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*Review Article*

# Molecular Epidemiology of Rotaviruses

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Molecular epidemiology of rotaviruses emerged a little over 25 years ago as a fascinating branch of science that utilized then cutting-edge technology of RNA polyacrylamide gel electrophoresis. Molecular epidemiology, as I have observed it closely almost since its dawn, is an ever-evolving discipline which has incorporated the advances of the related sciences including molecular evolutionary biology and ecology, while it is firmly and deeply rooted in the edifice of epidemiology of infectious diseases. Rotavirus is a non-enveloped virus possessing 11 segments of double-stranded RNA as the genome and belongs to the *Reoviridae* family. The consequences of rotavirus infections in terms of mortality are different depending on whether children live in the developing countries or they live in the developed countries, and this difference comes mostly from the availability of proper medical intervention. A second generation rotavirus vaccine has just been licensed in Mexico and will hopefully be used widely among countries where the burden of the disease is the highest. One potential threat to the existing and future rotavirus vaccines is the extreme diversity of strains circulating among children across the world, and it is the key to understand how rotaviruses maintain themselves in nature. Molecular epidemiology of rotaviruses helps address such questions and has demystified the way in which they evolve including interspecies transmission of rotaviruses. A few examples are provided from the work that my colleagues and I did over the course of my career in an attempt to give a feel of molecular epidemiology of rotaviruses.

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## Introduction

This year, 2004, has witnessed the licensure in Mexico of a second-generation rotavirus vaccine for the first time since the first generation rotavirus vaccine, Rotashield, was withdrawn in 1999 from the United States market because of a perceived association with intussusception in its recipients.[1-6] The newly-licensed vaccine, to be marketed under the commercial name of Rotarix, is a monovalent vaccine that contains a live attenuated human rotavirus strain RIX 4414, the precursor to which was the 89-12 strain carrying serotypes G1 and P1A[8].[7] As of this writing, optimism for the bright future prevails since this new vaccine has gone through rigorous safety tests involving over 60,000 infants[7] and the vaccine is to be given primarily to infants aged less than 3 months when cases of idiopathic intussusception are rarely observed.[8] It should also be welcomed that the Rotarix vaccine was licensed first in Mexico and ahead of the United States and Europe. It has been a common and repeated agenda in many international symposia and workshops how to shorten the interval between the use of rotavirus vaccines in developed countries and the use in developing countries where the vaccine is most needed.

In the back of such optimism there remains much unfinished business to be carried on before rotavirus vaccines are globally introduced and extensively implemented. Among such issues are the questions of how diverse strains of rotavirus are maintained in nature and how the dominance of particular genotypes, for example, G1P1A[8] which accounts for >50% of human rotavirus genotypes, will be affected by the extensive use of rotavirus vaccines. Such questions are most effectively addressed with the help of molecular epidemiology, a discipline of science that coincidentally emerged when I began my career and that has developed over the course of the last 25 years that my career spanned.

In this review I shall explain how I view the science of molecular epidemiology of infectious diseases, then overview the basic features of rotavirus and the disease it causes, and finally illustrate the way molecular epidemiology addresses the questions about rotavirus infection by presenting a few selected examples that my colleagues and I worked on before.

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## My view of molecular epidemiology of infectious diseases

The legend has it that Chargaff once defined molecular biologists as biochemists without license. Analogy may seem applicable to molecular epidemiologists, who may be defined as molecular biologists who do some epidemiological work without proper training in epidemiology. In reality, however, classic epidemiology is much more important to molecular epidemiology than it is generally perceived, because it forms the basis on which molecular epidemiology stands. Here, I will confine the discussion within the scope of infectious diseases keeping in mind those caused by rotavirus in particular. What matters first is therefore the classical approach based on the infectious disease epidemiology in which one tracks down the transmission of the virus by looking at the pattern of occurrence of the disease that it causes.

What comes next is an ecological approach in which it is studied how viruses are maintained under natural conditions. In many viral diseases, particularly those that have recently emerged, the host is rarely confined to the human species alone. The term zoonoses misrepresents what actually happens in nature, because what matters in zoonoses is the transmission of pathogens from animals to humans, and never the other way round, not to mention the transmission of pathogens between two different animal species. Thus, molecular epidemiology provides classic infectious disease epidemiology and ecology with the molecular tools without which today's outbreak investigations and infectious disease surveillance cannot be completed in most occasions.

Viruses, RNA viruses in particular, evolve at much faster rates than their hosts, which call for the molecular evolutionary approach in analyzing and predicting the range and direction of mutations. At the level of population at large, the occurrence of disease can be regarded as a statistical process, and a number of mathematical models have been proposed. Furthermore, an explosive advance in computer science makes it possible to simulate the transmission of infectious diseases in a complex society model. Molecular epidemiology is an ever-evolving discipline of science which has incorporated the advances of the related sciences, while it is firmly and deeply rooted in the edifice of epidemiology of infectious diseases.

## The basic features of rotavirus and the disease it causes

There are morphologically indistinguishable rotaviruses that possess distinct group antigens, but I shall discuss only Group A rotavirus, or *Rotavirus A*, which is taxonomically a species in genus *Rotavirus* within family *Reoviridae*.[9] The mature virion lacks an envelope and it has a characteristic double-shelled, wheel-like appearance measuring 70 nm in diameter when viewed with an electron microscope (the name of the virus is derived from the Latin rota, meaning "wheel").[10] Electroncryomicroscopy followed by electron-density data processing revealed, however, that these double-shelled particles actually consist of three layers of protein shells, hence the mature virions are now referred to as the triple-layered particles and formerly-called single-shelled particles correspond to the double-layered particles.[11] However, these terms are often used interchangeably in the literature and lectures. So, new comers and outsiders should take heed.

The outer surface of the mature triple-layered virion is made up of the trimers of the VP7 protein, and from this surface are protruding 60 spikes each of which comprises the dimer of the VP4 protein.[11] Both VP7 and VP4 independently induce virus neutralization antibodies and a dual nomenclature system has been adopted to define rotavirus serotypes, i.e., the G serotype defined by glycoprotein VP7 and the P serotype defined by protease-sensitive protein VP4.[11] The VP4 protein is protease-sensitive in that it is cleaved by trypsin into VP8* and VP5*, where an asterisk is a convention to denote that the protein is a post-translationally modified one. This cleavage of VP4 enhances the infectivity of rotavirus. The surface of the innercapsid or the double-layered particle is made up of trimers of the VP6 protein which is the most abundant viral protein, and diagnostic assays used in serology except neutralization assays primarily measure antibodies against this protein mass.[10] Antibodies against VP6 do not show virus neutralization activity in the test tube but it was demonstrated in a back-packed hybridoma model in mice that anti-VP6 IgA appeared to neutralize the viruses when they replicate in the epithelial cells.[12] In infected cells six virus-coded proteins are expressed and they are never incorporated into the mature virions, hence called nonstructural proteins. Among these, nonstructural protein 4 (NSP4) has captured much attention since it was shown to have an enterotoxin activity in newborn mice.[13] The enterotoxigenic function of NSP4 was confirmed and extended by other groups of workers,[14,15] but it remains to be determined what role NSP4 plays in the pathogenesis of natural rotavirus infection in man.

The genome of rotavirus comprises 11 segments of double-stranded RNA. Each viral genome segment basically codes for a single protein (i.e., monocistron) except the VP7 gene (genome segment 7, 8, or 9 depending on the strain) that encodes two in-frame VP7 proteins and genome segment 11 that encodes two mutually out-of-frame proteins called NSP5 and NSP6.[11] In total, the genome of rotavirus codes for six structural and six nonstructural proteins[11] (Figure 1).

The segmented nature of rotavirus genome affords ample opportunity for two strains to reassort upon coinfection and, when the exchange occurs in the gene segments coding for the G or P serotype, it will result in a sudden change in the neutralization specificity of the strain. Although there are 15 G serotypes and 22 P genotypes, and their genes can segregate independently under experimental and natural conditions,[10,16,17] there exist preferred combinations of G and P genotypes among naturally circulating rotavirus strains.[18,19] Two most marked examples are readily found among human rotavirus isolates. Serotype G1, the commonest human rotavirus G serotype, is almost always associated with P1A[8], while serotype G2 is associated with P1B[4].[18,19]

Rotavirus infection results in a various severity of gastroenteritis ranging from only a few bouts of vomiting and diarrhea to severe life-threatening diarrhea with dehydration.[10] It is believed that only

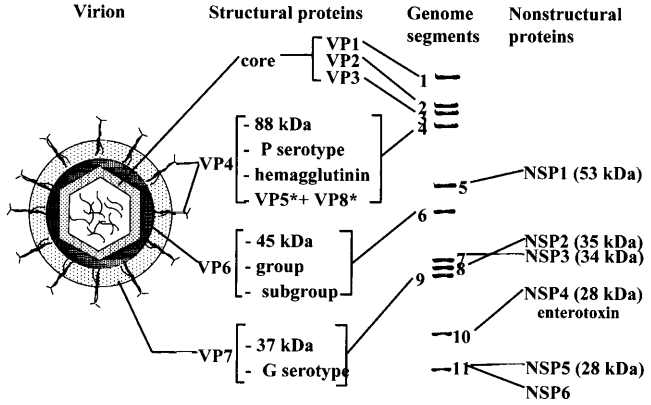


**Figure 1.** A schematic diagram showing the relationships among the structure of the virion (the location of structural proteins), the structural and non-structural proteins and the genome segments which encodes these viral proteins.

a tip of an iceberg (~2%) of rotavirus infection will end up with the severest form of diarrhea, which accounts for the staggering mortality in developing countries (~600,000 per year) and about a half of child hospitalizations due to acute diarrhea in developed countries.[20] The most recent estimate in Japan shows that approximately 790,000 physician visits of children under 6 years of age per year was attributed to rotavirus infection.[21]

One important feature of rotavirus as the etiological agent of gastroenteritis is that it is the single most important cause of severe diarrhea in both developing and developed countries.[10] For example, longitudinal studies in developing countries revealed that a median of 23% of episodes of diarrhea were associated with Enterotoxigenic *Escherichia coli* and that a median of 6.3% of episodes were associated with rotaviruses.[22] However, hospital-based studies showed that rotaviruses were detected in a median of 24% of the hospitalized patients whereas Enterotoxigenic *E. coli* was found only in a median of 9.3%.[22] This differential between longitudinal and inpatient studies provides evidence that rotavirus is the commonest agent consistently identified in severe diarrhea but that it may not be the most frequently-identified agent in diarrhea of any severity. Similar observation was made when a cohort of Finish children was followed from 2 months to 24 months of age. It was observed that 24% of 816 infants who had diarrhea of any severity shed rotavirus in the stool, whereas 68% of 56 infants who had severe diarrhea (14 or greater in the 20-point scoring system) shed rotavirus in the stool.[24]

Also important is the fact that both in developing and developed countries virtually all children get infected with rotavirus by the age of 5 years.[20] Thus, it is unlikely that the level of food hygiene and sanitation that we are enjoying in today's industrialized countries does not have significant impact on preventing rotavirus infections among children.[20] Thus, the vaccine is the most practical way of reducing the number of severe cases of and deaths from rotavirus infection.

It is now in order to briefly mention on what grounds rotavirus vaccine is feasible. Studies of natural history of rotavirus infections have provided researchers with the answer. First, natural infection does not provide complete protection against a subsequent infection or mild disease associated with it because humans are repeatedly infected with rotavirus from birth to old age. However, the majority of such infections are asymptomatic or associated with mild gastrointestinal symptoms.[20] Thus, while the simple idea of rotavirus vaccines being able to confer sterilizing immunity against subsequent infections is wrong, resistance to severe diarrhea is induced by naturally-acquired prior infection.[10] For example, Bishop et al.[25] showed that neonates who were infected in a newborn nursery during the first 14 days of life with what appeared to be a single strain of rotavirus experienced almost 50% fewer rotavirus diarrheal episodes and 100% fewer severe diarrheal diseases during the next three years than did a cohort of infants who were not infected with rotavirus during the first 14 days of life. In another prospective study by Bernstein et al.,[26] significantly fewer infants experienced prior infection developed a symptomatic infection compared with previously uninfected infants. Thus, it is now widely held that the goal of rotavirus vaccine is to prevent severe rotavirus gastroenteritis during the first two years of life, the period when rotavirus disease is most serious.[10,23,27]

## What molecular epidemiology of rotavirus is about: a few illustrative examples

Species is probably the single most important concept in biology, and it is defined as a group of individuals that are potentially interfertile. In contemporary virology the concept of species has much debated and has yet to be settled. However, the argument is mostly a conceptual one, and it has little practical impact than the concept of strain. This is because professional virologists usually work within the scope of only one species of virus (in my case *Rotavirus A*) and they have never been bothered by the definition of virus species in their daily research activities. By contrast, strain is an ambiguous term but what professional virologists deal with on the bench in their research activities is a some strain of virus, which may be defined as a collection of virus that originate from a given isolate from a given host animal at a given time. However, practical identification of strain is not always a simple task to do in many viral families. In this regard, rotavirus researchers are afforded unique opportunities in which they can use the characteristic banding pattern of genomic RNA produced upon polyacrylamide gel electrophoresis as the practically viable marker of strain.[18,28-30]

This characteristic banding pattern was termed electropherotype[28] and studies based on electropherotyping have contributed to our understanding of the molecular epidemiology of the rotavirus.[29] Major observations include: (i) Extensive heterogeneity occurs in the electropherotypes of rotavirus isolates. (ii) Despite this diversity, short and long RNA patterns are readily identified on the basis of the relative mobility of gene segments 10 and 11 (Figure 2). This two distinct RNA patterns are not only linked to two major subgroups of human rotavirus[31,32] but are shown by RNA-RNA hybridization to

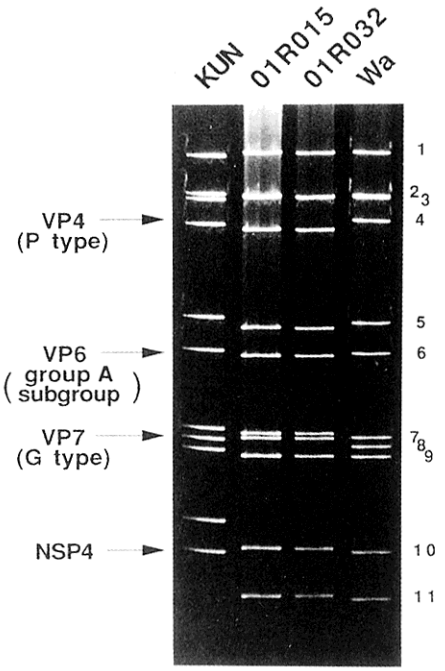


**Figure 2.** A representative polyacrylamide gel on which there are one strain with short RNA pattern (strain KUN at the left end) and another strain with long RNA pattern (strain Wa at the right end). Notice that genome segment 11 of the short RNA strain corresponds to genome segment 10 of the long RNA strain, and codes for the NSP4 protein. Genome segment 10 of the short RNA strain is resulted from rearrangement involving genome segment 11 of the long RNA strain. In this rearrangement process, the gene acquires some additional sequences either by repetition of its own sequence or from yet unidentified origin and this increase in size results in the reduced migration rate. The genomic RNA of 01R015 and 01R032 were derived from two different patients who were infected with rotavirus, but have identical electropherotypes. Thus, these two stool specimens contain a single rotavirus strain.

represent two major human rotavirus genogroups.[33,34] (iii) During outbreaks, one strain (one electropherotype) is predominant at any time and it is accompanied by co-circulating minor strains (less frequently found electropherotypes).

Serotype is an important attribute of rotavirus, as is in other viruses, because it is directly related to protective immunity against the virus. To address the relationships between rotavirus strain and serotype, we initially asked the question whether any given electropherotype corresponded to a single serotype, G serotype in the current terminology.[29] We determined both electropherotype and serotype of 291 rotavirus specimens, and showed that a given electropherotype always corresponded to a particular G serotype.[29] This was consistent with an earlier observation made by Coulson on 90 rotavirus specimens.[35] It was therefore proposed that once the electropherotypes present each year in a given location have been determined, serotyping of limited numbers of each electropherotype will be sufficient to ascertain the serotypes circulating in the population under study.[29,35] However, there were a few preceding papers describing examples of two rotavirus strains possessing identical electropherotypes yet belonging to different G serotypes.[36,37] During the decade that followed we did not come across such a strain, but one day in the late 1990s our colleagues at the Centers for Disease Control and Prevention, USA, brought us the news that they had identified two rotavirus strains that had apparently an identical electropherotype but belonged to different serotypes, i.e., G2 and G3. The collaborative work on these unusual strains resulted in the identification of naturally-occurring single VP7 gene reassortant in which a typical G2 rotavirus strain with short RNA pattern had acquired a G3 VP7 gene from a co-circulating strain in exchange of its own G2 VP7 gene[38] (Figures 3 and 4).

As is well illustrated in this naturally-occurring reassortants in

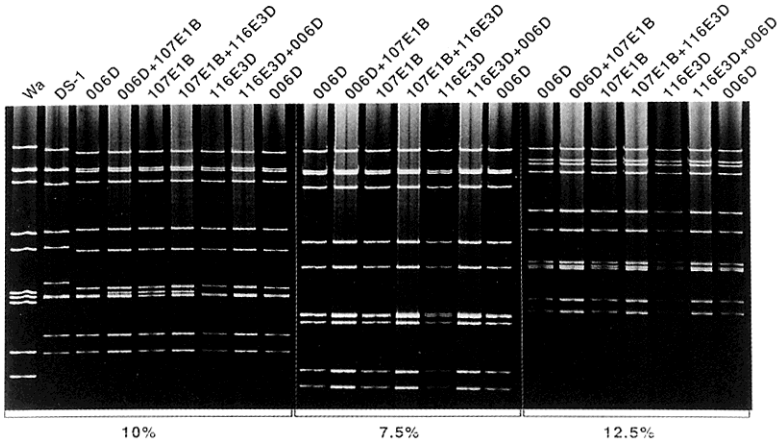


**Figure 3.** Strains 006D, 107E1B, and 116E3D were isolated in India and belonged to serotype G2P[4], G3P[4] and G2P[4], respectively. These three strains show a mutually indistinguishable electropherotype both on the 7.5% gel and the12.5% gel even after co-electrophoresis in which RNAs from two samples are run together on the same lane of the gel so that minor differences, if preset, will more easily be recognized than they are recognized by side-by-side comparison. On the 10% gel, however, co-electrophoresis of 006D and 107E1B produced four closely migrating bands, a three set of which comigrated with the corresponding genome segments of 006D and another three set of which comigrated with the corresponding genome segments of 107E1B. These two strains, 006D and 107E1B, are therefore concluded to be two different strains. On the other hand, 006D and 116E3D, are actually a single strain on the basis of possessing the same electropherotype under 3 different electrophorsis conditions. Because the difference is in one of the 7-9 genome segments, it is presumed that the one segment that does not agree with both stains is the gene coding for the VP7 protein which determines the G serotype specificity of the virus (from Ref. 38).

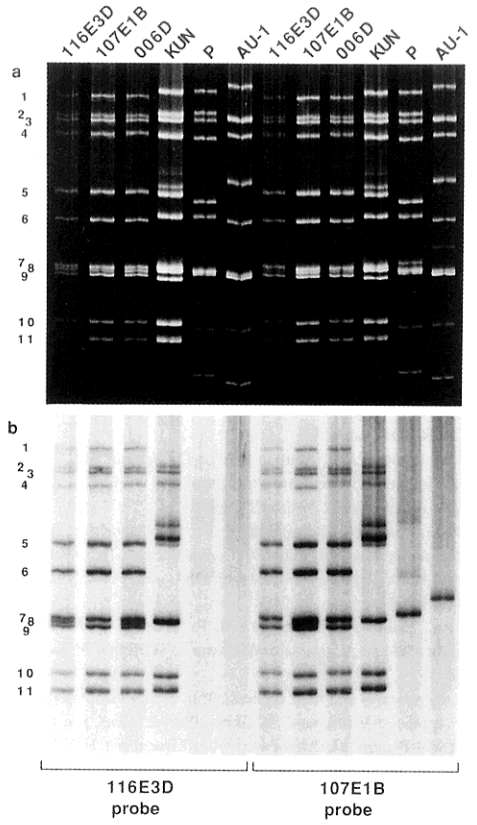


**Figure 4.** To further give support the hypothesis that 116E3D (=006D) and 107E1B are identical except their VP7 genes, RNA-RNA hybridization experiments were performed in which probes were made from both 116E3D and 107E1B. The 116E3D probe formed 11 hybrid bands with the genomic RNAs from 006D as well as those from 116E3D, while it formed 10 hybrid bands with the genomic RNAs from 116E3D. The difference is again in the area where genome segments 7-9 migrate. In this region of the gel, there are three hybrid bands on the lane of 116E3D and 006D but only two bands on the lane of 107E1B. By contrast, in the experiment in which the 107E1B probe was used, there were only two hybrid bands on the lane of 116E3D and 006D but three bands on the lane of 107E1B when attention was focused on the area where genome segments 7-9 migrated. These different patterns of hybrid formation completely agree with what G serotype each strain posseses, providing further support for the occurrence of the genome segment exchange, or genetic reassortment between strains. The G serotypes of 116E3D and 107E1B were finally confirmed after sequencing the VP7 gene of these strains (from Ref. 38).

India, RNA-RNA hybridization in solution plays a powerful role in solving many of the questions that molecular epidemiology addresses. Another example is a naturally-occurring reassortant between human and bovine rotaviruses that were isolated from an 8-month old infant in Cincinnati, Ohio, U.S.A.[39] The strain belonged to serotype G1P7[5] and contained 4 genes deriving from a bovine rotavirus carrying P7[5] VP4 gene and the remaining 7 genes from a human rotavirus strain which belonged to serotype G1P1A[8] and circulated concurrently at the time when the strain in question was isolated.[39]

Despite the presence of indirect evidence that rotaviruses reassort in nature such as the ones described above, there had been no evidence before 2002 that any one of field isolates of rotavirus was formed by direct reassortment between concurrently-circulating two parental strains. We compared the electropheroytpes of a large numbers of rotavirus specimens collected over a six year period with an aid of sequencing some selected gene segments, and identified two reassortants that were generated in nature between strains circulating co-dominantly in the same epidemic season.[40]

In quantitatively addressing the diversity of strains as defined by their electropherotypes, operational definitions of dominant and co-dominant strains are necessary. For the purpose of practicality, a strain is defined as dominant if the electropherotype of the strain is found in 50% or more of rotavirus specimens in one season and if its percentage is twice greater than those of any other electropherotypes detected in the same season.[30] Similarly, two strains are defined as co-dominant if the electropherotype of each strain is found in 25% or more of rotavirus specimens in one season and if its percentage is twice greater than those of any other electropherotypes seen in the same season.[30] When we examined the seasonal (yearly) distribution of electropherotypes (strains) over 11 seasons (a 10 year period), it was found that only one predominant electropherotype was seen in eight of the 11 seasons.[30] In the remaining three seasons, co-dominant electropherotypes were found. These dominant and co-dominant electropherotypes were different from each other. Thus, one predominant strain (occasionally two strains) emerged every season, and the predominant strain changed from one season to another. An interesting result emerged from the perspective of molecular epidemiology when we plotted the cumulative percentage of rotavirus specimens that belonged to each electropherotype according to the order of frequency of detection[30] (Figure 5). Only 14 dominant and co-dominant elctropherotypes accounted for 72.3% of electropherotypes that were observed during the 11 seasons. Since a rotavirus strain is defined by a virus specimen whose genome shows a single distinct electropherotype, the majority of the rotaviruses

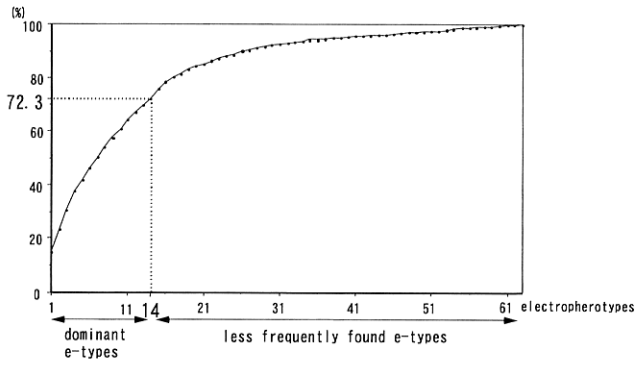


**Figure 5.** Cumulative percentage of rotavirus strains as defined by possessing a distinct electropherotype in the order of the relative frequency of detection. There were 61 strains (electropherotypes) identified over the surveillance period spanning 11 rotavirus seasons. In each season at least one new and dominant strain emerged and top 14 most-frequently appearing strains account for over 70% of all rotaviruses detected over the study period. This is the first study in which strain diversity was quantitatively addressed (from Ref. 30).

circulating among children in this study were derived from as few as 14 strains. The next logical question to be asked is whether or not dominant strains had any genetic advantages over less-frequently found strains and, if so, by what trait(s) they are determined. Answering this question will give tremendous insight into the rise and fall of rotavirus strains in nature and will perhaps help understand periodic yet unpredictable nature of changes occurring in the dominant serotypes of rotavirus in nature.

## Concluding remarks

Among a number of issues that we have addressed with the tools used in the molecular epidemiology of rotaviruses, perhaps the most important is the molecular identification of interspecies transmission of rotaviruses between humans and animals and between two different animal species. For the detailed treatment of this subject, interested readers are encouraged to refer to some of the review articles in the literature.[41-44] Here, I will confine myself only to mentioning that there are two distinct ways in which rotaviruses cross the host species barrier; i.e., the spread into a new host as whole virions and the spread into a new host through genetic reassortment with the rotavirus from the new host species. An increasing number of evidence indicates that such events pose a potential threat to the effectiveness of rotavirus vaccines that are intended to be used in developing countries.[45,46]

In closing this review, let me quote the very first few lines of the book that made a great impact on me when I embarked on this business more than 25 years ago. The book was the third edition of Natural History of Infectious Diseases by Sir MacFarlane Burnet.[47] Infectious disease is, and always has been, part of the everyday experience in life. In every generation men of affairs have had to cope as best they could with the practical problems it presents, while priest, philosophers, and, later, scientists have had perhaps the harder task of interpreting the significance of such disease in accordance with the intellectual outlook of the time. Over most of the historical period, the human attitude to epidemics and other aspects of infectious disease was a curious mixture of erroneous theory with a good deal of useful common sense. An appreciable remark one should keep in mind and it certainly is relevant to the molecular epidemiology of rotavirus.

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Molecular epidemiology, as is in classic epidemiology, requires collaboration of many workers possessing various aspects of expertise. This holds true in my case and I sincerely thank all those who worked with me and those whom I worked with.

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