</td>
<td>Gram-negative bacteria</td>
<td>Icosahedral</td>
<td>Circular</td>
<td>~10</td>
</tr>
<tr>
<td>STIV</td>
<td>Archaea (Crenarchaeota)</td>
<td>Icosahedral</td>
<td>Circular</td>
<td>17,663</td>
</tr>
<tr>
<td>MVV</td>
<td>Archaea (Euryarchaeota)</td>
<td>Unknown</td>
<td>Circular</td>
<td>12,039</td>
</tr>
<tr>
<td>TKV4</td>
<td>Archaea (Euryarchaeota)</td>
<td>Unknown</td>
<td>Circular</td>
<td>18,818</td>
</tr>
<tr>
<td>Adenoviridae</td>
<td>Eukarya (vertebrates)</td>
<td>Icosahedral</td>
<td>Linear</td>
<td>26–45</td>
</tr>
<tr>
<td>Iridoviridae</td>
<td>Eukarya (vertebrates and invertebrates)</td>
<td>Icosahedral</td>
<td>Linear</td>
<td>140–303</td>
</tr>
<tr>
<td>Asfarviridae</td>
<td>Eukarya (vertebrates)</td>
<td>Icosahedral</td>
<td>Linear</td>
<td>170–190</td>
</tr>
<tr>
<td>Phycoadnaviridae</td>
<td>Eukarya (unicellular)</td>
<td>Icosahedral</td>
<td>Linear or circular</td>
<td>160–560</td>
</tr>
<tr>
<td>Mimivirus</td>
<td>Eukarya (unicellular)</td>
<td>Icosahedral</td>
<td>Linear</td>
<td>1,181,404</td>
</tr>
<tr>
<td>Poxviridae</td>
<td>Eukarya (vertebrates and invertebrates)</td>
<td>Oval</td>
<td>Linear</td>
<td>130–230</td>
</tr>
<tr>
<td>Ascoviridae</td>
<td>Eukarya (invertebrates)</td>
<td>Oval</td>
<td>Circular</td>
<td>120–180</td>
</tr>
</tbody>
</table>

*If a virus has not been ascribed to a family, its name is provided. STIV, Sulfolobus turreted icosahedral virus.
Viruses with double β-barrel proteins
The PRD1–adenovirus lineage comprises icosahedral tailless viruses with dsDNA genomes. The hallmark of these viruses is their characteristic trimeric MCP, which has a double β-barrel structure (FIG. 1a). After trimerization of the MCP, hexagonal capsomers are formed, which are then arrayed as triangular plates to form the icosahedral virus shell (FIG. 1b). Members of the PRD1–adenovirus lineage infect cells in all three domains of cellular life. General characteristics of the current members of this lineage are briefly described below and summarized in TABLES 1, 2.

Bacterial viruses. The best characterized bacterial member of the PRD1–adenovirus lineage is bacteriophage PRD1, the type member of the Tectiviridae family, which infects a range of Gram-negative bacteria. The PRD1 virion consists of an icosahedrally organized proteinaceous capsid that surrounds a protein-rich lipid membrane. This membrane encloses the linear dsDNA genome, which is replicated by the phage-encoded type B polymerase (PolB) in a protein-primed manner. The structure of the PRD1 MCP (FIG. 1a), and subsequently the entire 66 MDa PRD1 virion, was solved by X-ray crystallography, which revealed an interaction between the amino-terminal α-helices of the MCP subunits and the internal phage membrane. This interaction forces the viral membrane to obey icosahedral symmetry. Another tectivirus, Bam35, that infects Gram-positive Bacillus spp. was found to be different from PRD1 genetically, but nearly identical structurally.

Bacteriophage PM2, the type member of the Corticoviridae family, infects marine Pseudoalteromonas species. The PM2 virion and its major structural proteins have been analysed by both X-ray crystallography and cryo-electron microscopy (EM). Interestingly, X-ray analysis revealed that the double β-barrel MCP of PM2 (FIG. 1a) lacks the bottom part, the typical N-terminal α-helix, which contacts the viral membrane in the PRD1 virion (discussed below).

Archaeal viruses. Viruses that infect the archaeal domain are represented by the crenarchaeal Sulfolobus turreted icosahedral virus (STIV). Cryo-EM analysis of the STIV virion revealed similarities to bacteriophage PRD1 (REF. 33). Subsequent X-ray analysis of the STIV MCP revealed that three-dimensional structures of PRD1 and STIV MCPs are nearly identical (FIG. 1a). Although STIV is the only archaeal virus isolated so far,

Several structure-based viral lineages have been proposed to date. One of these lineages, the PRD1–adenovirus lineage, unites dsDNA tailless viruses that use similar architectural principles and are present in the largest number of different viral families; this lineage will be used in this Opinion as an example of a structurally defined viral lineage. We discuss the available virion and capsid protein structures to show how far the lineage hypothesis can be extended and what it tells us about virus origin and evolution in this particular lineage.

Figure 1 | Structural features common to members of the PRD1–adenovirus lineage. | A comparison of the available X-ray structures of the double β-barrel major capsid proteins (MCPs) and the pseudoatomic model of the putative MCP of Maverick 1 from sea squirt Ciona intestinalis (Mav-1_CI). STIV MCP (Sulfolobus turreted icosahedral virus MCP) is coloured cyan, human adenovirus MCP is coloured purple, bacteriophage PRD1 MCP is coloured green, bacteriophage PM2 MCP is coloured blue and Paramecium bursaria Chlorella virus type 1 MCP (PBCV-1 MCP) is coloured red. The putative MCP of Mav-1_CI is coloured red, whereas the insertions with respect to the corresponding PBCV-1 sequence are white. Protein sequences of Mavericks were downloaded from the Repbase Update database of repetitive elements (see Further information). BioInfoBank Meta Server was used to predict the tertiary structure of the putative MCP of Mav-1_CI. The structure of PBCV-1 MCP was determined to be the best template for structural modelling. The sequence of the PBCV-1 MCP was aligned with the corresponding protein sequences of Mav-1_CI, and a three-dimensional model of the putative Mav-1_CI MCP was built. b | Architecture of the PRD1 virion (triangulation number of 25). Apices of the red triangle combine the fivefold vertices (light shading) outlining the triangular virus facet. Grey hexagons depict the morphology of the double β-barrel trimers. The inset shows a close-up X-ray model of one trimer (top view) in which individual monomers are shown in different colours.
two putative proviruses, TKV-4 and MVV, have been recently identified in the genomes of archaeal species from the second major phylum of Archaea, the Euryarchaeota.46

Eukaryotic viruses. Adenoviruses infect higher eukaryotes and have linear dsDNA genomes that are replicated by a protein-prime mechanism. Various adenoviruses have been subjected to intensive EM and X-ray analyses.47,48. Unlike other members of the PRD1–adenovirus lineage, adenoviruses do not have an internal membrane (discussed below). In addition, a high-resolution X-ray structure of the MCP, as well as a cryo-EM-based reconstruction of the entire virion, is available for Paramecium bursaria Chlorella virus type 1 (PBCV-1; a member of the Phycodnaviridae family).49 [FIG. 1a]. The Phycodnaviridae family unites genetically diverse viruses that infect both marine and freshwater algae.50 Some members of the Phycodnaviridae can lysogenize their host cells by integrating into the cellular chromosome.49

Comparative genome analyses of large eukaryotic dsDNA viruses exposed a conserved set of genes for four virus families — Phycodnaviridae, Iridoviridae, Asfarviridae, Poxviridae — and the recently isolated Mimivirus, which suggests that these groups share a monophyletic origin. It has been proposed that these groupings should be collectively called the nucleo-cytoplasmic large DNA viruses (NCLDV)s.50,51 Phycodnaviruses, asfarviruses, iridoviruses and the Mimivirus all possess a large icosaheiral capsid that is surrounded by a large icosaheiral capsid,52,53,54 whereas poxviruses are brick shaped.46 However, vaccinia virus (Poxviridae family) encodes a highly conserved scaffolding protein (D13) that is homologous to the MCPs of other NCLDV55,56, and is known to play an essential part during the formation of genome-containing, spherical immature virions.57,58. Subsequent transition from immature to mature brick-shaped virions seems to be associated with the loss of D13 [REF. 49]. Intriguingly, D13 was shown to trimerize and form an external, honeycomb-like lattice that covers the lipid bilayer of the immature virion.59 Similarly, a three-dimensional density map of the trimeric D13 homologue in Orf virus (Poxviridae family) was found to accommodate the electron-density map of the PBCV-1 MCP, indicating that D13 also adopts a double β-barrel fold. These observations suggest that the immature virions of poxviruses resemble the mature icosaheiral virions of the PRD1–adenovirus lineage.46,50

Similar morphological transitions might have given rise to viruses of the Ascoviridae family. Ascoviruses attack lepidopteran larvae and pupae, and vary in shape from ovoid to bacilliform depending on the species.52,53 Comparative genomic and phylogenetic analyses unexpectedly revealed that ascoviruses are likely to have evolved from an iridovirus ancestor.52,53

Mavericks: the missing link? As discussed above, members of the Adenoviridae are outliers in the PRD1–adenovirus lineage (BOX 1). On the one hand, the adenoviral MCP is much larger than the MCPs of the other members of the PRD1–adenovirus lineage (FIG. 1a) and adenoviruses lack lipids as structural components. On the other hand, adenoviruses have the same genome organization and mode of replication as tectiviruses. We assume that the large MCP of adenoviruses evolved from a smaller version that is still found in NCLDV and poxviruses. Identification of a virus with an NCLDV-like capsid protein and the genome organization of the adenovirus would be the best proof of such a hypothesis.

A class of self-synthesizing DNA transposons was recently identified in a wide range of eukaryotic organisms (from nematodes to vertebrates).54,55. These transposable elements were named Mavericks54 or Polintons54. Interestingly, Mavericks possess several features that are common to tectiviruses and adenoviruses, including genome organization and gene content.55,56 The most conserved set of encoded proteins includes a protein-primed PolB, an adenoviral cytostome protease, an ATPase (similar to the packaging ATPases of internal membrane-containing viruses) and an integrase.55,56. It is important to note that the ATPase is the second protein that is common to all membrane-containing members of the PRD1–adenovirus lineage.57 These ATPases form a distinct clade in the FtsK–HerA superfAMILY of cellular chromosome-pumping ATPases that is evolutionary distinct from the packaging ATPases of tailed bacteriophages and herpesviruses.58,59. In addition

Glossary box

**Biosphere**
The global ecological system that integrates all living organisms.

**Capsomer**
The basic structural unit of the capsid of a virus.

**Convergent evolution**
The process by which organisms that are not monophyletic independently evolve similar traits as a result of adaptation to ecological niches or similar environments.

**Divergent evolution**
The accumulation of differences between groups that can lead to the formation of new species, usually as a result of the adaptation of different groups of the same species to different environments.

**Transposon**
A genetic element that can move from one locus of a chromosome to another through a recombinase-mediated reaction.

**Triangulation number**
(T). Used to describe the number of subunits that exist in a capsid: an icosaheiral capsid of triangulation number T will possess 60 T subunits.

**Virosphere**
The total sum of all viruses.
to Walker A and Walker B motifs, ATPases of these internal membrane-containing viruses contain a conserved and essential P9/A32 motif, which was proposed to be specific to membrane-containing viruses\(^6\). Adenoviruses seem to use the ATP-binding cassette (ABC)-type ATPase for genome packaging, which might have been acquired from the host together with loss of the internal membrane\(^9\). Interestingly, the ATPases encoded by Mavericks possess a P9/A32 motif. In addition to the four proteins mentioned above — PoB, cysteine protease, ATPase and integrase — Mavericks encode four conserved hypothetical proteins, which have been named PX, PY, PW and PZ\(^5\). Using Structure Prediction Meta Server\(^6\), the MCP of PBCV-1 was found to be the best template for structural modelling of PY proteins\(^8\). In a subsequent analysis, the high stereochemical quality of a three-dimensional model of a selected PY protein (FIG. 1a) indicated that the Maverick protein PY has the potential to adopt the same fold as the MCP of PBCV-1: the double β-barrel fold (M.K. and D.H.B., unpublished observations).

So what are these large transposable DNA elements of eukaryotes? It is possible that Mavericks represent the remnants of ancient viruses, as proposed by Pritchard and colleagues\(^8\). Based on the presence of the putative MCP and the packaging ATPase, the progenitor virus is proposed to be a member of the PRD1–adenovirus lineage. Retention and high conservation of these two genes as part of the Maverick genome, as well as the restricted size of the genome itself, are intriguing. One explanation might be that Mavericks are still capable of forming virions and can sometimes spread as genuine viruses. An alternative scenario is that the possible ancestor virus of Mavericks, in contrast to the two evolutionary schemes proposed above for NCLDV\(^+\)s and adenoviruses (BOX 1), evolved a third tactic to avoid the immune system of the evolving eukaryotic host (see the figure in BOX 1). Instead of coding for a larger capsid that would accommodate a larger genome, Mavericks changed their life style to persist inside the host cell without destroying it. However, unless this change in life style occurred recently, this scenario does not explain the conservation of the two putative genes that encode for structural virion proteins or explain the apparent conservation of genome length, which is likely to be constrained by capsid size.

**Divergent versus convergent evolution**

The viral lineage hypothesis assumes that the origin of viruses in each lineage is monophyletic and that diverse viral families in a lineage arose as a result of \textit{divergent evolution}. Unfortunately, a lack of significant sequence similarity between viral genomes of the PRD1-like viruses prevents us from verifying such an assumption. Although it is not possible to reject with certainty that \textit{convergent evolution} led to the appearance of the double β-barrel fold, it should be realized that the protein fold is not the only feature shared by viruses in the lineage. The most conserved feature of viruses of the PRD1–adenovirus lineage is the use of double β-barrel MCPs to build the protein shell: pseudohexameric trimers of the MCP are arrayed as triangular...
Figure 2 | Genome organization of selected members of the PRD1–adenovirus lineage from all domains of life. Presented are the genome organizations of members of the PRD1–adenovirus lineage from the Bacterial domain (PRD1, Bam35, PM2 and a corticoelement in the Vibrio splendidus 1ZB01 genome), the Archaeal domain (Sulfolobus turreted icosahedral virus (STIV), and proviruses TKV4 and MVV) and the Eukarya (Frog adenovirus). The genomic organizations of Maverick-1_CI and Maverick-2_XT are also provided, which reside in the genomes of Ciona intestinalis and Xenopus tropicalis, respectively (see Further information). Genomic sequences were aligned using major capsid protein (MCP)-coding genes. Arrows indicate the direction of transcription. MCP-coding genes are coloured blue, ATPase-coding genes are coloured yellow, replicase-coding genes are coloured red, recombinase-coding genes are coloured green and adenoviral protease-coding genes are coloured purple. The last five genomes are arbitrarily linearized for a more convenient alignment. Asterisks denote the viruses for which MCP X-ray structures are available. The genomes of Paramecium bursaria Chlorella virus type 1 (PBCV-1) and other nucleo-cytoplasmic large DNA viruses are much larger than those shown and were therefore omitted from the alignment. Int, integrase; PolB, polymerase B; Pro, protease; RCR, rolling circle replication.

**Virus or element**

<table>
<thead>
<tr>
<th>Virus or element</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog adenovirus*</td>
<td>includes genes that encode the key structural components of the virion. Such components eventually determine how the virion is constructed and are specific to a given viral lineage, which indicates that they are inherited vertically. The probability that so many similarities between viruses within a viral lineage would arise as a result of convergent evolution is low.</td>
</tr>
<tr>
<td>Maverick-2_XT</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
<tr>
<td>Maverick-1_CI</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
<tr>
<td>PRD1*</td>
<td>By contrast, the other category, the viral self genes, includes genes that encode the key structural components of the virion. Such components eventually determine how the virion is constructed and are specific to a given viral lineage, which indicates that they are inherited vertically. The probability that so many similarities between viruses within a viral lineage would arise as a result of convergent evolution is low.</td>
</tr>
<tr>
<td>Bam35</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
<tr>
<td>STIV*</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
<tr>
<td>TKV4</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
<tr>
<td>MVV</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
<tr>
<td>PM2*</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
<tr>
<td>Corticoelement</td>
<td>for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.</td>
</tr>
</tbody>
</table>

Structurally similar viruses that can infect hosts from different domains of life have different gene contents, as interactions with the host cell change the gene content of the virus. We therefore find it useful to divide viral genes into two groups. One category, referred to as the non-self genes, includes viral genes that are involved in the interaction with the host and can be exchanged between unrelated viruses or even between the virus and the host chromosome through horizontal gene transfer. Consequently, their evolutionary histories cannot be followed with certainty. This category includes genes for DNA and RNA metabolism (replication, recombination and transcriptional factors), host recognition, evasion of the immune system and release of the virus progeny.

By contrast, the other category, the viral self genes, includes genes that encode the key structural components of the virion. Such components eventually determine how the virion is constructed and are specific to a given viral lineage, which indicates that they are inherited vertically. The probability that so many similarities between viruses within a viral lineage would arise as a result of convergent evolution is low.

**Evolutionary origin of the lineage**

A number of hypotheses have been put forward to explain the origin of viruses (see, for example, REFs 4, 5, 21, 65). These hypotheses converge to one common feature: the viral capsid protein is the only component that is unique to the viral world. Extending this idea, Raoult and Forterre have even proposed to divide biological entities into ribosome-encoding organisms, which include eukaryotic, archaeal and bacterial organisms, and capsid-encoding organisms, which include viruses. Acquisition of the protein shell by a replicon was a cornerstone in the origin of viruses. This event seems to have occurred multiple times, giving rise to diverse viral lineages, some of which still exist today. The origin of capsid proteins is speculative. One possibility, as suggested by Koonin et al., is that viral hallmark genes (one of which is the MCP-coding gene) might have descended directly from a primordial gene pool, as they are missing from the cellular gene content.

Structural and genomic data allow us to extrapolate back in time and suggest an evolutionary trail of the double β-barrel proteins and consequently viruses that use these proteins for their assembly. Figure 3 combines information about a number of viral families that infect hosts from all three cellular domains. It should be noted, however, that the scenario presented is highly hypothetical, and is meant only to stimulate further research and discussions on virus evolution. The numbers represent the possible major turning points in the evolution of the members of the PRD1–adenovirus lineage and will be discussed in numerical order.

The double β-barrel fold seems to arise through gene duplication,62,31,34. In line with this proposal was the recent identification of SH1 and P23-77, two membrane-containing viruses that infect archaeal and bacterial hosts, respectively.76,78. The structure of the SH1 and P23-77 virions has been determined by cryo-EM and image reconstructions to 9.6 Å and 14 Å resolutions, respectively. Analysis of the two virions revealed that hexagonal capsomers are similarly arranged in a T = 28 (T represents the triangulation number).
Figure 3 | Possible origin and evolution of viruses that belong to the vertical β-barrel superlineage. The schematic represents the hypothesized evolutionary relationship between double-stranded DNA viruses that use vertical β-barrel pseudohexamers for icosahedral capsid shell assembly. It should be noted that the diagram is not a tree that is inferred from phylogenetic analysis but rather a summary of the available data on the depicted viral families. The same diagram is coloured with different shades to reflect the major capsid protein-based (part a) and genome replication machinery-based (part b) relationship between different virus families that are unified into the vertical β-barrel superlineage. The numbers denote the key turning points in the evolution of this virus superlineage. The coloured circles represent the different domains of life from which the corresponding hosts reside. Bacteria are coloured red, Archaea are coloured green and Eukarya are coloured blue. The organization of the viral genomes (circular or linear) is indicated. NCLDV, nucleo-cytoplasmic large DNA virus; PolB, polymerase B; STIV, Sulfolobus turreted icosahedral virus.

Icosahedral lattice. The subnanometre resolution structure of the SH1 virion, together with biochemical data, suggested that hexagonal capsomers are formed from six individual β-barrels that are oriented normal to the capsid surface: the central axis of the barrels was orientated perpendicular to the capsid surface and was stabilized by additional decorating proteins. Importantly, SH1 and P23-77 both encode a putative packaging ATPase with the P9/A32-specific motif that is common to all membrane-containing members of the PRD1–adenovirus lineage (M. Jalasvuori and J. K. Bamford, personal communication). On the basis of these common virion assembly principles, we propose that such single β-barrel viruses should be grouped together with the double β-barrel viruses into a vertical β-barrel superlineage (FIG. 3). Such a superlineage would include all viruses that use similar virion architecture regardless of whether the hexagonal capsomer is composed of six monomers or three dimers.

Duplication and subsequent fusion of the gene that encodes for a vertical β-barrel capsid protein probably led to the emergence of viruses with the double β-barrel MCPs (FIG. 3, branching point 1). The use of double β-barrel trimers instead of single β-barrel hexamers reduces the number of protein subunits needed to build the hexagonal capsomer (from six in SH1 to three in PRD1) and would account for a lower assembly error probability, as proposed by Jäälinnoja and colleagues. Furthermore, double β-barrel trimers are likely to be more stable than hexamers, which could account for the absence of decorating or stabilizing proteins. The simplest double β-barrel is found in corticovirus PM2 and was designated the minimal double β-barrel (FIG. 1a). Structural comparison of the two PM2 β-barrels with those of other viruses from the same lineage revealed that the two barrels in PM2 are more similar to each other than to any other coat protein, which supports the hypothesis that the protein arose by gene duplication.

Importantly, an interaction with the internal membrane in PM2 is achieved by separate viral integral-membrane proteins. All other membrane-containing viruses of the lineage possess larger and more elaborate MCPs that directly interact with the underlying viral membrane, which suggests that they diversified from the minimal double β-barrel fold (FIG. 3, branching point 2).

PM2, and all other corticoviral elements that can frequently be found integrated into the genomes of aquatic bacteria, seem to possess circular genomes that are apparently replicated through four different mechanisms. Circular dsDNA genomes are also characteristic of cherenarchael virus STIV and the euryarchael proviruses, TKV4 and MVV. Interestingly, the integrated genome of MVV is only slightly larger than that of PM2, and replication of both genomes seems to be carried out through a rolling-circle mechanism. Notably, none of these (pro)viruses encodes a DNA polymerase for independent genome replication, but rather they depend on the cellular replication machinery. This also seems to be true for SH1, for which no replication protein could be identified, and P23-77, which encodes a putative replication initiation protein (M. Jalasvuori and J. K. Bamford, personal communication). By contrast, all other members of the PRD1–adenovirus lineage, including those that infect bacteria or eukaryotes, encode PolB (FIG. 3b). It is possible that the polB gene was acquired only once, and then diversified into the protein- and RNA or DNA-primed PolB, whereas all the other members of the PRD1–adenovirus lineage, the NCLDVs’s and ascoviruses, encode an RNA or DNA-primed PolB.

Diversification of bacterial tectiviruses from adenoviruses, Mavericks and NCLDVs was probably associated with the emergence of the Bacteria and Eukarya domains (FIG. 3, branching point 4). This scenario is supported by a structure-based phylogenetic analysis of currently available double β-barrel MCPs, which revealed that MCPs of PBCV-1 and adenovirus cluster together. It is hard to predict the order in which the radiation from the common
ancestor of adenoviruses, Mavericks and NCLDV s might have proceeded. The monophyletic origin and relationship between individual virus families of the NCLDV group, as well as the connection between iridoviruses and ascoviruses, was thoroughly discussed by Iyer et al. and Staak et al., respectively, and will not be repeated here. In contrast to the evolution of NCLDV s, which advanced by increasing their capsid T number, adenoviruses modified their ancestral PBCV-1-like MCP and seem to have sacrificed their internal membrane to build larger shells and consequently obtain the ability to increase their gene content (Box 1). This idea is supported by the identification of Mavericks, which possess genome organization and gene content similar to adenoviruses and tectiviruses, but an MCP that is more similar to that of iridoviruses and ascoviruses, was modified their ancestral PBCV-1-like MCP. This led to the realization that the previous structural data on diverse viruses that infect hosts from all three domains of life, are under positive selection, possibly owing to the benefit of incorporating additional genetic elements to enhance survival and propagation in more complex eukaryotic cells.

**Conclusions**

Many technological advances have been achieved during the past decade, which together with genomic data analyses, have allowed us to propose a scenario of how these icosahedral dsDNA viruses might have evolved. These findings not only provide order to the entire domain of viruses but also shed light on the origins and evolution of early life, when viruses may have substantially contributed to the composition of the biosphere by facilitating genetic exchange.

**References**


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e-Science: relieving bottlenecks in large-scale genome analyses

Tracy Craddock, Colin R. Harwood, Jennifer Hallinan and Anil Wipat

Abstract | The development of affordable, high-throughput sequencing technology has led to a flood of publicly available bacterial genome-sequence data. The availability of multiple genome sequences presents both an opportunity and a challenge for microbiologists, and new computational approaches are needed to extract the knowledge that is required to address specific biological problems and to analyse genomic data. The field of e-Science is maturing, and Grid-based technologies can help address this challenge.

By 30 September 2007, 708 bacterial genome sequences had been submitted to publicly accessible databases, such as those at the European Molecular Biology Laboratory’s European Bioinformatics Institute (EMBL—EBI), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the National Center for Biotechnological Information (NCBI) (see Further information). Many other genomes are currently being sequenced and several have not yet been placed in the public domain. The number of publicly available genomes has doubled approximately every 16 months since 2000. At this rate, more than 2,000 bacterial genomes will be available by the end of 2010 (Fig. 1).

In most cases, the genome of only a single representative of a species has been sequenced. Increasingly, however, replicate genome sequences are appearing in the public domain (Fig. 1). For example, there are now 14 publicly available Staphylococcus aureus genomes, with 2 additional strains at the ‘finishing’ stage. Using replicate genomes, direct comparisons can be made between closely related strains, providing unprecedented insights into the mechanisms involved in genome plasticity and rates of evolution. As

Further information

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Virus survival in the environment

E.C. PIRTLE and G.W. BERAN *

Summary: Viruses pass into the environment from clinically ill or carrier hosts; although they do not replicate outside living animals or people, they are maintained and transported to susceptible hosts. Population concentrations and movement, both animal and human, have been steadily increasing in this century, enhancing transmission of respiratory and enteric viruses and compounding the difficulty of preventing environmental transmission.

Studies on environmental survival factors of viruses have been most definitive for polioviruses, foot and mouth disease viruses and Aujeszky's disease virus. In addition, heat resistance studies have been reported on adenoviruses, African swine fever virus and the Norwalk virus. Resistance to disinfectants has been studied for many viruses, including picornaviruses, papovaviruses, reoviruses and retroviruses. Survival of viruses in and on a variety of fomites has been studied for influenza viruses, paramyxoviruses, poxviruses and retroviruses. The subacute spongiform encephalopathy agents, under extensive current studies, are being found to have incredible stability in the environment.


INTRODUCTION

In the triad of infectious disease transmission involving aetiological agents, susceptible hosts and the environment, the role of the environment is the most ambiguous. The environment receives, maintains or protects and transports aetiological agents to susceptible hosts. Viruses may enter the environment in enormous quantities from clinically ill or inapparent carrier hosts; when extant outside the hosts which support their replication, they are the least understood of infectious agents. The greatest prospects for disease control for the future, however, lie in environmental measures to halt or reduce transmission. Conversely, failure to break the chains of transmission will result from failure to protect the environment or to modify it beneficially.

The increase in respiratory disease transmission through population concentrations, in cities in the case of humans and in confinement production units in the case of animals, is the most striking example of disease prevalence. A greater contemporary awareness of the role played by the confined environment in increasing the transmission of some diseases while reducing that of others has prompted more serious study of the environment.

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This review of the survival of viruses in the environment attempts to consolidate data from reported studies. Further investigation of how numerous viruses survive in the environment is necessary. Current knowledge is fragmented and the fragments differ widely according to the infectious agents; this fact highlights the need for more comprehensive studies in the future.

This article discusses viral entry, survival and transport as they relate to any nonliving substances or living organisms which do not support viral replication. Viruses are considered by families and are ordered alphabetically. When more than one virus is discussed in a chapter, they are considered alphabetically in relation to the first virus mentioned in the chapter.

**ADENOVIRIDAE**

Thermal inactivation studies have been reported for adenovirus 12, reovirus 1 and herpes simplex virus in raw milk, sterilized homogenized milk, raw chocolate milk and raw ice cream mix, with minimum essential medium (MEM) as control suspending fluid (55, 57). From approximately 10,000 plaque-forming units (PFU) per ml of each suspending medium, inactivation curves at 40°C-60°C were asymptotic to the baseline, indicating that small amounts of these viruses survived, even at the higher temperature. At 65°C, the inactivation curves approached first order reactions, indicating that temperatures near pasteurization standards were effective in inactivating these three viruses. In the same studies, influenza A and Newcastle disease viruses showed stability in raw and sterilized milk equivalent to that in MEM. Thermal inactivation of Maloney virus, Rauscher leukemia virus and Rous sarcoma virus assayed in mice showed Rous sarcoma to be the most resistant.

**ARENAVIRIDAE**

The hallmark of all rodent-borne arenaviruses is persistent infection in the rodent host in the presence of immunological response (38). Persistence is established in the natural host if virus transmission occurs *in utero* or shortly after birth. Most persistently infected rodents have permanent viruria and viremia. Viral persistence is a highly efficient means of virus perpetuation in most rodent offspring; it is also the most important source of contamination of the external environment and leads to transmission of infection.

**HERPESVIRIDAE**

Extensive studies have been published on assays of potentially contaminated fomites for both human and animal herpesviruses. Additional studies of virus survival on and in experimentally-contaminated fomites have been reported. In studies on the alphaherpesvirus, HSV2, assays of spa water failed to yield the virus (45). To
simulate the conditions of survival of HSV2 on plastic-coated benches and seats in spa facilities, HSV2 (10^{4.2}\text{CCID}_{50}/0.5 \text{ ml}) was placed on plastic surfaces in a humid atmosphere at 37°C-40°C. The virus was found to survive for up to 4.5 h under these conditions. The results of a study with HSV1 and HSV2 viruses demonstrated that HSV obtained directly from ulcerative or vesicular genital lesions was able to survive for several hours on fomites and hard surfaces and for several days on dry cotton gauze (35). HSV2 has been shown to survive for short periods outside the host; it is the opinion of some workers, however, that while the virus can persist on certain surfaces and porous items, such as towels, for relatively long periods of time, fomites are not particularly significant in transmission (22).

There has been considerable interest in environmental transmission of the betaherpesvirus, cytomegalovirus (CMV), among infants and personnel in medical facilities. During a four-month study, CMV was found in the urine of eight infants (54). Three of the isolates were found to be identical by restriction endonuclease analysis, which suggests that the three infants in question were infected with the same CMV strain. The evidence indicates that CMV was transmitted from one infant to the other two infants through unidentified fomites within the nursery. Samples from the immediate environment of these eight CMV-infected infants were obtained and submitted for virus assay in cell cultures (23). CMV was isolated from those objects which had come in direct contact with infected secretions, i.e. from six of eight oronasal suction bulbs, one feeding tube, four dry diapers in contact with genitalia, and from a pair of gloves worn by a nurse. While the conclusion was reached that CMV could be isolated for several hours after natural contamination, it was not determined that fomites were an important source of nosocomial CMV transmission. That personnel should wash their hands during patient care, however, was considered as essential.

Nosocomial transmission of CMV was investigated in a chronic care unit (CMV excretion prevalence 16%) and a neonatal unit (CMV excretion prevalence 0.7%) (19). In the chronic care unit, two infants were infected with homologous strains of CMV. No infants acquired CMV in the neonatal unit of the hospital, though seroconversion did occur in two nurses. CMV was isolated from diapers and from the hands of patients and personnel, but not from environmental surfaces.

In an epidemiological study of bovine herpes mammillitis (BHM) virus (27), pseudocowpox was confirmed in dual infections in nine of eleven BHM-positive milking herds. The pattern of BHM spread was not related to the incidence of pseudocowpox or to the order in which cows were milked. Meteorological data suggested that BHM occurred more frequently in those years conducive to the increase of large populations of insect vectors, and that the direction in which BHM spread to herds within a locality was related to the predominant wind direction.

Fomites were experimentally identified as a risk in the transmission of CMV (53). Urine and saliva inoculated with 1 \times 10^4 \text{ PFU/ml} of either a wild or laboratory strain of CMV could be recovered, at 37°C, for 48 h and 2 h, respectively.

Survival studies on Aujeszky's disease virus (ADV) in this unit have focused on experimental contamination of fomites and vehicles present in and around swine operations. Virus suspended in saliva, nasal-washing or saline-glucose control fluids
was assayed for survival on solid fomites kept moist at 25°C. What follows are the maximum times needed to reach 99.99% inactivation:

- control fluid (no fomite) 58 days
- steel 18 days
- concrete 4 days
- plastic 8 days
- rubber 7 days
- denim cloth <1 day
- loam soil 7 days
- green grass 2 days
- shelled corn 36 days
- pelleted swine feed 3 days
- meat and bone meal 5 days
- alfalfa hay <1 day
- straw bedding 4 days
- sawdust bedding 2 days
- swine faeces 2 days.

Mean survival time of ADV in contact with fomites in saliva suspension was 45% of virus in saline-glucose suspension; in nasal-washing suspension, the mean survival time was 30% of that for virus in saline-glucose suspension (52).

ADV suspended in liquid fomites at 25°C reached 99.99% inactivation at the following times (52):

- well water 7 days
- chlorinated water 2 days
- urine from swine on subtherapeutic medicated feed 14 days
- manure pit effluent <1 day
- anaerobic lagoon effluent 2 days.

ADV in aerosol decayed logarithmically with half-lives of 17.4-36.1 min at 22°C and 27.3-43.6 min at 4°C, with longer survival times at 55% relative humidity than at 25% or 85% relative humidity (51).

ADV fed to houseflies (Musca domestica) could be recovered for as long as seven days in the internal organs but for only a short time from the body surfaces. The longest survival time for virus was obtained when 3-day-old flies were exposed (compared to survival in 5- to 13-day-old flies), and when exposed flies were kept at 10°C as compared to 20°C or 30°C (61). Virus placed on the feet of pigeons and
starlings could not be recovered, even when suspended in mucin, placed on washed surfaces of the toes and sampled promptly after exposure (M.A. Schoenbaum and G.W. Beran, unpublished data).

ADV mixed into 80% lean ground pork sausage, pH 5.85, could still be recovered after 14 days at 4°C storage and after 40 days at -20°C storage (E.C. Pirtle and T.A. Proescholdt, unpublished data).

**IRIDOVIROIDAE**

African swine fever virus (ASFV) is the only virus in the Iridoviridae family which is known to infect mammals. ASFV survives over a wide range of pH values and is particularly resistant to alkaline conditions. In the presence of protein, some infectious virus may survive for 7 days at pH 13.4 and for several hours below pH 4.0 (49).

**NORWALK VIRUS**

The Norwalk group of viruses is tentatively described as calici-like, but has not been definitely classified (32). In experiments, the Norwalk virus retained infectivity to volunteers producing gastroenteritis following exposure to pH 2.7 for 3 h at room temperature and after heating at 60°C for 3 min. Both the Norwalk and “W” (Wollan boarding school) agents were stable following treatment with 20% ether at 40°C for 18-24 h.

**ORTHOMYXOVIRIDAE**

Orthomyxoviruses, influenza A and B ($10^{3.5}$ and $10^{4.2}$ infectious doses/0.1 ml, respectively) were spread over stainless steel, plastic and absorbent material surfaces (30 to 50 mm) (4). These objects were subsequently sampled by rubbing with sterile swabs or “clean” fingers. Both influenza A and B viruses survived on hard surfaces for 24 to 48 h, but for less than 8 to 12 h on porous surfaces. The conclusion was therefore reached that, under conditions of heavy environmental contamination, the transmission of influenza virus by fomites may be possible.

**PAPOVAVIRIDAE**

Objects used in the treatment of patients with human papillomavirus (HPV) infections were tested for recovery of HPV-DNA (25). HPV-DNA was identified by hybridization on 8 of 16 (50%) surgical gloves, on 23 of 62 (37%) and 1 of 62 (1.6%)
biopsy forceps before and after sterilization in 30% tincture of Savlon for 30 min, and on 5 of 22 (23%) and 1 of 22 (4.5%) cryoprobe tips before and after cleaning with 90% ethanol for 1 min. Whether the HPV on fomites remained infectious was not evaluated; however, extreme caution on the part of patients and personnel was suggested.

PARAMYXOVIRIDAE

In studies on paramyxoviridae obtained from infants, a suspension of respiratory syncytial virus (RSV) containing approximately $10^{5.5}$ CCID$_{50}$/ml was used to contaminate Formica counter tops, cloth gowns, rubber gloves, paper facial tissues and hands (28). The virus was recovered from counter tops for as long as 6 h, from rubber gloves for 1.5 h, from cloth gowns and paper tissues for 30 to 45 min, and from skin for up to 20 min. Self-inoculation by contact with contaminated infant secretions was therefore considered a potential method of nosocomial transmission of RSV.

Dilutions of parainfluenza viruses (PIV), types 1, 2 and 3, were experimentally studied on nonabsorptive and absorptive surfaces (9). Virus was recovered for up to 10 h from nonabsorptive surfaces and for up to 4 h from absorptive surfaces. Interpretation of the results suggests that fomites should be considered as sources of PIV transmission inside and outside hospitals.

Newcastle disease virus was isolated from the carcasses of frozen poultry for over 730 days and from buried carcasses for 121 days (31).

Rinderpest virus was reported to inactivate rapidly outside the body (48) and to be most stable at pH 4.0-10.2 at 4°C. Infected meat stored in a chilled state between 2°C and 7°C was still infective after seven days.

PICORNAVIRIDAE

Extensive investigations have been conducted on the survival, under natural and experimental conditions, of several viruses belonging to the Picornaviridae family (small RNA viruses). Results of these picornavirus investigations are given herein.

The Mengo encephalomyocarditis virus, a picornavirus of the Cardivirus genus, was examined for survival at various temperatures (21). The virus suspended in saline at $1 \times 10^{6.6}$ infectious doses/ml was still detectable at titres of $\geq 10^2$ infectious doses/ml on the 25th day at 37°C, on the 102nd day at room temperature, and on the 117th day between 2°C and 10°C.

In studies in this unit, encephalomyocarditis virus (Florida strain) was mixed into 80% lean ground pork sausage, pH 5.85, held at 4°C and -20°C and assayed at regular intervals. By the 56th day, viral titres had decreased 99% in sausage stored at 4°C but not at all in sausage stored at -20°C (E.C. Pirtle and T.A. Proescholdt, unpublished data).
Foot and mouth disease (FMD) virus, a picornavirus of the genus Aphthovirus, has been widely studied for its strong environmental stability. Virus shed from infected mammary glands was incorporated into milk micelles and fat droplets, thus affording thermal resistance (7). A portion of the viral population was found viable in contaminated milk after pasteurization at 72°C for 15 seconds or after acidification to pH 4.6 (11). FMD virus has been recovered from cattle stalls 14 days after removal of infected cattle, from urine after 39 days, from soil after 28 days in autumn and after 3 days in summer, and from dry hay at 22°C after 20 weeks storage. Salting, curing and drying of hides of infected cattle has not been effective in inactivating the virus (15). Carcasses of infected animals at 4°C have reached a tissue pH \( \leq 5.3 \) within a few days when chilled, thus inactivating FMD virus; however, virus has been recovered from bone marrow and lymph nodes after six months of refrigeration (1) and from swine blood after two months of refrigerated storage (15).

Numerous human pathogenic viruses exist among the enterovirus genus of the picornaviruses. Banker (3) has emphasized the detection of more than 100 different types of enteric viruses in drinking water, wastewater, sea water, as well as in soil, crops, foods and aerosols. Also emphasized was the possibility that viruses which are more resistant than indicator bacteria to conventional purification procedures, and which thus have greater potential for survival, may be contained in drinking water which meets bacteriological standards.

Three enteroviruses — polioviruses, echoviruses and coxsackieviruses — were used to contaminate soil and vegetables; their survival times, under various storage conditions, were then recorded (2). The concentration of the viruses employed varied from \( 1 \times 10^4 \) to \( 1 \times 10^5 \) CCID\(_{50}\)/ml. Depending on soil type, moisture content, pH and temperature, the viruses survived for 150 to 170 days in soil. When added to uncooked vegetables and stored under household conditions, the viruses survived for as long as 15 days.

Three of the numerous studies conducted on insects as possible survival hosts of enteroviruses are cited here. In one study (16), poliovirus types 1 and 3 were fed to two groups of blowflies (Phoenicia sericata) via sugar cubes contaminated with \( 10^{7.5} \) PFU/cube. The flies were then alternately incubated between 40°C and 4°C. Dead flies were removed and titrated for the polioviruses. Viruses were recovered from flies for 13 days (type 3) to 15 days (type 1). Virus titres decreased during the study period; this indicated that the virus did not replicate in the flies, and that mechanical means accounted for survival and potential spread.

In the second study (17), cockroaches (Periplaneta americana) were fed poliovirus type 3 at approximately \( 10^{6.5} \) PFU/roach. Virus survived in roaches for as long as 50 days with no evidence of virus multiplication; as in the blowfly study, the cockroaches were considered to be potential mechanical spreaders of the surviving poliovirus.

In studies conducted on the MF strain of swine enterovirus on the initial isolation farm, houseflies which had been in contact with the swine environment were collected and then assayed in nine pools of 25 flies each. The virus was recovered from all pools at mean concentrations between 19 and 186 CCID\(_{50}\)/fly. Wash fluids from surfaces of the flies yielded a mean of 124 CCID\(_{50}\) per fly, while a mean of 66 CCID\(_{50}\) was obtained from ground tissues of the washed fly bodies. The authors concluded that contaminated houseflies could readily transport the virus between swine units. However, with regard to the widespread viral shedding in the environment by adult
swine and the widespread early infection of young pigs, flies did not seem to play any essential role in the maintenance of the endemic state of infection (60).

Poliovirus type 1 and coxsackieviruses, types B1 and B6, were added to foods at total concentrations of $1 \times 10^5$ or $1 \times 10^6$ CCID$_{50}$, then held at room temperature, 10°C and -20°C (37). The viruses were still viable after one week, one month and five months, respectively. Decomposition did not affect virus viability at room temperature and antibiotics controlled bacterial contaminants during laboratory assay.

In a study of the survival of coxsackievirus B2 experimentally inoculated on ground beef, a 75% recovery rate (7,000 PFU) was achieved by suspension, elution and inoculation of monkey kidney cell cultures (55). The same technique was used to recover naturally contaminating polioviruses and echoviruses from three of twelve purchases of market-purchased beef at levels between 1 and 195 PFU per 5 g. One loaf of beef yielded poliovirus type 1 and echovirus type 6, one yielded poliovirus type 2, and one yielded poliovirus types 1 and 3.

In a study on experimental contamination during the preparation of Thuringer sausage, approximately 85% of the coxsackievirus A9 ($10^4$ to $7.5 \times 10^5$ CCID$_{50}$) which was added to the sausage was lost during 24 h fermentation at 30°C (30). Additional heating of the prepared sausage at 49°C resulted in further progressive loss of virus; after 6 h at 49°C, however, an average of $1.1 \times 10^3$ CCID$_{50}$/g of sausage still remained of the initial $7.5 \times 10^5$ CCID$_{50}$/g. Despite the counts of spoilage bacteria which rose progressively, coxsackievirus A9 suspended in ground beef was found to survive, at levels considered as infectious, for up to eight days at both 23°C and 4°C.

So that parameters for virus isolation from environmental samples might be better defined, the effects of inoculum size and cell culture density on cytopathic effect (CPE) or plaque assay were assessed with poliovirus type 1 and Buffalo green monkey (BGM) cells (47). A linear relationship was obtained with an inoculum volume of 1.0 ml/25 cm$^2$. Depending on the sensitivity of the cell cultures, maximum titres were obtained when 25,000 to 75,000 cells/ml were incubated for six days before exposure to virus.

Survival of enterovirus 70 (EV70), the aetiologic virus of acute haemorrhagic conjunctivitis, was assayed at various temperatures (20°C to 35°C) and relative humidities (20, 50, 80 and 95%) (50). Ultrahigh relative humidity (95%) best protected EV70 from inactivation at 20°C, but the virus was least stable between 33°C and 35°C at this humidity level.

Human enterovirus 72, the aetiologic virus of human hepatitis A, has shown a relative stability which is typical of the majority of enteroviruses. Rapidly developing epidemics of hepatitis A have often followed faecal contamination of a single source, such as drinking water, food, or milk, with human enterovirus 72 (40, 42, 43, 58, 59).

A swine enterovirus was tested for stability to dimethyl ether, arsenic acid, penicillin, dihydrostreptomycin, butyl parasept and oxytetracycline. It was also tested at pH values 5 to 9 and at incremental temperatures between 23°C and 70°C (5). No depression of infectivity titre was obtained with any of the chemicals or compounds tested. The virus was stable at pH values 5 to 9 at 4°C for 28 days, at 23°C for two weeks, and at 37°C for four days. It was inactivated within 30 min at 50°C; inactivation was immediate at 56°C and higher.
Swine enterovirus, which was excreted in large amounts in the faeces, was shown to survive in faeces at ambient temperatures for periods up to 27 days (20).

Swine vesicular disease virus (SVDV) has been isolated from infected faeces after 28 days (18). It was stable over a wide pH range (2 to 12) and survived at pH 5 to 10 at 4°C for long periods. SVDV has been isolated from infected premises for as long as eleven weeks after swine herd depopulation and disinfection of the premises. Only processes which involved heat treatment at or above 60°C for at least 30 min effectively inactivated SVDV. Contaminated meats, which had been imported and refrigerated, were found to remain dangerous almost indefinitely; for example, SVDV at -20°C yielded viable virus for 300 days.

Domestic sewage was found to contain about 500 enteric virus units/100 ml, while polluted surface water contained no more than one unit/100 ml (12). The ratio of enteric viral density to coliform bacterial density in human faeces was approximately 15 virus units for every 106 coliforms. The activated sludge process removed 90 to 98% of enteric viruses in raw sewage. Hypochlorous acid (HClO) was shown to be effective in inactivating viruses in water, with the rate depending on the virus, pH, temperature and contact time. Polioviruses, coxsackieviruses and hepatitis A viruses were more resistant to HClO than coliform or enteric pathogenic bacteria.

Coxsackieviruses in human stools were thermally inactivated at 55°C for 15 min or at 71.1°C for 15 seconds in water. Dairy products offered these viruses a measure of protection against thermal inactivation, but minimum conditions recommended for pasteurization (61.7°C for 30 min or 71.1°C for 15 seconds) proved adequate for inactivation of the faecal strains (34).

Surface sea water from the Mediterranean inactivated $10^3$ CCID$_{50}$/ml of poliovirus type 1 in six to nine days (39). The sea water lost part of this virucidal activity after boiling, but Seitz filtration was found to have no such activity. Artificial sea water was less deleterious to the virus. It was therefore concluded that the virucidal activity was partly due to heat-labile substance(s) in sea water.

A study was conducted in the Philippines on the survival of enteroviruses in sea water alone, or in sea water containing oysters. In sea water alone, 99.99% of the decay in virus titre occurred for poliovirus type 1 in fourteen days and for poliovirus types 2 and 3 in seven days. The same percentage occurred for coxsackievirus B4 in three days, for coxsackieviruses A9, B1 and B6 in seven days, for coxsackieviruses B3 and B5 in fourteen days, for echoviruses 5 and 7 in seven days and for echoviruses 1, 12, 13, 17 and 20 in fourteen days. The same survival periods were recorded for poliovirus types 1-3 in sea water with living oysters; however, at three days the oysters were found to have concentrated poliovirus type 1 at titres of $10^{5.5}$ CCID$_{50}$/g of digestive tract and $10^{6.5}$ CCID$_{50}$/g of flesh compared to $10^{3.5}$ CCID$_{50}$/ml of sea water. At seven days, $\geq 99.9%$ of the poliovirus type 1 was gone from the digestive tracts and tissues of the oysters; at fourteen days, virus levels in the oysters were at nearly the same levels as those found in their ambient sea water. Poliovirus types 2 and 3 were not taken up from the contaminated sea water by the oysters (G.W. Beran and J.C. Nakao, unpublished data).

In a flow-through sea water system, oysters were found to concentrate poliovirus type 1 at rates of 10 to 60 times the concentration of the virus in sea water. The oysters later cleansed themselves of virus in UV-treated fresh sea water. E. coli assayed as indicator showed that experimental conditions were optimal for the study (41).
The results of another study indicated that poliovirus added to ground beef may be more resistant to the levels of heat commonly employed when cooking if the meat contains fat levels approaching 30% (26).

In studies on the survival of virus during the manufacture of Cheddar cheese, 98% of poliovirus type 1, and nearly 100% of influenza A virus and vesicular stomatitis (VS) virus, were inactivated (13). Poliovirus persisted in cheese throughout the aging process, but was inactivated $10^6$-fold when milk which was to be made into cheese was given minimal prior heat treatment.

When suspended in water, milk or ice cream, poliovirus in faecal material from poliomyelitis patients was inactivated at pasteurization temperatures, 61.7°C for 30 min or 71.1°C for 15 seconds (33). Poliovirus in the spinal cords of infected monkeys suspended in the same media was also destroyed at pasteurization temperatures.

When cattle were inoculated in tongue epithelium with $10^5$ bovine ID$_{50}$ of foot and mouth disease virus (FMDV) (15), thyroid, adrenal and rumen yielded 1.2, 3.2 and 1.2 log$_{10}$ PFU, respectively, at eight days post inoculation. FMDV was recovered from bone marrow in titres of 1.2 to 1.5 log$_{10}$ PFU after seven months storage at 1°C to 4°C.

**POXVIRIDAE**

In studies involving one- to two-day-old chicks, avipox virus strain used as modified live virus (MLV) vaccine with $10^6$ CCID$_{50}$/ml of drinking water proved more efficacious than $10^4$ CCID$_{50}$/ml when administered either by drinking water or by aerosol (44). This was probably due to the better survival rate of avipox virus in the higher initial concentration of $10^6$ CCID$_{50}$/ml.

The persistence of capripoxvirus in sheep flocks has been attributed in large part to the survival of infectious virions in skin scales which fall on pasture plants or the soil (24).

**REOVIRIDAE**

Reovirus type 1, influenza A, and parainfluenza 3 viruses survived for three days or less in low moisture processed food. However, poliovirus and echovirus 6 survived in such food for more than two weeks at room temperature and more than two months at 5°C (14).

In studies on the survival of rotaviruses, approximately $10^7$ PFU of human rotavirus suspended in faecal matter were placed on disks of stainless steel, glass and plastic (36). Twenty-seven disinfectants were tested for ability to inactivate these viruses. Only nine of the twenty-seven formulations proved to be effective. The results indicated a relative resistance of human rotaviruses to a wide range of chemical disinfectants; they also emphasized the need to evaluate thoroughly the virucidal potential of formulations employed so as to prevent and control outbreaks of rotavirus diarrhoea in human patients.
It has been demonstrated that bluetongue is a noncontagious disease transmitted primarily by insects (46). The bluetongue virus has been shown to lose infectivity at pH 3.0, and lipid solvents to reduce infectivity approximately tenfold.

RETROVIRIDAE

The constantly increasing number of cases of human immunodeficiency virus (HIV) infections and the recognition of retroviral infections in animals has led to a search for effective methods to disinfect contaminated materials. Hypochlorite-releasing disinfectants have been evaluated against HIV (8). Sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) were tested by quantitative suspension. HIV suspensions of $10^4$ to $10^5$ syncytial forming units/ml were prepared in isotonic NaCl or NaCl plus 10% plasma. Results indicated that disinfection ($10^3$-10$^4$ reduction in 2 min) could be achieved using NaDCC and NaOCl at concentrations of 50 ppm and 2,500 ppm available chlorine (Av Cl$_2$) for virus in NaCl and NaCl plus plasma, respectively. Spilled blood required disinfection with high Av Cl$_2$ concentrations of NaDCC and NaOCl solutions of 5,000 ppm of blood to effect complete inactivation within 2 min.

RHABDOVIRIDAE

As rabies virus is transmitted by bites — unless accidentally injected under highly unusual conditions — and thus circumvents the need to survive in the environment, it is epidemiologically unimportant even when viable in the environment. The lipoprotein envelope and glycoprotein projections subject the virions to oxidative or ultraviolet light inactivation as well as to desiccation. Rare occurrences of aerosol transmission have involved high concentrations of virus through laboratory accidents or in caves where depleted oxygen levels, combined with a relative increase in virus-protecting nitrogen, allowed the accumulation of virus excreted by infected, cave-dwelling bats (6).

The vesiculovirus, vesicular stomatitis virus (VSV), is resistant to marked pH changes and is moderately resistant to heat and chemical inactivation (29). The virus remains infective for up to three weeks, depending on the medium in which it is suspended; frozen VSV has remained viable for several years.

SCRAPIE AGENT

The scrapie agent remains unclassified. Scrapie agent-infected brains from infected hamsters were ground, made into suspensions and centrifuged, after which the supernatant fluids were suspended in soil (10). The soil suspensions were placed into petri dishes and buried for three years. Of the $10^3$ hamster infectious units thus interred, between $10^2$ and $10^3$ of infectious prions were recovered after three years in the soil.
Further investigations of the viruses which enter the human and animal environments are needed, especially as soil and water receive increasing concentrations of these infectious agents. Determinations of the viability of viruses identified in the environment are important, as such viruses do not replicate. In the absence of applicable laboratory culture procedures, differentiating viable virions from degraded ones may be difficult. Nucleic acid hybridization is a promising method of detecting viral DNA.

Of specific importance are hepatitis A virus, the calici- and calici-like viruses (Norwalk, Ditchling or W, Cockle, Parramatta and Snow Mountain), astroviruses (Marin County), Sapporo and Otofuke agents, parvoviruses, coronavirus and the enteric adenoviruses. Emerging in importance are the agents of subacute spongiform encephalopathies in humans and animals, the unique protein structures of which are being termed prions. These agents are highly resistant to any degradation or inactivation.

In the future, more emphasis needs to be placed on the survival of these viruses in specific environments where intimate human and animal contact exists. The movement of viruses over long distances in air, particularly in aerosols of different droplet sizes, in droplet nuclei and by adherence to dry particles, is a critical area which has yet to be adequately delineated. The survival of viruses in water remains a priority, while the survival of viruses in foods is emerging as a new priority. If properly developed, environmental virology will clearly be a significant discipline in the future.

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SURVIE DES VIRUS DANS L'ENVIRONNEMENT. — E.C. Pirtle et G.W. Beran.

Résumé: Les virus se transmettent dans l'environnement à partir de porteurs sains ou malades. Bien que leur réplication ne se produise que dans l'organisme des animaux ou des hommes, ils peuvent être transmis à des hôtes sensibles. L'augmentation régulière, depuis un siècle, des concentrations et des déplacements des populations tant animales qu'humaines, facilite la transmission des virus à tropisme respiratoire et entérique, et en rend la prévention plus complexe.

L'étude des facteurs liés à la survie des virus dans l'environnement a donné des résultats concluants dans le cas des poliovirus, des virus de la fièvre aphteuse et du virus de la maladie d'Aujeszky. La résistance à la chaleur des adénovirus, du virus de la peste porcine africaine et du virus de Norwalk a fait l'objet d'études dont les résultats sont également connus. La résistance aux désinfectants de nombreux virus, dont les picornavirus, les papovavirus, les réovirus et les rétrovirus, a été étudiée. La survie des virus sur ou dans divers vecteurs passifs a aussi été étudiée, en particulier pour les influenzaEUR, les paramyxovirus, les poxvirus et les rétrovirus. Les agents responsables des encéphalopathies spongiformes subaiguës font preuve d'une stabilité extraordinaire dans l'environnement, comme le démontrent des recherches en cours.

MOTS-CLÉS: Environnement - Inactivation - Résistance - Stabilité - Survie - Vecteurs passifs - Virus.

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SUPERVIVENCIA DE LOS VIRUS EN EL MEDIO AMBIENTE. – E.C. Pirtle y G.W. Beran.

Resumen: Los virus son transmitidos al medio ambiente por portadores sanos o enfermos. Aunque su replicación se produce únicamente en los organismos de los animales o del hombre, los virus pueden sobrevivir fuera de ellos y transmitirse a huéspedes sensibles. En el siglo presente, las concentraciones y los movimientos de poblaciones, tanto animales como humanas, han aumentado regularmente, favoreciendo así la transmisión de virus respiratorios y entéricos y haciendo más difícil su prevención.

Los estudios relativos a los factores de supervivencia de los virus en el medio ambiente han sido concluyentes para los poliovirus, los virus de la fiebre aftosa y el de la enfermedad de Aujeszky. También se tienen informes sobre estudios de la resistencia al calor de los adenovirus, del virus de la peste porcina africana y del virus de Norwalk. La resistencia a los desinfectantes se ha estudiado en numerosos virus, incluidos los picornaviruses, papovavirus, reovirus y retrovirus. Así mismo, se ha investigado acerca de la supervivencia de los virus dentro o sobre diversas fomites, sobre todo en lo relativo a los influenzavirus, paramixovirus, poxvirus y retrovirus. Se están llevando a cabo estudios que demuestran una increíble estabilidad en el medio ambiente de los agentes responsables de las encefalopatías espongiformes subagudas.

PALABRAS CLAVE: Estabilidad - Fomites - Inactivación - Medio ambiente - Resistencia - Supervivencia - Virus.

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REFERENCES


A REVIEW

Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection

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1. SUMMARY

Enterically infecting viruses are ubiquitous agents, mostly inducing silent infections. Several are however associated with significant diseases in man from diarrhoea and vomiting to hepatitis and meningitis. These viruses are drawn from a variety of virus families and have different structures and genetic material, yet all are suited to this means of transmission: Normally they are shed in high numbers (assisting environmental transit) and exhibit great particle stability (permitting survival both outside the body and on passage through the stomach). Human activities particularly associated with food and water processing and distribution have the capacity to influence the epidemiology of these viruses. This review provides a description of viruses spreading by these means, their significance as pathogens and considers their behavior in these human-assisted processes.

2. INTRODUCTION

The term virus stems from the Latin *virus* meaning ‘poison’, and in some ways virus contamination of food resembles toxic contamination more than contamination with other micro-organisms. Viruses are not free living; they are dormant between hosts and have an absolute requirement for living cells in which to replicate. Human viruses require human cells in which to replicate, these are not present in our food and thus such viruses cannot increase in number during storage. The amount of any contaminating viruses should actually decline during storage, and this can be assisted by treatment with chemicals, heat or irradiation. Human viruses do not cause food spoilage and contamination may provide no visible clues to its presence. These features mean that measures to control bacteria will not...
necessarily control viruses and could actually preserve them. Food-borne viruses are infectious at very low doses and could be introduced at any point in the food chain. Many are difficult (or currently impossible) to culture and detection is no simple task. Outbreaks and sporadic occurrences of food-borne virus infection continue throughout the world. There are simply too many to mention and there is no complete data set. It seems likely that the documented outbreaks are limited only by our ability to document them.

The cost of these events is likely to be phenomenal to the community; a single food-borne outbreak of hepatitis A virus (HAV) exposed up to 5000 persons in Colorado. In this case the costs for medical treatment of those infected amounted to approx. $50 000 whilst the cost of tracing and controlling this single outbreak cost over half a million US$ (Dalton et al. 1996). The burden of infectious intestinal disease (IID) in its broader sense is likewise huge, in the UK the cost per case of norovirus (NoV)-induced gastroenteritis involving a GP visit is estimated at £176 and two persons in every 1000 will make such a visit each year (FSA 2000). The interested reader is referred to several recent reviews (Lees 2000; Seymour and Appleton 2001; Sair et al. 2002; Koopmans and Duizer 2004).

As enteric viruses cannot replicate outside their hosts, all such virus transmission is in effect person-to-person. Environmental transit time between hosts may be brief or prolonged. Long-distance travel may take place, e.g. through water systems or even the air. Long-distance travel is accompanied by exposure to the environment and dilution; thus viruses having prolonged environmental transit times must be very stable to survive and are (usually) shed in very large numbers. Enteric viruses meet both requirements; they are acid stable and replicate to prodigious titres in the gut before being shed in concentrated doses directly into the sewage system. All potentially food-borne viruses can also be transmitted directly from person to person via faecal contamination of the environment and viewed in this way food is simply another kind of fomite in environmental transmission, it occupies a special niche simply because of its privileged position in terms of its introduction to the body and the potential it may offer for widespread distribution through trade and commerce.

The relative importance of food-borne vs more direct person-to-person transmission is unclear; enteric infections are ubiquitous, single occurrences are far too numerous to mention and statistics usually record only outbreaks (when several people are infected in one location or through one common vehicle). However any one outbreak may involve different types of spread; these viruses have a high secondary attack rate and person-to-person transmission will probably follow even if the virus was actually introduced to that setting by food. This can potentially mask food-borne introductions and it is likely therefore that food-borne transmission is underestimated.

### 3. ENTERICALLY INFECTING VIRUSES AND THEIR TARGETS

There are two types of enterically infecting virus – the first are capable of spreading elsewhere in the body. Infection by these viruses is often subclinical but they may induce signs and symptoms of disease in nonintestinal tissues. These viruses include enteroviruses (e.g. polio or Coxsackie, which may spread to the meninges, central nervous system; skeletal/heart muscle or pancreas) and hepatitis viruses A and E spreading to the liver. The second type of virus are true gut inhabitants. These replicate in the enteric tract, specific symptoms when they occur, are those of a gastrointestinal infection; usually diarrhoea and vomiting but the extent of each component is variable.

### 4. THE VIRUSES

Table 1 lists the main viruses associated with enteric infection and summarizes their key properties. The most important are illustrated in Fig. 1. Enteric viruses are drawn from a variety of virus families, they range approximately 10-fold in diameter and 20-fold in terms of genome size and complexity. The major enterically transmitted and thus potentially food/waterborne agents comprise (alphabetically) the human adenoviruses (AdV), astroviruses (HAstV), caliciviruses, hepatitis E virus (HEV), paroviruses, picornaviruses [including enteroviruses, kobuviruses and hepatitis A (HAV)], and the rotaviruses (RV). Most enteric viruses are childhood infections; spreading largely person-to-person and assisted by the lower hygiene levels in this group. Food-borne transmission may be negligible (AdV) or insignificant (RV) in the developed world. However in the undeveloped world spread of these agents by these routes is poorly characterized. Childhood infection leaves residual immunity that may prevent (or mollify) infection over the rest of an individual’s lifetime. Although this is not universally true, in general viruses causing childhood illness are not significant pathogens in healthy adults previously exposed as children. The IID survey in England (FSA 2000) estimated the incidence of GP consultations for intestinal disease by patient age and causative organism. Data from this survey have been reanalysed (Fig. 2) to show the proportion of consultations made for each virus in the age groups <5 and >5 years. As expected GP consultations induced by these viruses were biased towards children <5 years; GP visits by older persons comprised only a small proportion of the total consultations. There were two exceptions to this observation; some 25–50% of GP consultations for NoV infection were made by older
<table>
<thead>
<tr>
<th>Name</th>
<th>Virus family (genus)</th>
<th>Food-borne</th>
<th>Size (genome)</th>
<th>Features</th>
<th>Associated illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio, Coxsackie, echo, enterovirus</td>
<td>Picornaviridae (enterovirus)</td>
<td>Yes, mainly water, present in shellfish</td>
<td>28 nm (ssRNA)</td>
<td>Little surface detail</td>
<td>Mainly asymptomatic, can induce muscle pains (Bornholm disease), cardiomyopathy, meningitis, CNS motor paralysis</td>
</tr>
<tr>
<td>Aichivirus</td>
<td>Picornaviridae (kobuvirus)</td>
<td>Yes, shellfish</td>
<td>28 nm (ssRNA)</td>
<td>Knob-like projections</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Picornaviridae (hepatovirus)</td>
<td>Yes</td>
<td>28 nm (ss RNA)</td>
<td>Little surface detail</td>
<td>Hepatitis, mild in the young</td>
</tr>
<tr>
<td>Hepatitis E virus</td>
<td>Unclassified</td>
<td>Mainly water</td>
<td>34 nm (ssRNA)</td>
<td>Calicivirus-like structure</td>
<td>Hepatitis, severe in pregnancy</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Reoviridae</td>
<td>Rare often water</td>
<td>70 nm (dsRNA)</td>
<td>Multilayered Segmented genome (11 pieces) Cultivable Ma104 cells</td>
<td>Diarrhoea – common in the young, incidence decreasing with age, but increases in the elderly</td>
</tr>
<tr>
<td>Adenovirus group F, types 40 and 41</td>
<td>Adenoviridae</td>
<td>Not reported</td>
<td>100 nm (dsDNA)</td>
<td>Distinctive icosahedral</td>
<td>Mild diarrhoea, shedding may be prolonged, mainly affects children</td>
</tr>
<tr>
<td>Saporovirus</td>
<td>Caliciviridae (saporovirus)</td>
<td>Yes (rare), mainly shellfish</td>
<td>34 nm (ssRNA)</td>
<td>Cup-like depressions on surface; none are cultivable</td>
<td>Gastroenteritis – common in children believed to be milder in effect</td>
</tr>
<tr>
<td>Norovirus</td>
<td>Calicivirus (norovirus)</td>
<td>Yes</td>
<td>34 nm (ssRNA)</td>
<td>Fuzzy surface structure; not cultivable</td>
<td>Explosive projectile vomiting in older children/young adults</td>
</tr>
<tr>
<td>Human astrovirus</td>
<td>Astroviridae (mamastrovirus)</td>
<td>Occasionally, water and shellfish</td>
<td>28 nm (ssRNA)</td>
<td>Eight serotypes 5 or 6-point star motif</td>
<td>Mostly infect children, higher serotypes seen in adults. Relatively mild gastroenteritis, but probably underestimated</td>
</tr>
<tr>
<td>Wollan, ditchling, Paramatta and cockle agents</td>
<td>Parvoviridae?</td>
<td>Yes, shellfish</td>
<td>25 nm (ssDNA)</td>
<td>Smooth featureless, poorly characterized noncultivable</td>
<td>Gastroenteritis – widespread shellfish-associated outbreaks, largely controlled through cooking</td>
</tr>
</tbody>
</table>
children/adults; the highest of any virus, with astroviruses close behind.

4.1 Adenoviruses

There are 51 serotypes of AdV; all are large icosahedral DNA-containing viruses. About 30% of the serotypes are pathogenic in man; most being upper respiratory tract pathogens spreading primarily via droplets. However, even the respiratory strains grow well in the gut and are present in the faeces. Only types 40 and 41 induce gastroenteritis and these are shed in larger numbers. AdV are frequently found in faecally polluted waters and have been identified in shellfish (Girones et al. 1995; Pina et al. 1998a; Vantarakis and Papapetroupoulou 1998; Chapron et al. 2000), but have not been appreciably associated with food-borne illness.

Fig. 1 Food-borne viruses. Electron micrographs of the most important enterically infecting and food-borne viruses found in clinical samples (human faeces). All panels are reproduced at the same magnification; bar represents 100 nm. Panels show: human rotavirus; 2, enteric adenovirus; 3, astrovirus; 4, norovirus; 5, sapovirus

Fig. 2 Virus identifications in GP consultations for infectious intestinal disease by age of patient. Viruses identified following GP consultations have been segregated into the percentage of cases that involve children below 4 years and those involving older children and adults. Bias for infection of the young emerges clearly, even in the case of noroviruses. Data are re-analysed from FSA (2000) IID survey in England. ■, NoV; □, HastV; ●, AdV; ●, RV; □, SaV

presumably because most adults are immune and children do not commonly eat shellfish. However outbreaks of other strains associated with conjunctivitis (shipyard eye) and pharyngitis are commonly associated with exposure to polluted water, normally through recreational use (Crabtree et al. 1997).

Adenoviruses 40 and 41 account for 5–20% of US hospital admissions for diarrhoea, mainly in children below age 2 years (Uhnoo et al. 1984; Kotloff et al. 1989). Incubation lasts 3–10 d, and illness (usually a watery diarrhoea) may last a week. As children age, experience with AdV infection gradually increases the levels of population immunity. Only 20% of children below 6 months have antibody to these viruses, but by age 3 for this has risen to 50%. In the IID survey in England AdV infections were confined largely to children under 5 years and accounted for approx. 12% of all viruses identified (FSA 2000). Incidence was determined as 400 and 800 per 100 000 person years in the age groups 0–1 and 1–4 years. Infection is not significant in healthy adults although it may increase in significance again in the elderly (Dupuis et al. 1995); fewer than 4% of enteric AdV GP consultations involved persons over 5 years (Fig. 2). AdV are associated with tumours in mice but no such association has ever been made in man.

4.2 Astroviruses
Astroviruses are usually described as 28 nm rounded particles with a smooth margin. In their centres they may bear a 5 or 6-pointed ‘star’ motif from which they are named (Astron, a star) (see Fig. 1). However, the appearance of these agents is certainly variable, sometimes showing surface projections (Appleton and Higgins 1975) and at other times resembling caliciviruses (Willcocks et al. 1990). Morphology was subsequently used to present a classification scheme for many of these small viruses associated with enteric infection and including the caliciviruses (below) (Caul and Appleton 1982). Cryo electron microscopy studies have now confirmed the presence of surface projections (Matsui et al. 2001). Human astroviruses comprise eight serotypes (HAst1–8); types 1 and 2 are rapidly acquired in childhood; by age 7 years 50% of children are seropositive for type 1 and 75% by age 10 years (Lee and Kurtz 1982; Kurtz and Lee 1984). Exposure to the higher serotypes (4 and above) may not occur until adulthood.

Illness is generally mild, lasting 2–3 d after an incubation period of similar length. This has led many to dismiss these viruses as causative agents of significant disease in humans. However astroviruses are the second most commonly identified virus in symptomatic children (Herrmann et al. 1991) and account for 5% of US hospital admissions for diarrhoea – almost entirely of children (Ellis et al. 1984). Adults may be infected by higher serotypes and childhood antibody may not prevent clinical disease: in Japan (1995), 1500 older children and teachers were affected in a widespread food-borne outbreak of HAstV type 4 (Oishi et al. 1994). Finally, astrovirus identification often relies on electron microscopy but virus appearance is not always clear. Astroviruses may be frequently mistaken for small round (parvovirus)-like agents (Willcocks et al. 1991) and even for NoVs (Madore et al. 1986). In England the IID survey conducted between 1992 and 1995 (FSA 2000) found astroviruses comprised 12% of all virus identifications with incidences of 125 and 550 per 100 000 person years in the age groups 0–1 and 2–4 years respectively. However, 16% of GP consultations for astrovirus infection were made by older children and adults (Fig. 2). As this survey identified HAstV only by EM it remains possible that astrovirus infections in the adult population were underestimated. Culture conditions have been described (Willcocks et al. 1990) and recently an ELISA-based detection kit has been produced.

4.3 Caliciviruses
Caliciviruses appear under the electron microscope as if covered in cup-like depressions, from which the virus takes its name (calix = a cup). The family includes two genera that infect humans, the NoV and the sappoviruses (SaV). To date neither of these can be cultured in the laboratory. The nomenclature of these viruses has changed several times recently. Formerly they were known by names derived from their morphology (see Fig. 1): the small round structured viruses, e.g. Norwalk virus appeared fuzzy and indistinct whilst the human caliciviruses, e.g. sapporo virus, had a more obvious calicivirus-like appearance (Caul and Appleton 1982). Classification then moved to genomic organization and the groups were renamed the Norwalk-like viruses and the sapporo-like viruses. The nomenclature is now hopefully settled with the refinement of these names to the NoV and SaV respectively.

4.3.1 Noroviruses. Analysis suggests that the NoV are the single most significant cause of IID in the developed world. NoVs were first identified following an outbreak of enteric illness amongst children and adults in the town of Norwalk, OH (Alder and Zickl 1969). Although samples were first collected in 1968, viruses were not clearly identified until 1972 when antibody was used to clump the particles (Kapikian et al. 1972). NoVs are now routinely detected by PCR amplification of the RNA-polymerase gene and by commercial ELISA kits, electron microscopy is used as a back up. Sequence analysis of the PCR products divides the NoV into two genogroups; group 1 exemplified by Norwalk virus itself and group 2 by Hawaii virus (Lambden and Clarke 1994; reviewed in Clarke and
Infections occur around the globe and throughout the year but may be more common in winter giving rise to its former name ‘winter vomiting disease’. Incubation lasts up to 48 h and is followed by a self-limiting illness lasting 24–48 h. NoV infection is not regarded as severe in otherwise healthy adults, but it is debilitating and very unpleasant. In vulnerable groups, the malnourished or elderly it can be serious and may even precipitate death. It was thought that subclinical and childhood infection was rare but recent studies have shown this does occur in very young children (Carter and Cubitt 1995). The IID study in England estimated that 1% of children < 1 years would contract NoV (FSA 2000).

Norovirus differs from other agents of gastroenteritis in three ways: first, it causes disease in adults (teenagers and above), thus NoVs are the most significant diarrhoeal virus in terms of working/education days lost. Secondly, it induces a high level of explosive projectile vomiting that may be the first obvious sign of infection. Many cases are identified at work with serious implications if a food handler should be infected. Thirdly, although there are probably multiple serotypes of NoV, immunity to all seems to be short-lived. Thus individuals may be protected for only a few months following an infection before they become infectable once more by the same virus (Parrino et al. 1977). Some people appear to have an inherent resistance to infection; community outbreaks that stemmed from communal exposure by swimming pool contamination showed familial clustering of symptomatic illness, and even in middle age population antibody levels are only 50%. Many of these seronegative individuals remain symptom-free and it is now thought likely that they lack the cell-surface receptor (a carbohydrate antigen) to which the virus must bind to initiate infection (Hutson et al. 2003). Susceptible persons require several bouts of infection by the same virus before antibody levels are boosted sufficiently to afford some protection. In the recent IID survey in England NoV accounted for 30–40% of all viruses identified, they were the most commonly identified agent in the community study, and the third most common agent that caused persons to seek consultation with their GP (FSA 2000). Several reports across the world have indicated a rise in NoV detection during 2002–03. These included shipboard outbreaks, multistate occurrences in the US, a sudden rise in outbreaks in Canada and numerous hospital outbreaks throughout the UK that forced many to close wards or cease new admissions. These have been attributed in part to the emergence of a new strain of NoV across the world characterized by mutations in the polymerase gene (Lopman et al. 2003, 2004). This might be more infectious than previous strains and if such an event has occurred then the mechanism underlying this process requires investigation: food-borne transmission, perhaps via international trade should be considered.

4.3.2 Sapoviruses. Sapoviruses induce symptomatic infections mainly in children. They account for some 3% of hospital admissions for diarrhoea in both the UK and US. Most children are sero-positive by age 12 and seem to become infected between 3 months and 6 years of age. SaV were found most frequently in children below age 4 in the England IID with an incidence of 460 per 100 000 person years in those aged <1 years that fell to 150 in those between 2 and 4 years (FSA 2000), <2% of GP consultations for SaV infection took place in those >5 years (Fig. 2). Infection is particularly common in institutional settings such as schools and day care centres. Incubation is between 24–48 h and illness is usually mild and short-lived with diarrhoea predominating. However in those cases when SaV have been seen to infect adults then symptoms are very similar to those of NoV (Cubitt 1989).

4.4 Hepatitis A and E viruses

Epidemic hepatitis has been recognized since ancient times, and its infectious nature was appreciated in the middle ages. However the causative agent was identified only in 1972 when the newly developed technique of immune electron microscopy permitted the particles to be identified (Kapikian et al. 1972). Subsequently named HAV to distinguish it from serum hepatitis (hepatitis B), this agent was found to be responsible for the bulk of infectious hepatitis. Symptoms were seen mainly in older children and adults but infection was common in younger children although in these it tended to be symptom free. HAV was found to be a member of the Picornavirus family (see below) and was initially placed in the genus Enterovirus. However it possessed some unique properties in relation to its genetic structure and replication procedure and it was subsequently removed to a new genus (Hepatovirus) of which it is the only member. HAV can be cultured in FRhK-4 cells but this is slow and difficult, especially for primary isolates.

Although responsible for most enterically transmitted hepatitis in the developed world, it was clear that, HAV alone could never account for all enterically transmitted hepatitis in the undeveloped world. Thus the concept of enterically transmitted non-A, non-B hepatitis grew up. This gap in knowledge was filled in 1990 when a new agent, HEV was identified by molecular means (Reyes et al. 1990; reviewed Bradley 1990, 1992). The genetic organization and particle structure of HEV resembled the Caliciviruses and HEV was initially classified in this family. However the detail of the genomic organization and the enzymic capacities encoded are such that it could not remain in this family. Consequently it
now forms the only member of a group called the ‘hepatitis E-like viruses’ (Green et al. 2000). HEV is rare in the developed world with cases generally limited to travellers.

4.5 Parvoviruses

Parvoviruses are poorly characterized as agents of enteric infection. Diagnosis is based solely on electron microscopy. Although associated clearly with gut infection in animals (e.g. canine parvovirus), the only infectious human parvovirus characterized to date is a nonenteric agent B19, causing a maculopapular rash in children. Parvoviruses have been associated with gastroenteritis in primary and secondary schools in the UK (Wollan and Ditchling agents) and Australia (Paramatta agent) (Paver et al. 1973; Christopher et al. 1978; Appleton 1987, 2001). The cockle agent was identified following a large outbreak in England (Appleton and Pereira 1977), and was associated with consumption of contaminated seafood. There is a strong argument that some/most of these viruses may actually be misidentified astroviruses, caliciviruses (Willcocks et al. 1991).

4.6 Picornaviruses

Picornaviruses infecting the gut were formerly all contained in the Enterovirus genus. Enteroviruses have a rather featureless appearance under the electron microscope and include the ECHO Coxsackie and polioviruses. Most grow well in laboratory cell cultures such as HeLa or Vero. Formerly much feared, the success of vaccination has controlled polio and allowed the WHO to target this virus for global elimination early this century. Picornaviruses were thought not to be usually associated with diarrhoeal symptoms in humans but this changed in 1993 when Aichivirus was discovered as the agent responsible for an outbreak of shellfish-associated gastroenteritis (Yamashita et al. 1993). Aichivirus could be grown in Vero cells and study showed that it had a genome organization typical of the picornaviruses (Yamashita et al. 1998). However the particle shows differences in structure from other picornaviruses and bears surface projections similar to those of astroviruses. These viruses have now been recognized as a new genus in the picornavirus family termed the kobuviruses.

4.7 Rotavirus

Rotaviruses are large RNA-containing viruses belonging to the family Reoviridae. Their particles are multilayered and complex, replicative functions may be built into the shell. Particles are readily visible and distinctive in the EM where the outer layer of the capsid can appear like the spokes of a wheel from which the virus is named (rota, a wheel).

Illness develops after an incubation period of 4–7 d and usually presents as diarrhoea and vomiting lasting approx. 7 d. Viruses are shed in extremely high numbers (perhaps over 10⁹ per gram of stool) and diagnosis is a relatively simple matter. Virus is readily detected by direct examination by EM or PAGE (Moosai et al. 1984), antibody-based bead-agglutination or ELISA systems. RV account for some 3.5 million cases of diarrhoea p.a. in the US equating to 35% of hospital admissions for diarrhoea (Ho et al. 1988). Approximately 120 children die each year in the US from this virus and fatalities in the undeveloped world may amount to millions (Parashar et al. 2003).

Rotaviruses occur in five groups (A–E) but only groups A–C infect humans. Group A is by far the most common with sporadic episodes due to group C, group B is limited largely to China. Only group A viruses can be cultured, these are grown in MA104 cells. Within each group RV are divided into serotypes based on their surface-exposed proteins. Within group A there are 14 types of VP7 (termed G types) and approx. 20 of VP4 (termed P types). This generates great antigenic diversity permitting serial infections which may be symptom free. The peak age for illness is between 6 months and 2 years, by 4 years most persons have been infected. Immunity to rotavirus is long-lasting, thus sequential exposure leads to accumulated immunity and frequency of illness decreases with age. Silent secondary re-infections can occur (as in parents caring for infants) and this provides another means for the virus to spread in the community. RV were the most commonly identified enteric pathogen in children <4 years in England and Wales and comprised 25–35% of the virus identification made in this study (FSA 2000). Only 11% of GP consultations for rotavirus were made by persons >5 years. However this raises two points, first not all adult infections are mild and secondly this small percentage may actually reflect a greater number of adult GP consultations for rotavirus infection than for NoV (FSA 2000).

5. PATHOGENICITY OF FOOD-BORNE VIRUSES

5.1 Gastroenteritis

Gastroenteritis viruses replicate and destroy the mature enterocyte covering the upper third of the intestinal villi. Undifferentiated, immature cells do not support virus replication. Destruction of functional mature cells disrupts the reabsorption of water from the gut and diarrhoea ensues. The villi retract in response to damage and decrease the surface area available for absorption. At the same time the crypt cells undergo rapid division and soon repopulate the villi with young, as yet undifferentiated cells. These immature cells are resistant to virus infection but cannot
replace the function of those that have been lost; they require time to mature. Thus malabsorption continues until the cells can develop the necessary ion uptake capabilities. This exuberant cell division in the crypt is central to recovery. Fortunately in humans viruses do not attack the crypt cells themselves. When such infections do occur (e.g. canine parvovirus) they result in bloody diarrhoea from which recovery may not be possible (especially in a young animal).

5.2 A novel virus toxin

Although most viruses do not manufacture toxins some RV are exceptional and induce the synthesis of a toxin-protein termed NSp4 that can induce diarrhoea if administered alone (Ball et al. 1996). NSP4 has no similarity to bacterial toxins (Tian et al. 1995). It stimulates transepithelial chloride secretion via a calcium ion-dependent path; it has no effect on cAMP formation and is independent of the cystic fibrosis channel. The mechanism involves stimulation of inositol production (Dong et al. 1997). The protein also has direct effects on brush border transport mechanisms (Halaihel et al. 2000). It is proposed that at least one form of soluble NSp4 is released from infected cells and binds to neighbouring cells (Zhang et al. 2000).

5.3 Hepatitis

Hepatitis A and E viruses enter via the gut and may replicate there, however both move rapidly to the liver and invade the hepatocytes. Clinical features of both viruses are similar although HEV is more severe and may have a fatality rate of 20–40% in late pregnancy. HEV has a longer incubation period than HAV (60 d vs 48 d) and a more prolonged viraemia (Clayson et al. 1995b; Scharschmidt 1995; Reid and Dienstag 1997). The long incubations make identification of the source of infection problematic as contaminated food will usually have been eaten or disposed of before illness arises. Haemoglobin breakdown by the liver is impaired and a bilirubin (normally shed in the bile and thence in the faeces) overflows into the blood. The skin and whites of the eye turn yellow (jaundice) and faeces become pale. Bilirubin is filtered from the blood by the kidneys, and urine becomes dark. Virus particles are shed into the bile and thence in the faeces, but in contrast to the cell destruction caused by gastroenteritis viruses there is little virus-induced liver cell damage. HAV interferes only weakly with host cell activities and new viruses are released inside membrane-bound packets without necessity to lyse the cell. An immune response develops 2–3 weeks after infection and leads to immune attack on infected liver cells. It is this host response rather than the virus itself that causes the signs of liver damage. Eventually the immune response eliminates all infected cells (and thus the virus) from the body. Convalescence may be prolonged (8–10 weeks) and some 15% of HAV cases may follow a relapsing course over 12 months or more.

Hepatitis viruses A and E have been affected by human activities. In former times, infection occurred early in life, often whilst still protected by maternal antibody. Such endemic infections tended to be mild or subclinical. Both viruses are rare where sanitation is improved. This has reduced exposure and so increased the age at which first infection occurs; in Hong Kong, 30% of those under 30 were seropositive in 1979; by 1989 this had fallen to only 9%, although seropositivity in the elderly remained high. In contrast, in France where sanitation has been good for many years, 80% of persons over 30 have no antibody to HAV (i.e. have never been infected).

This shift in age at infection increases the severity of infection: below 3 years, HAV infection is virtually always subclinical; but symptomatic infections predominate by 5 years and severity worsens with age (Hadler et al. 1980). Persons over 50 years of age account for only 12% of the cases of HAV but have a case-fatality rate sixfold higher than average (CDC 1994; Fiore 2004). This delay in infection allows a pool of susceptible individuals to accumulate in the community and establishing conditions for epidemic spread. Analysis of annual incidence figures in the US reveals evidence of epidemic behaviour (Fiore 2004). The Centers for Disease Control, USA estimates 267 000 cases occurred on average per year between 1987 and 2001, most were mild or symptom free but 10–30 000 acute cases were registered annually. Mead et al. (1999) estimate that 5% of cases are food-borne. The situation in the UK has been summarized (Crowcroft et al. 2001). The existence of susceptible adults in some parts of the world is significant in the context of food-borne infection since trade that could bring virus-contaminated food grown/produced in areas of high endemicity to areas of low prevalence could pose a threat for adults in those areas (see below).

Hepatitis E virus is not significant in the UK or US; most infections are limited to returning travellers. Epidemics of HEV are known, often spread by contaminated water; the worst cases involved 30 000 people in New Delhi (1955); 100 000 in Xinjiang Uighar, China (1986), and 79 000 in Kanpur India (1991) (Grabow et al. 1994; Scharschmidt 1995). More limited shellfish-associated outbreaks occur sporadically around the Mediterranean. HEV replicates in pigs (Balayan et al. 1990) and has been found in both wild and domestic cows, goats and pigs (Clayson et al. 1995a). Replication also occurs in laboratory rats (Maneerat et al. 1996; Meng et al. 1996). These findings mean that animals might act as reservoirs for infection (Kabrane-Lazizi et al. 1999; Wu et al. 2000). Seropositivity has been estimated at 2–10% even in areas free from human disease; 15% of homeless persons in Los Angeles revealed antibody to HEV, possibly through contact with infected urban rats (Smith et al. 2002). Similarly
HEV has been detected in sewage from areas in which clinical disease is absent (Pina et al. 1998b).

6. VIRUS STABILITY

The stability of an enteric virus is of fundamental significance to its transmission. First, it may limit the period for which virus contamination remains a threat in the environment whether in sea or fresh water or dried onto a surface, and secondly, it governs the efficiency of attempts to deliberately destroy a virus, e.g. in food or water processing (mainly sensitivity to temperature and chlorination). Many enteric viruses are nonenveloped and contain RNA as their genetic material (exceptions AdV and parovirus that contain DNA). RNA is labile, hydrolysed at both acid and alkaline pH and destroyed by radiation or enzymic processes. DNA is more stable but is still sensitive to UV irradiation. This emphasizes the crucial role of the virus coat proteins that must protect the nucleic acid. The relative stabilities of some of the enteric viruses are assembled from a variety of sources and presented in Table 2. There is no central data set for all viruses and their frequently used surrogates; most studies use only a few viruses under restricted conditions and have not considered the effects of combined treatments. The environment in which inactivation proceeds will certainly affect the values determined and this has central significance when considering survival in different foodstuffs (e.g. inside shellfish or in milk) or in the environment (marine vs fresh water etc.). As each study has used slightly different conditions, this table should be regarded as only indicative.

Although all these viruses are destroyed by boiling, the thermal stability of some is remarkable. Temperatures above 90°C are required to inactivate HAV (Millard et al. 1987) and others suggest 100°C may be necessary (Croci et al. 1999). Inactivation is often biphasic and a residue of resistant virus may survive. This probably represents virus in a protected microenvironment, e.g. aggregated or combined with materials exerting a protective/stabilizing effect (Tierney and Larkin 1978; Larkin and Fassolitis 1979; Bidawid et al. 2000a).

Virus persistence in dried matter depends on the surface onto which it is dried, the presence of extra (faecal) material and the temperature/humidity of storage. Many enteric viruses survive for long periods on common surface types requiring up to 60 d for a 2 log reduction in titre (Abad et al. 1994a). In general reducing the temperature and adding faecal contamination promotes virus survival but viruses may respond differently to relative humidity; HAV and rotavirus are stabilized at low relative humidity whilst enteroviruses are stabilized at higher values (Mbithi et al. 1991). This probably depends on the type of surface onto which the viruses are dried (Abad et al. 1994a).

Values for inactivation times/persistence in water vary widely with reported T90 values (i.e. time for 1 log titre reduction or 1 TLR) for enteroviruses of 14–288 h. A 4 log reduction times in sea water are likewise variable but are probably measured in weeks (Chung and Sobsey 1993; Callahan et al. 1995). Persistence in artificially contaminated water has been demonstrated for >1 year (rotavirus and poliovirus) (Biziagos et al. 1988) and 300 d for AdV 41 (Enriquez et al. 1995). These variations probably reflect differences in the conditions used particularly, type of water, illumination, turbidity and pH, and emphasize the need for standardized studies of all relevant viruses (and common surrogates) under directly comparable conditions. Despite this variation all these viruses are probably capable of survival for weeks/months at environmental temperatures and at low temperatures, sheltered from UV irradiation some may persist for years.

7. WATERBORNE VIRUS INFECTION

All these viruses enter the sewerage system and may survive wastewater treatment to contaminate receiving waters. Here they could pose a threat to recreational users or to consumers of shellfish or other produce eventually destined for the food chain. In addition, where such waters are later abstracted and treated for use as drinking water, contaminating viruses might survive this treatment too. Drinking water can also be contaminated after treatment if supplies are not adequately separated from untreated sources.

7.1 Wastewater treatment and virus survival

Enteric viruses are usually shed in large numbers, rotavirus, titres can exceed 10⁹ particles per ml and could comprise up to 2 mg in every gram of stool. A value between 10⁹–10¹⁰ per ml is common for astrovirus and AdV. Caliciviruses, entero- and hepatitis viruses are often shed in lower (but still appreciable) numbers; cultivable enteric viruses are ubiquitous in human populations and levels in sewage can exceed 10⁸ PFU per litre. Water treatments lacking a tertiary step (e.g. UV treatment) reduce this load only poorly. Sewage sludge production reduces numbers by 95% but many viruses survive giving levels in receiving waters of up to 100 PFU per litre if contamination is serious and 1–10 PFU per 100 l where it is less so (Gerba et al. 1985; Bloch et al. 1990; Jothikumar et al. 2000; Scipioni et al. 2000; Pina et al. 2001). Most enteric viruses have been detected in wastewater, treated water and receiving waters over time, usually by PCR techniques even if viruses are cultivable, e.g. HAV (Dubrou et al. 1991; Goswami et al. 1993; Graff et al. 1993; Tsai et al. 1993; Jaykus et al. 1996; Schwab et al. 1996) and astrovirus (Le Cann et al. 2004). AdV have consistently been detected in raw sewage and approx. 80% may be enteric.
Table 2  Virus stability values; assembled from references below. No study has included all these viruses under identical conditions and thus data are not always comparable; values presented here should be taken as indicative of stability only. Log titre reduction (LTR), measured by infectivity unless otherwise stated. Bacteriophage MS2, a common surrogate for enteric viruses is included for comparison.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Persistence/inactivation conditions</th>
<th>pH stability</th>
<th>Thermal inactivation</th>
<th>Free chlorine (unless stated)</th>
<th>UV irradiation (mJ cm(^{-2}))</th>
<th>Persistence (dry)</th>
<th>Persistence food matrices</th>
<th>Persistence sea water</th>
<th>Persistence fresh water</th>
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<tbody>
<tr>
<td>Adenovirus</td>
<td>Stable</td>
<td>pH 6–9*</td>
<td>Inactivated</td>
<td>56 deg, 10 min* (6)</td>
<td>2 LTR at 0.5 mg ml(^{-1}) for 10 min (20)</td>
<td>4 LTR at dose 216 (23)</td>
<td>2.5 LTR on drying, further 3 LTR 10 d (19)</td>
<td>Not significantly food-borne</td>
<td>1 LTR in 40 d 15 deg (27)</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>Resists</td>
<td>pH 3 (4)</td>
<td>Resists</td>
<td>50 deg 1 h, 60 deg 5 min (4)</td>
<td>2 LTR at 1 mg l(^{-1}) for 10 min, 20 LTR at 0.5 mg l(^{-1}) for 20 min (18)</td>
<td>No data</td>
<td>1 LTR on drying then stable for 60 d at 4 deg (19)</td>
<td>No data</td>
<td>Reportedly lower stability than fresh water Drinking water: 2 LTR at 4 deg, 3.2 LTR at 20 deg both 60 d</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Resists</td>
<td>pH 10 for 2 h (9)</td>
<td>Resists 60 deg for 10 min, cations stabilize to 81 deg</td>
<td>Inactivated</td>
<td>98–100 deg (7)</td>
<td>4 LTR at dose 16–39 (23); 1 LTR at dose 36.5 (30); 1 LTR in 1:3 min at 42 mW cm(^{-2}) in sea water (25)</td>
<td>Survives &gt;1 month at 25 deg in 42% humidity (10, 11); 3 LTR in 8 d and then stable to 32 d (16)</td>
<td>Resists 60 deg for 19 min in oysters (12)</td>
<td>2 LTR in 28 d at 25 deg (11) Estuarine water: 2 LTR in 28 d at 25 deg (11) PBS: 1 LTR in 56 d at 25 deg</td>
</tr>
<tr>
<td>Norovirus</td>
<td>Resists</td>
<td>pH 27 3 h (14)</td>
<td>Resist 60 deg for 30 min (14)</td>
<td>Resists exposure to 6.25 mg l(^{-1}); 30 min (CT 187.5) inactivated at 10 mg l(^{-1}) (CT 300) (15); others suggest CTs 30–60 required (see text)</td>
<td>1 LTR (PCR) at 2 mg l(^{-1}) chloramine for 3 h (25)</td>
<td>No data – FCV surrogate T90 value 47.85 (30)</td>
<td>NoV Recoverable from disinfected hospital surfaces; FCV 2 LTR in 15 d at 4 deg; or in 30 d at 20 deg, inactivated in 1 d at 37 deg (31)</td>
<td>Infectivity in shellfish not reduced after 1 month storage at 4 deg or over 4 months frozen. Stable in ice</td>
<td>1 LTR in 24 h at 10 deg (illuminated) FCV suspension – 1 LTR per day at 37 deg, 4 LTR in 60 d at 4 deg (31)</td>
</tr>
<tr>
<td>Virus</td>
<td>Persistence/inactivation conditions</td>
<td>pH stability</td>
<td>Thermal inactivation</td>
<td>Free chlorine (unless stated)</td>
<td>UV irradiation (mJ cm(^{-2}))</td>
<td>Persistence (dry)</td>
<td>Persistence food matrices</td>
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<tr>
<td>Picornavirus/enterovirus</td>
<td>Resists pH 3-0 (7)</td>
<td></td>
<td>Destroyed 42 deg, cations stabilize to 50 deg (8)</td>
<td>2 LTR at free chlorine 1-1-2.5 mg l(^{-1}) (28)</td>
<td>1 LTR in 1:3 min at intensity 42 mWs cm(^{-2}) in sea water (25)</td>
<td>2 LTR on drying, further 3 LTR in 60 d at 4 deg</td>
<td>&gt;3 LTR in water or milk at 63 deg, 30 min</td>
<td>1 LTR in 96 h at 12 deg; 1 LTR in 22 h at 22 deg (22); 1 LTR &gt;670 d at 4 deg (26)</td>
<td>1 LTR 24-31 h in river water in situ 12-20 deg (21)</td>
</tr>
<tr>
<td>Picornavirus/kobuvirus</td>
<td>Resists pH 3-5 (7)</td>
<td></td>
<td>Resists 50 deg (3)</td>
<td>CT values for 2 log inactivation 0.01-0.05 (17)</td>
<td>4 LTR at 56 (23)</td>
<td>1 LTR on drying; 1 further LTR in 30 d 4 deg (19)</td>
<td>Bovine Rotavirus – decay 0.5 LTR per day natural sea water (29)</td>
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<tr>
<td>Rotavirus group A</td>
<td>Stable pH 3-9 (5)</td>
<td></td>
<td>1 LTR by monochloramine 2 mg l(^{-1}), 3 h (25)</td>
<td>&gt;3 LTR by 10 min in 2 ppm CLO2 (16)</td>
<td>4 LTR at dose 750 253 nm (24)</td>
<td>App 3 LTR in 4 d (16)</td>
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<tr>
<td>MS2</td>
<td>Tolerates low pH</td>
<td></td>
<td>&gt;1 LTR in 15 s at 72 deg in water or milk (16)</td>
<td>1 LTR by monochloramine 2 mg l(^{-1}), 3 h (25)</td>
<td>1 LTR at dose 230 4 (30)</td>
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*Nonenteric virus strains.
forms. These appear to survive sewage treatment well and are frequently detected even in waters that lack enterovirus contamination. This has led many to suggest that AdV may be appropriate sentinels for the indication of faecal pollution in water (Enriquez et al. 1995; Pina et al. 1998a; Wyn-Jones and Sellwood 2001).

Noncultivable viruses have also been detected. NoV have been found in sewage influent and effluent waters using PCR techniques (Lodder et al. 1999), serial dilution of influent waters suggested that levels may exceed $10^7$ detectable virus units although this cannot be readily correlated with particle numbers or infectivity (Wyn-Jones et al. 2000). Estimates using quantitative PCR obtained similar values (Laverick et al. 2004). A study of treated water suggested that NoV levels were virtually undiminished in primary treatment and were reduced by only a little over 1 log in the final (secondary) effluent (Cross 2004). Similarly astroviruses have been detected in sewage treatment plant inlet and effluent waters, indicating a reduction of approx. 2 log during processing; effluent waters still contained $10^5$ detectable astrovirus genomes per litre (Le Cann et al. 2004). HEV was found in sewage by molecular means (Pina et al. 1998b), and survives at least some wastewater treatments although its removal has not been quantitiated (Jothikumar et al. 1993).

Viruses surviving water treatment and entering receiving waters could persist and pose a risk for recreational users of this water and studies have inferred potential exposure to both NoV (Gray et al. 1997) and HAstV (Myint et al. 1994) from this source. Studies have indicated that the risks of disease resulting from exposure to recreational water may be as high as 1/1000 for AdV (Crabtree et al. 1997) and perhaps higher for rotavirus (Gerba et al. 1996).

The mechanisms of virus inactivation/removal during wastewater treatment are not clearly understood and the environment itself probably alters the effectiveness of each process. Physical removal seems to be significant and much virus (perhaps 95%) appears to be removed via activated sludge or complexing with mineral particles. Active enterovirus has been recovered from sludge (Albert and Schwa-rzbod 1991) and HAV was detected by both antigen capture and PCR suggesting that particles were intact (Graff et al. 1993). EU requirements for heat-treatment before sludge is spread onto land should control virus contamination from this source if followed [EU directive on sewage sludge in agriculture (86/278/EEC) implemented under the sludge (use in agriculture) regulations 1989] but there may be fewer controls in the less developed world and these might contaminate crops destined for export.

### 7.2 Potable water treatment and virus survival

Tap water may account for some 14–40% of gastrointestinal illness and thus efforts to ensure its safety are vital (Payment et al. 1988; Payment et al. 1997). The assurance of drinking water quality begins with the source water, which should be obtained from sources as far removed from potential contamination as possible. In the US subsequent treatment aims to reduce levels of contaminating virus by 99-99%. Filtration can achieve an initial 10-fold reduction with a further 1000-fold achieved by active disinfection (e.g. chlorine, chlorine dioxide, ozone or UV irradiation). Of these, chlorination is the most widespread; peak levels are usually around 1 mg l$^{-1}$ for 60–240 min (Grabow 1990; Thurston-Enriquez et al. 2003) and water entering the US distribution system should have a residual level of 0.2 mg l$^{-1}$ to comply with requirements to control coliform bacteria. Exposure to disinfectant is expressed by the contact time (CT) parameter, concentration of agent (mg l$^{-1}$) multiplied by time (min). CT values for a 4 log inactivation of enteric viruses fall in the range of 4–400 mg·min l$^{-1}$ at a contact concentration of 0.4 mg l$^{-1}$ at 5°C. However the rates of virus destruction and thus CT values required are temperature dependent, doubling for every 10°C rise and potentially defective around 0°C. Increasing the pH from 6 to 9 reduces the efficiency of free chlorine threefold, ozone or chlorine dioxide are unaffected. Turbidity has the greatest effect, shielding viruses from UV radiation, promoting aggregation and also ‘mopping up’ viricide. Increasing the turbidity from 1 to 10 Nephelometric Turbidity Units decreases free chlorine effectiveness eight times (LeChavallier et al. 1981; Hoff 1986; HECS 2003). Thurston-Enriquez et al. (2003) re-evaluated the efficiency of chlorine and found viruses to be 30 times more resistant when aggregated. If this is so then procedures commonly in use in the US should destroy most but possibly not all aggregated viruses (Thurston-Enriquez et al. 2003).

Norovirus was found relatively insensitive to chlorine; in volunteer studies the virus survived exposure to 6.25 mg l$^{-1}$ for 30 min (CT 187:5) and required 10 mg l$^{-1}$ for destruction (CT 300 mg·min l$^{-1}$) (Keswick et al. 1985). These values imply that NoV could survive some water chlorination procedures. However, these experiments are difficult to do and rely on clinical samples as NoV cannot be cultivated. Keswick et al. (1985) used a crude sample of very high titre that may well have protected the virus. A protective effect due to such factors was later noted (Meschke and Sobsey 2002). When purified, NoV has the same sensitivity to chlorine as poliovirus (Shin and Sobsey 1998; Meschke and Sobsey 2002). A PCR-based study found a CT value of 30–60 to be sufficient to inactivate the virus (Shin and Sobsey 1998). However it is difficult to determine which of these findings is the more relevant as viruses are not found ‘pure’ in the environment, or even in the same form in all locations; exogenous material and aggregates are always likely to be present. Rotavirus is inactivated efficiently by chlorine (CT 112:5 at 3.75 mg l$^{-1}$) and for
this virus waterborne infections in the developed world are usually linked to contamination of ‘clean’ water post-treatment (e.g. Hopkins et al. 1984).

Enteric viruses have been found in drinking water (1–20 PFU per 1000 l) (Payment 1988; Gerba and Rose 1990; Payment et al. 1997). Studies in France showed that the presence of astrovirus RNA in tap water was correlated with an increased risk of intestinal disease (Gofti-Laroche et al. 1990; Payment 1988; Gerba and Rose 1990; Payment et al. 1997). However it is not at all clear what an acceptable level of virus contamination might be; a risk of infection of $1 \times 10^{-4}$ per person per year has been accepted in both the US and the Netherlands (USA EPA 1991; Staatscourant 2001). Using a model based on rotavirus dose–response data, Regli et al. (1991) calculated that $2.22 \times 10^{-7}$ viruses l$^{-1}$ would be consistent with this risk factor. A 4 log reduction achieved from even highly rotavirus-contaminated source water (Raphael et al. 1985) would meet this level (Gerba et al. 1996). Estimates based on enterovirus levels suggest that higher reductions might be required (Regli et al. 1991). Such assessments are variable, poor culture efficiency would underestimate the levels present and increase risk to the consumer; uneven distribution of contamination would cause an uneven risk across the country and differences in levels of consumption (and the proportion that is boiled before drinking) also affect risk. Finally models based on infection may be less relevant than models based on symptomatic illness and could thus exaggerate the risk (Gerba et al. 1985; Gale 1996; Payment et al. 1997; Haas et al. 1999; Hurst et al. 2001).

Most documented instances of drinking water contamination by viruses have been attributed to contamination of previously ‘clean’ water. This can result simply from contamination of drinking water supplies, e.g. through heavy rainfall or flood overwhelming treatment works and permitting untreated or partially treated sewage to contaminate wells (Cannon et al. 1991; Kukkula et al. 1997, 1999). Failures in treatment processes themselves can also allow contamination, e.g. pressure failure (Kaplan et al. 1982), insufficient disinfection, or exceptionally high levels of virus overwhelming a correctly applied procedure (Payment 1988; Gerba and Rose 1990; Payment et al. 1997; Bitton 1999; Gofti-Laroche et al. 2003). Virus leakage from sewers or septic tanks can contaminate clean water (Hedberg and Osterholm 1993). Waterborne outbreaks of enteric virus infection are common; between 1980 and 1994 the US recognized 28 outbreaks and 11 195 cases of waterborne illness, of these 9000 cases were NoV and nearly 400 HAV (HECS 2003). None of these viruses are appreciably affected by freezing (which may actually preserve infectivity for long periods) and thus infection is also spread when ice is manufactured and distributed using contaminated water (Cannon et al. 1991).

### 8. Food-borne virus transmission

Food-borne transmission may be divided into two areas: in primary contamination food materials are already contaminated before they are harvested, e.g. shellfish grown in contaminated waters, or soft fruits irrigated/sprayed with contaminated water. Secondary contamination occurs at harvest or during processing and emphasizes the role of the food handler preparing foods for others with whom he/she does not come into direct contact. Food handlers in this context include field harvesters, production plant workers right through to professional chefs and caterers. Contamination from these persons involves not only transfer of virus from infected persons but also their use of polluted water or materials in processing. It is worth noting that transfer of virus from an infected individual to food would be counted as a food-borne infection if it takes place in the commercial/industrial setting, but as person-to-person infection if it occurs in the home.

It is difficult to assess the extent of food-borne transmission in the community, first because person-to-person and food-borne routes may overlap (above), but secondly because it is technically difficult to detect viruses in foods, apart from shellfish most are not routinely tested. This has led to two assumptions, first most food-borne outbreaks in which a vehicle is not demonstrated are likely to have arisen by contamination from food-handlers – at or close to the point of serve – and the second is that most (if not all) cases occurring in the community (i.e. outside outbreak situations) probably arise from person-to-person transmission. These assumptions should be reconsidered because both disguise or negate potential instances of primary or distant secondary contamination.

Estimates of the incidence of food-borne disease have relied on statistics obtained from outbreaks. In recent years, reports of viral gastroenteritis, particularly NoV outbreaks have shown a dramatic increase in England and Wales; 418 cases were identified in 1992 and 2387 in 1996. In 1994 UK reports of NoV outbreaks outnumbered those of Salmonella for the first time. This increase may be partially explained by increased awareness and improvements in virus identification techniques, thus more recently attempts have been made to estimate the extent of community illness. Mead et al. (1999) estimated that 67% of food-borne illness in the US was caused by viruses and that 40% of NoV infections; 1% astrovirus, 1% rotavirus and 5% of HAV infections, respectively, were food-borne. NoVs were responsible for 23 million cases per year with over 9 million food-borne events. NoV then emerged as the most significant food-borne infection in the US. Annually NoV was estimated to be responsible for 20 000 hospitalizations and 120 deaths.

8.1 Shellfish

Enterically transmitted viruses are shed into sewage and become rapidly diluted as they travel through the water system. Filter-feeding mollusc shellfish (particularly cockles, mussels and oysters) partially reverse this dilution because they are filter-feeders. They are harvested from close-to-shore locations where water will contain virus inputs from sewage effluents; viruses become concentrated within the shellfish and may be retained for some time. Levels of virus in shellfish may be 100–1000-fold higher than concentrations of viruses in their surrounding water.

European regulation divides shellfish harvesting waters into different categories depending on the levels of faecal pollution (Table 3). Very badly contaminated waters may not be used to harvest shellfish for human consumption at all, whilst intermediate levels may be used provided that the shellfish are relayed into cleaner waters, heat-treated or depurated before harvest. Depuration is a process in which the animals are kept in a recirculation tank; water is passed over the shellfish, disinfected by UV treatment and recycled. The shellfish gradually purge their bodies of faecal indicator bacteria and may be sold when these reach a target level. It is now clear that bacteria are not an adequate indicator of the presence of enteric viruses, and numerous virus infections have been documented from shellfish compliant with these regulations (Chung et al. 1996; Griffin et al. 1999; reviewed by Lees 2000). Bacteria are purged more rapidly than viruses. Schwab et al. (1998) found that a 95% reduction in Escherichia coli was achieved by 48 h depuration but NoV was reduced by only 7% in the same period. The efficiency of virus removal is influenced by a number of factors chiefly temperature (Dore et al. 2000). Most gastroenteritis viruses are more common in winter (even if not shellfish transmitted) and increased community illness coupled with less efficient purging by the shellfish could increase the risks associated with shellfish consumption at these times. Viruses retain infectivity well in shellfish and no loss of infectivity was observed over 1 month in refrigerated storage (Tierney et al. 1982) or 4 months when frozen (Di Girolamo et al. 1970).

Trade in shellfish permits long-distance transmission: in 2002 oysters from Cork Bay spread NoV infection around the world to Hong Kong (ProMED-mail 2002a; 20020331.3850), and in 2004 Chinese frozen oysters were implicated in a (presumed NoV) outbreak in Singapore (ProMED-mail 2004; 20040107.0075). In 1993 a multistate outbreak of NoV illness occurred in the US affecting up to 186 000 people (Berg et al. 2000). This was traced to contaminated oysters harvested in the Gulf of Mexico. Oysters in Europe accumulate a mixture of all viruses present in their environment, but in these Gulf oysters only one strain was present. This indicated that input virus must have originated from a restricted number of infected persons and was possibly attributable to incorrect disposal of faeces or vomitus at sea. This remarkable occurrence indicates firstly just how efficiently the shellfish can accumulate virus from their environment and secondly just how significant commercial trade and distribution can be in the dissemination of infection. It further demonstrates the power of molecular epidemiology in linking occurrences that would otherwise not necessarily have been connected. Sporadic outbreaks occur throughout the developed world via consumption of virus-contaminated shellfish (reviewed by Lees 2000).

Many shellfish are subject to minimal cooking (if any), linking this to their known ability to concentrate environmental viruses the fact that they are the most commonly identified source of food-borne virus infection should not be surprising. The most significant viruses in this context are NoV first reported as an Australia-wide outbreak (Murphy et al. 1979), and hepatitis viruses. Recently Aichivirus has been associated with infection from this source in Japan but reported cases are few. However the viruses most commonly detected in shellfish are enteroviruses (reviewed by Gerba and Goyal 1978). These seldom give rise to symptomatic disease although some cases have been reported (Cliver 1997).

Virus concentration by shellfish is nonspecific and thus their consumption can expose the consumer to a cocktail of viruses that might have different effects when present together. Limitations on current technology mean that during investigations it is usual to seek only a few defined virus types (those that succeed in inducing clinical disease). However the possibility of multiple simultaneous infection brought about by the consumption of shellfish should not be overlooked, viruses might act synergistically to increase the

<table>
<thead>
<tr>
<th>Class</th>
<th>Standard</th>
<th>Requirements for marketing for consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class A</td>
<td>&lt;230 E. coli or 300 faecal coliforms</td>
<td>May be consumed directly</td>
</tr>
<tr>
<td>Class B</td>
<td>90% compliance &lt;4600 E. coli and 600 faecal coliforms</td>
<td>Depuration or relaying until class A standard is met, or apply heat-treatment</td>
</tr>
<tr>
<td>Class C</td>
<td>&lt;60 000 faecal coliforms</td>
<td>Relay for 2 months to meet class A or heat-treat when class B standards met. Or heat-treat</td>
</tr>
<tr>
<td>Prohibited</td>
<td>&gt;60 000 faecal coliforms</td>
<td>Prohibited</td>
</tr>
</tbody>
</table>

severity of symptoms or to present confusing clinical pictures.

Shellfish transmission of HAV is well documented and includes the largest ever outbreak that of Shanghai (1988); 300,000 persons were believed to have been infected through consumption of contaminated clams (Halliday et al. 1991). Studies around the world have consistently identified shellfish consumption as a major risk factor for the contraction of hepatitis. In some studies the risk was equivalent to that of contact with an infected person (Koff et al. 1967; Kiyosawa et al. 1987). HAV is rarely detected in food itself, largely because of the long incubation period for this illness which means that no samples of suspect food are likely to be available for testing. Even so shellfish are believed to be the major vehicle for HAV infection and Salamina and D’Argenio (1998) estimated that up to 70% of all hepatitis A in Italy was contracted from shellfish. In the UK it is recommended that shellfish flesh be raised to 90°C for 1.5 min (Millard et al. 1987) and continuous flow methods now ensure that all shellfish are subjected equally to this treatment. Since the implementation of these recommendations, there have been no reports of outbreaks in the UK of either viral gastroenteritis or hepatitis A associated with shellfish heat-treated in this way (Appleton et al. 2005). This was unusual in that it affected older persons and was attributed to a higher serotype virus. Astroviruses are frequently detected when sought in oysters and the apparent lack of resultant illness is presumably attributable to prior immunity remaining from childhood infection. Similar considerations apply to rotavirus contamination.

8.2 Soft fruit and salad vegetables

After shellfish, food-borne virus illness is most commonly linked to salads and soft fruits. These items are almost always subject to handling immediately before serving and this presents an opportunity for contamination. In virtually all cases where this type of food is implicated an infected food handler is suggested as the origin but in only a small proportion of such cases is this link actually proved. Soft fruits and salad vegetables, like shellfish are eaten raw but also share other features; first, they all have a high water content – absorbed from groundwater during growth; secondly, many are eaten without peeling which would remove external contamination. Consequently, primary contamination is possible externally by spraying or internally by uptake of viruses from contaminated irrigation water or fertilizer. Surface splash may be significant for fruits close to the ground (e.g. strawberries) and multi-state outbreaks of HAV may have arisen in this way (Niu et al. 1992; Hutin et al. 1999). Wastewater may be used for irrigation, especially in dry areas where water is more precious, and these communities show elevated incidence of hepatitis virus infection although reasons have not been identified (Katznelson et al. 1976).

Viruses can enter plants through root damage (Katzenelson and Mills 1984), and this occurs universally through root abrasion with soil particles. Other workers found virus uptake from roots to the leaves (but not the fruits) of tomato plants even when virus was introduced below the soil surface eliminating the possibilities of aerial contamination (Oron et al. 1995). Our own data also show that internal contamination can occur but is probably of low level compared with external contamination (M.J. Carter, unpublished observations). However, a lower amount of virus inside a crop plant could potentially have an effect equivalent to a much higher external dose because it cannot be removed by either peeling or washing and may persist for longer because it is shielded from UV inactivation. Washing is not always efficient as a means of removing contamination, for instance HAV was found to adhere well to produce, especially lettuce (Croci et al. 2002). It is difficult to detect viruses in plant material and in only a few cases has contamination been shown directly, e.g. NoV in raspberries (Gaulin et al. 1999). Most associations are made by epidemiological connection and identification of the infectious agent in clinical samples from those affected (reviewed by Seymour and Appleton 2001).

Fruit and salad vegetables are traded around the world, originating from areas where sunlight permits growth but where water quality is not always assured. This trade either as raw materials or as ready-to-eat products can aid widespread virus dissemination. In 2003 an outbreak of hepatitis A occurred in the US, this affected several hundred persons in four states and resulted in four deaths. The source was eventually traced green onions imported from Mexico although virus was not identified in the food; identification was possible only by analysis of the chain of importation and transport (ProMED-mail 2003; 20031128.2946). In 1997 contaminated strawberries were used in a school lunch preparation that was distributed to 17 states in the US and may have exposed up to 9000 children (ProMED-mail 1997; 19970402.0689). A total of 200 cases
of HAV were reported in Michigan. Similarly, pre-prepared salads distributed across the US were responsible for a multi-state outbreak of NoV affecting over 300 persons (ProMED-mail 2000; 20000318.0377). Imported raspberries were responsible for an outbreak in Canada (Gaulin et al. 1999). Viruses are not destroyed by freezing or freeze-drying, although there are few studies of the latter process in contaminated food, in the laboratory freeze-drying is an efficient means of preserving virus infectivity and should be expected to preserve viruses in produce. Both hepatitis A (Reid and Robinson 1987) and NoV (Ponka et al. 1999) have been transmitted by frozen fruit. The growth in the worldwide trade in freeze-dried soft fruits (particularly raspberries and strawberries) offers another potential vehicle for transmission.

There are no data concerning levels or persistence of potential internal virus contamination of fruits and vegetables, although Sadovski et al. (1978) recovered poliovirus from cucumbers that had been irrigated with contaminated water 8 d earlier an upper limit to virus persistence was not established. In areas of high UV illumination surface contamination may be short-lived in the field, Badawy established. In areas of high UV illumination surface water 8 d earlier an upper limit to virus persistence was not achieved in just 8–10 h in the summer, slightly longer in the winter (16–24 h). However viruses can survive well on the surface of produce once harvested, a number of studies have indicated survival times of longer than a week and sometimes up to 30 d for a range of enteric viruses under typical storage conditions (Konowalchuk and Speirs 1974, 1975a,b; Badawy et al. 1985; Pirtle and Beran 1991; Kurzdziel et al. 2001; Croci et al. 2002). Increasing temperature decreased virus survival times but conditions usually associated with produce storage were conducive to virus survival. It is not clear what effect microenvironments on a plant surface might have, waxy cuticles, hairy projections and curled, crinkled or convoluted leaves can all modify the way that virus contamination might behave, the amount of wetting will affect the way that a droplet dries whilst convoluted leaf surfaces could shield a virus from UV or increase local humidity. These effects are difficult to replicate experimentally. Data from Kurzdziel et al. (2001) and Ward and Irving (1987) show that there is variability in the times that poliovirus may survive on different artificially contaminated plants stored at 4°C. Similarly these potentially sheltered environments suggest that washing processes will likewise vary in efficiency even over sections of the same plant.

These data suggest that contaminated fruit and vegetables might account for a large number of food-borne infections. A study in the UK indicated that these might present a significant opportunity for virus infections (O’Brien et al. 2000). However, the criticism frequently levelled at this implication is that these infections are simply not observed. To some extent this may arise from the assumption that community illness is entirely or mainly spread by the direct person–person route even though supporting data for this assumption is weak. When investigations have attempted to distinguish between person-to-person and food-borne illnesses in the community the latter can be identified by statistical means and suggest that there are potentially many thousands of such instances per year (see below).

8.3 Other foods

Few other foods are associated with primary contamination but a recent example is the consumption of raw liver from wild boar or venison. Both have resulted in the transmission of HEV to humans (Matsuda et al. 2003; Tei et al. 2003; Tamada et al. 2004). Consumption of raw liver is rare but virus contamination of utensils used in handling it should be borne in mind. Supermarket pork liver has also tested positive for HEV (Yazaki et al. 2003) – even in countries with no significant HEV infection. This suggests that pork liver is not a significant source of infection for humans.

8.4 Food handlers

Food handlers may contaminate foods at any point from harvest to serving. Toilet arrangements (including hand washing) should be provided for harvesters in the field to prevent contamination of produce as it is picked. Infected food handlers may or may not be symptomatic at the time of contamination. HAV infections may be mild or asymptomatic but large numbers of particles are nonetheless shed in the faeces. PCR technology has shown that even in asymptomatic illness, NoV shedding may precede symptoms by 10–30 h and may continue (albeit it at a much lower level) for 1–2 weeks afterwards (Graham et al. 1994; Okhuysen et al. 1995; Estes and Leparc-Goffart 1999). NoV transmission by pre-, post- or asymptomatic food handlers has been demonstrated (Patterson et al. 1993; Parashar et al. 1998; Gaulin et al. 1999). Cowden et al. (1995) recommend a 48-h period of exclusion following the cessation of symptoms but as virus shedding is likely to continue for some weeks returnees must follow strict hand washing procedures. Inadequate hand washing/personal hygiene has been implicated in outbreaks of both NoV and HAV (Bean et al. 1990; Bidawid et al. 2004) and recently its effectiveness has been demonstrated. Bidawid et al. (2000b) considered the likely levels of hand contamination by HAV, and then estimated the resultant transfer of viruses to foods by handling. They concluded that up to 10% (or approx. 1000 particles) could easily be transmitted via the fingers to food items (lettuce). If proper washing procedures were followed this transfer fell to 0.3–0.6%. Other experiments have shown that virus transfer rate is high immediately after
hand contamination but reduces as the virus dries (Larson 1985; Ansari et al. 1988; Springthorpe and Sattar 1998). Whilst this might imply that the window for contamination closes rapidly, it should be borne in mind that virus does survive drying onto the skin and reapplication of moisture (as in handling washed and wet foods) could then lead to increased transfers once more. Findings such as these emphasize the importance of proper hygiene in controlling food-borne infection from this source. However, given the likelihood that this will not be achieved in all cases, Koopmans and Duizer (2004) attempted to estimate the risks associated with food contaminated in this way. Using the value of 1000 particles for transfer (above), they reasoned that a 3 log reduction in virus viability would be required to ensure safety after such a contamination event. They then estimated the effects of various common food-processing procedures that might follow handling (e.g. freezing, fermentation, acidification, pasteurization, etc.). With the exception of boiling (and other high temperature treatments) no procedure achieved a 3 log reduction suggesting that viable virus would remain in almost all cases to present a threat to the consumer. Mariam and Cliver (2000) also showed that HAV would survive common pasteurization procedures for milk (30 min at 63°C and 15 s at 72°C).

The sudden onset of projectile vomiting and associated aerosolization of virus particles is particularly problematic when this occurs in a food handler. As vomiting may be the first overt sign of infection such a situation is virtually impossible to prevent. Given the survival characteristics of these viruses all uncovered food should be destroyed or cooked even if at some distance from the event. Decontamination of catering premises presents similar problems to that of hospitals. A sink into which an infected food handler had vomited was thoroughly cleansed with chlorine bleach. The following week the same sink was used to prepare salad and resulted in an outbreak of NoV amongst the consumers (Patterson et al. 1997). Food handlers certainly can contaminate foods but this is usually proved in only a minority of cases. Attempts were made to assign transmission as person-to-person or food-borne in an analysis of 2149 outbreaks occurring between 1992 and 1995 (ACMSF 1998). About 33% were attributed to NoV, 2% to RV and 0-5% each to astroviruses and small round (parvovirus)-like agents. Although not robust, this analysis suggested that many of the NoV outbreaks were food-borne and a food vehicle was suggested in 35 cases. Virus was detected in only two of the suspect foods (both oysters), in the remaining cases contamination was attributed to a food handler but subsequent investigation confirmed food handler infection in only 20%. Food vehicles implicated were diverse but included a variety of fresh fruits and salads as well as other items that would commonly either contain or be served with a salad or fresh fruit garnish. These included items such as sandwiches, pies, vegetable salads, gateaux, fish, lobsters and prawns. Consequently there was potential for primary (or at least remote secondary) contamination in 80% of these cases.

9. PERSON-TO-PERSON TRANSMISSION

Most enteric viruses are highly contagious, for most the infectious dose is believed to lie between 10 and 100 particles and for NoV possibly lower. Given rotavirus shedding at a level of 10^9 per ml, as little as 0.1 μl of stool contamination could contain an infectious dose. Such tiny quantities are invisible and easily retained on skin. Although hand washing is effective as a disinfectant (particularly alcohol-containing washes) in the case of rotavirus (Estes et al. 1979) infections are easily transmitted from person to person either directly or via contamination of objects and surfaces.

Vomit is also significant; 30 million particles may be shed by this route (Reid et al. 1988; Caul 1994) and RV may survive for up to 9 d in the air (Sattar et al. 1984). Proximity to vomit at the time of vomiting may be more significant than exposure later on; in a hotel NoV outbreak infection was related to distance between the secondary cases and the primary case at the time of the event (Marks et al. 2000) and in a hospital setting, persons in the proximity of a patient who vomited were at greater risk of acquiring NoV than those who actually cleaned up the vomit (Chadwick and McCann 1993). This implies that aerosolization is a potent means of infection of those in the immediate vicinity. Airborne transmission is distinct from respiratory transmission as the route of infection remains via the gut rather than the respiratory tract. Infection could result from the trapping of aerosolized droplets containing virus in the nasal passages and subsequent swallowing of virus. Alternatively aerosolized virus may travel some distance before settling and contaminating surfaces or uncovered food. Spread may be assisted by artificial ventilation (Chadwick et al. 1994).

Person-to-person transmission is clearly favoured by closed settings, persons cannot leave cruise-ships, hospitals or care homes and once a virus has been introduced by whatever route efficient spread within that setting is to be expected. In 2002 the CDC, Atlanta, recorded 21 outbreaks of NoV on 17 ships docking in the US (ProMED-mail 2002b; 20021212-6049); this included one vessel on which more than 200 cases were reported. Once a virus is present it can be very hard to eliminate even by thorough disinfection (Chadwick et al. 2000; Barker et al. 2004). Some ships showed serial outbreaks amongst each new set of passengers coming aboard. This is not surprising as even cleaning in a hospital context may fail – serial astrovirus infections were experienced in a bone marrow transplant unit despite vigorous attempts to disinfect the unit between patients
9.1 Person-to-person or food-borne?

Unsurprisingly, contact with an infected person is a major risk factor for contraction of these viruses: secondary attack rates of up to 95% have been reported for HAV within the home (Villarejos et al. 1982). HEV appears to infect less well in the domestic setting and secondary attack rates are lower (Purcell 1997) even though virus shedding may be more prolonged (Clayson et al. 1995b). Contact with an infected person was found to constitute the greatest single risk for infection by NoV in studies in the UK, US and the Netherlands (Mead et al. 1999; FSA 2000; De Wit et al. 2003). However this does not mean that all community spread must be via the person-to-person route, because unlike other viruses (which may be maintained entirely by this route), enteric viruses can also spread through food or water. It is difficult to estimate the contribution of each route to the total burden of infection in the community. Most figures consider only outbreaks, but the IID survey in the UK revealed the true extent of these 'missed' community infections for the first time (FSA 2000). This survey estimated that for every case of NoV that is laboratory confirmed, over 1500 occur in the community. The report attributes community cases (almost) entirely to person-to-person transmission but presents no data on this point. Recently De Wit et al. (2003) have made a thorough attempt to determine the extent of food-borne transmission in the community. They found that person-to-person transmission was indeed common, much of it occurring in the home and in many cases actually involving contamination of food prepared for others. This is not counted as 'food-borne' infection (see above). The analysis also showed for the first time that person-to-person transmission alone could not account for all community illness and the authors concluded that 12–16% of infections could be attributed to contaminated food/water entering the household. In the UK there are some 9·5 million cases of IID annually and has been estimated that some 16% (1·52 × 10⁶) are caused by NoV. Assuming that all of these took place in a household of four persons and that all persons in each household were infected, this would correspond to a minimum of 380 000 infected households. Lastly, if 12–16% of these resulted from incoming contaminated food as above, then contaminated food could instigate directly some 45–60 000 food-borne cases in the UK per year as a minimum estimate. Estimates in the Netherlands using less conservative assumptions suggest 80 000 food-borne NoV infections could occur annually even in this smaller country (De Wit et al. 2003).

10. VIRUS DIAGNOSIS AND DETECTION

10.1 Clinical samples

An assessment of the impact of these viruses in human disease requires the provision of a sensitive and reliable detection method for application to clinical samples. Routine reagents for the detection of rotavirus, HAV and poliovirus have been available for some time but technology for the other viruses of interest has only recently moved from the specialist laboratory into widespread use. As a result the burden of disease contributed by other gut-infecting viruses has not been so clearly established. Diagnostic reagents have formerly been hard to provide and in their absence perhaps the simplest method has been to examine the faeces using electron microscopy. This is a catch-all method and requires no prior knowledge of the virus in order to detect it but it is expensive, makes high operator demands and its efficiency depends on a virus being readily recognizable; Viruses that are large, of distinctive appearance, and present in significant numbers (>10⁶ particles per ml) are relatively easily observed. However, small, diffuse or fuzzy viruses can often be overlooked. Identification makes high demands on the operator and this is even worse when a virus may have several distinct appearances (see astroviruses). Historically it is thought that this method has identified AdV and rotavirus infections quite well but has been less successful in the cases of caliciviruses, astroviruses and parvoviruses.

Most gut-dwelling bacteria are routinely identified by culture; however, this route has not been open to the detection of viruses. All viruses require living cells as hosts and are totally dependent on the processes that their host cells are able to provide, e.g. for protein synthesis and protein processing, but the gut is a very specialized habitat in virological terms. It contains a multiplicity of different cell types, each having a different enzyme content and surface protein composition; they may even vary in these properties at different stages of their differentiation. Furthermore, they are all bathed in a solution of the various secreted products produced by other specialized cells, notably of course proteases and bile salts. These features make the gut a very difficult cellular environment to mimic in culture. A virus may replicate in one type of cell, at one stage of its differentiation and may require soluble products released from quite different cells entirely. It is probably for this reason that gut-replicating viruses have been very difficult to culture in the laboratory. Those that can be cultivated often require that the culture be supplemented with proteases (usually trypsin) and in one case (porcine enteric calicivirus) with duodenal juice, bile salts being the active ingredient (Chang et al. 2004). Specialized cells are often needed, e.g. differentiating colonic carcinoma cells for astroviruses. Viruses that penetrate beyond the gut, invading...
other tissues are often (but by no means always) simpler to cultivate. The significance of this point cannot be overstated, most extracellular parasites will grow reasonably efficiently in a broth which mimics the gut contents, but this is not true of viruses and there are very good reasons why the characterization and detection of the viruses of gastroenteritis have been problematic. Culture for viruses is not generally used even to identify agents present in a faecal sample. The chances of using such a procedure for the detection of the lower quantities of virus present in food are still more remote.

In view of the above difficulties in EM detection, reagent availability in the past and difficulties in culture most workers have acknowledged the existence of a ‘diagnostic gap’ represented by the large proportion of infections for which no obvious cause could be found. Even in the IID survey in England and Wales, when causative agents were vigorously pursued, target organisms were not identified in 63% of community samples and 45% of General Practice samples (FSA 2000). Given the difficulties in virus diagnosis, it was always likely that a large part of this diagnostic gap would probably be caused by viruses, but probably by the smaller, less well-characterized and less-readily observed viruses. The extent of any such bias in detection has recently become clearer with the development of objective and sensitive methods for the detection of many viruses: the development of recombinant antigens for astroviruses and especially NoV, and the development of ELISA systems for both have at last provided a reproducible supply of antigen from which to prepare and validate diagnostic serological reagents as well as to conduct serosurveys of the incidence and age-first-infection profiles of these viruses. This type of detection method has been used in the IID survey in England and Wales – although not all tests in all cases (FSA 2000), and in similar studies in Europe (Koopmans et al. 2000) and the US (Mead et al. 1999). These studies all support the conclusion that virus-associated enteric illness has been greatly underestimated and currently suggest (in the absence of concrete data) that most, if not this entire diagnostic void is made up of NoV infections. Similarly data from the FSA survey in England (2000) suggested that for every case of NoV actually detected in the diagnostic laboratory, on average 1562 have probably occurred in the community.

10.2 Food samples

Virus detection in food and water (i.e. preinfection) is even more problematic than detection postinfection, in clinical samples. Levels of virus are orders of magnitude lower and the challenge is to detect a potentially infectious dose (perhaps as low as 10 particles) in a quantity of a size likely to be consumed as a single portion. As these viruses can either not be cultivated or cultivated only with difficulty, most detection methods have concentrated on PCR technology. This has proved extremely successful when applied to NoV detection in water and shellfish and can even provide quantitative information (Laverick et al. 2004; Le Cann et al. 2004). However PCR-based detection has two problems, the first is that most of these viruses are RNA viruses, PCR cannot be used unless the RNA has been reverse transcribed (RT) to manufacture a DNA copy and the efficiency of this RT step is notoriously variable. Secondly PCR targets only a short section of the virus genome (usually 200–300 bases) and reveals nothing at all about the presence or integrity of the rest of the genome. Strictly then, even quantitative PCR for such a virus detects only the levels of cDNA to a specific region of the virus, it cannot tell us how the numbers of DNA copies detected relate to the number of RNA copies actually present in each case and nor can it tell us whether the target detected was present as an intact genome inside a viable particle or present as a short section of free RNA released by virus degradation. The first of these difficulties can be tackled by careful incorporation of internal controls, although these may of themselves interfere with target amplification. The second is harder to address. It is often assumed that RNA degradation occurs in two phases; virtually none at all whilst the particle is intact followed by rapid and total degradation once the particle is breached. Consequently levels of free RNA should be insignificant and most detectable RNA would be contained inside an intact particle. However it is not clear that this assumption is valid; whilst Slomka and Appleton (1998) used FCV as a model for NoV and found that RNA was indeed rapidly degraded after inactivation of the virus in shellfish they also confirmed that some PCR-positive samples were not cultivable. Similar findings were reported for poliovirus and bacteriophage MS2 (Shin and Sobsey 1998; Sobsey et al. 1999). Recently a study by Nuanualsuwan and Cliver (2003) suggests that the situation could be yet more complex. These workers inactivated calicivirus and picornaviruses (including hepatitis A) by exposure to high temperature (72°C), UV radiation and hypochlorite disinfection. They found that infectivity of all viruses could be vastly reduced by these means. However, the RNA inside the particle appeared to survive, the reduction in infectivity being attributed to a loss of capsid function. Inactivated particles could no longer attach to host cells and were no longer recognized by antibodies. Despite this failure the RNA remained intact within them and continued to be detected by PCR; furthermore it was still protected from degradation by hydrolytic enzymes suggesting that the disjunction between PCR detection and virus viability would become worse with time. These findings are of great significance as they suggest that PCR-based detection and quantitation would also detect virus particles
that are inactive and pose no threat to a consumer. This would overestimate the extent of virus contamination. Antibody-based detection methods (or PCR detection following an antibody-mediated concentration step) should overcome this effect and this has also been observed (Sobsey et al. 1999). A technical account of the primers and conditions required for virus detection is beyond the scope of this review but have been summarized elsewhere (Sair et al. 2002).

One drawback of PCR-based detection is that the test consists of the observation of amplification or failure to amplify. Specificity relies on the choice of primers which should amplify only the selected target viruses. This approach is intrinsically unsuitable for the identification of multiple viruses simultaneously. It yields no information on the presence of other virus types not specifically sought by the procedure, or more distantly related to the ‘known’ viruses from which primers were designed. PCR for NoV for instance can identify only 90–95% of EM-positive samples, presumably because of inherent sequence variability within the virus family. However microarray technology now offers the ability to use multiple and redundant PCR primers to target and amplify a range of viruses present in a sample. Specificity is then achieved at the array hybridization stage when hybridization to a microarray plate containing several hundred probes for individual viruses or groups of viruses detects each simultaneously. Such detection technologies offer the chance of defining at last the true extent of virus contamination of both food and the environment.

11. ALTERNATIVE INDICATORS OF VIRUS CONTAMINATION

In view of the difficulties in detecting viruses above, much use has been made of indicator organisms to provide clues to the likelihood of virus contamination (reviewed by Kator and Rhodes 1987). Cultivable enteroviruses such as poliovirus have been extensively used. This agent has been ubiquitous in sewage-polluted environments because of widespread vaccination in the community and the resultant faecal shedding from vaccinees. However it is difficult and expensive to routinely extract and assay poliovirus from contaminated food and water, further the WHO aim to eliminate this virus from the world and work with it is already subject to control. As a substitute many workers now suggest AdV (see above). However an alternative approach has been to use nonhuman viruses such as the bacteriophages (Power and Collins 1989, 1990) and in particular these are the male-specific bacteriophages of the Llevivirus family such as MS2. These bacteriophages contain an RNA genome of similar size to the picorna-, calici- and astroviruses and have particle sizes in a similar range. Bacteriophage are frequently found in faecally contaminated materials (and shellfish associated with gastroenteritis outbreaks) and are thought to show similar stabilities to the enteric viruses. These data suggest that they may be good models for virus contamination in these systems (Dore et al. 1998, 2000). However it should be borne in mind that indicator organisms such as these can only indicate the possibility of human virus contamination; they cannot prove either its presence or its absence. F+-specific bacteriophage can be specifically enumerated using an engineered pilus-bearing Salmonella typhimurium host that cannot be infected by non-RNA coliphage (Havelaar and Hogeboom 1983). Use of this host yields data relatively free of contaminating somatic coliphage but does not easily separate contamination of animal origin from that of human. Further work would be necessary to make such a distinction using oligonucleotide hybridization (Beekwilder et al. 1995; Hsu et al. 1995). At present there are no data concerning the use of these organisms as potential indicators of faecal contamination for soft fruits and vegetables. Mariam and Cliver (2000) attempted to use bacteriophage to determine the extent of virus inactivation likely in response to certain treatments in various food processes and Dawson et al. (2005) used phage to monitor the survival of viruses on soft fruits. Slomka and Appleton (1998) used feline calicivirus in a similar manner. However these experiments require careful analysis as neither are exactly equivalent to the viruses of interest. Mariam and Cliver (2000) found that both MS2 and ΦX174 were more labile than HAV in their analysis. However they nevertheless concluded that these organisms could be useful if appropriate relationships could be determined between phage inactivation rates and those of viruses of concern. Such work demands parallel experiments using the actual viruses of concern to ‘calibrate’ the response of the indicators in a variety of circumstances and such data is urgently required.

12. CONCLUSIONS

Food-borne viruses are ubiquitous; it is likely that their incidence has been underestimated in the past not only in terms of the number of infections but also in terms of the frequency with which they are food-borne. Sewage treatment without disinfection should be recognized as inadequate and UV treatments of final effluents should be encouraged to protect both recreational waters and especially shellfish harvesting areas. Intrinsic contamination of foods other than shellfish needs to be considered and more work is needed to characterize the uptake, survival and removal properties of viruses in plant tissues. Given the difficulty of excluding (or even identifying) food handlers likely to be shedding virus at any one time and the growth in national/international trade in foods requiring minimal cooking we may expect food-borne viruses to increase in significance in the future. This effect may be of most significance in the case of HAV where the divergence in
infection patterns between producer and consumer countries is greatest.

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14. REFERENCES


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