REVIEWS

Rotavirus typing methods and algorithms†

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SUMMARY

Vaccination is the current strategy for control and prevention of severe rotavirus infections, a major cause of acute, dehydrating diarrhoea in young children worldwide. Public health interventions aimed at improving water, food and sanitation are unlikely adequately to control the disease. The development of vaccines against severe rotavirus diarrhoea is based upon homotypic or heterotypic protection provided against either a single common G serotype (monovalent vaccines) or against multiple serotypes (multivalent vaccines). Rotavirus strain surveillance has a high priority in disease control programmes worldwide. The continued identification of the most common G and P serotypes for inclusion in vaccines is an important priority. And subsequent to the introduction of a vaccine candidate, not only monitoring of circulating strains is recommended, but also surveillance of potential reassortment of animal rotavirus genes from the vaccine into human rotavirus strains is critical. Conventional methods used in the characterisation of rotavirus strains, such as enzyme immunoassay serotyping and reverse-transcription PCR-based genotyping often fail to identify uncommon and newly appearing strains. The application of newer molecular approaches, including sequencing and oligonucleotide microarray hybridisation, may be required to characterise such strains. The present paper presents a brief overview of the variety of standard methods available, followed by suggestions for a systematic approach for routine rotavirus strain surveillance as well as for characterisation of incompletely typed rotavirus strains. Improved detection and characterisation of incompletely typed strains will help to develop a comprehensive strain surveillance that may be required for tailoring effective rotavirus vaccines. Published in 2004 by John Wiley & Sons, Ltd.

INTRODUCTION

The need for the development of new rotavirus vaccines for prevention of severe, acute dehydrating diarrhoea among young children worldwide is underscored by the prospect of preventing approximately 600 000 childhood deaths annually, most of which occur in developing countries [1]. These prospects explain the high priority given to rotavirus vaccine development by major health organisations worldwide, such as the WHO and the Children’s Vaccine Initiative and Global Alliance of Vaccination and Immunization (GAVI). Prevention through vaccination seems to be the only effective option because rotavirus infections demonstrate a similar incidence in children throughout the world, regardless of hygiene and development standards [2].

The current vaccine development strategy is based on oral administration of live attenuated rotaviruses that will mimic the protection conferred by natural rotavirus infection [3]. Although the efficacy of early monovalent bovine and simian rotavirus vaccines was variable [4–6], evidence from some studies indicated that the induction of serotype-specific or homotypic immunity may be substantial [7,8]. This recognition prompted the development and testing of polyvalent reassortant vaccines [8,9] containing either all or selected VP7 proteins of the four most common VP7 (G) serotypes worldwide (G1–G4), and in one case the VP4 protein of the most common P serotype

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Abbreviations used
GITC, guanidinium isothiocyanate; IT, incompletely typed specimens, either partially typed (PT) (with either a G or a P type identified) or non-typeable (NT) (with neither a G nor a P type identified); PAGE, polyacrylamide gel electrophoresis; RT-PCR, transcription polymerase chain reaction

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(P[8]) recognised worldwide [3,4,7]. A promising tetravalent recombinant rhesus rotavirus vaccine, based upon the four globally most common G types (G1–G4), was licensed by the U.S. Food and Drug Administration in 1998 and shortly thereafter introduced to the National US Childhood Vaccine program, but the vaccine was voluntarily withdrawn from the market in 1999 after being used for less than a year, due to its association with intussusception among vaccine recipients [10]. The increasing evidence of the importance of serotype-specific immunity [11] has stimulated strain surveillance in countries considering a rotavirus vaccine programme, in order to determine whether the distribution of prevalent strains reflected the serotypes selected for incorporation into experimental vaccines. Rotavirus strain surveillance has proven to be a very worthwhile effort, since recent studies have demonstrated a surprisingly high diversity of rotavirus strains in most developing [12,13,15–19,21–23] and many developed countries [14,24–28]. These findings have raised important questions concerning the ability of the current rotavirus vaccines to protect against serotypes other than G1–G4. Also, it has prompted vaccine manufacturers to construct additional reassortants as candidate vaccines containing antigens of strains such as G9, found through surveillance to be more important than previously recognised [26,29–31]. Once rotavirus vaccines are introduced for routine use in individual countries it would be important to continue strain surveillance to evaluate the impact of newly introduced rotavirus vaccines and subsequently relate variation of naturally circulating strains to any changes in vaccine efficacy which may occur.

The present paper gives a brief overview of the standard methods available for characterising rotavirus specimens and suggests a systematic approach to be used in routine rotavirus strain surveillance. The paper also suggests approaches for characterising incompletely typed (IT) specimens. For applicable methodological step-by-step procedures, we refer to a recently published book by Gray and colleagues [32].

**ROTAVIRUS CLASSIFICATION AND STRUCTURE**

Rotaviruses are classified as a genus in the *Reoviridae* family. The virus is characterised by its wheel-like shape (rota is Latin for wheel). The genome consists of 11 segments of double-stranded RNA that encode six structural and six non-structural proteins [11]. Rotavirus is a non-enveloped, triple-layered icosahedral virus consisting of an inner core containing proteins VP1, VP2 and VP3, encoded by RNA segments 1–3; a middle capsid made up of protein VP6, encoded by segment 6; and an outer capsid made up of a VP7 shell encoded by segment 9 (or 7 or 8, depending on strain), and a VP4 spike protein encoded by RNA segment 4 [11,33].

Rotaviruses possess at least three important antigenic specificities based on VP6 groups (A–E), and subgroups (I, II, I+II, or neither I nor II) within group A, and VP7 (glycoprotein, designated G) and VP4 (protease-sensitive protein, designated P) serotypes. Most human infections belong to group A, but epidemics of severe group B rotavirus diarrhoea in adults in China as well as sporadic infections elsewhere (i.e. in India) are known to occur [34–37]. Rare outbreaks of diarrhoea predominantly in children due to infection with rotavirus group C [38–43] have been reported. A Swedish study reported a relatively high prevalence (38%) of rotavirus antibodies against rotavirus group C in older children and adults [44].

To date, 14 G serotypes have been defined by neutralisation assays, and 10 of these were isolated from humans, whereas only 11 of the 20 reported P types have been isolated from humans [2]. Based on nucleotide sequence characterisation of a bovine rotavirus strain, a 15th G serotype and a 21st P genotype have recently been proposed [45]. The majority of G serotypes identified in humans belong to serotype G1–4 and G9, and most P types belong to three P-genotypes P[4], P[6] and P[8], corresponding to P serotypes 1B, 2A and 1A, respectively [46]. Uncommon G and P types, such as G5 and G8 and P[6] as well as mixed infections are increasingly found in tropical countries [19,47,48].

**STRAIN CHARACTERISATION METHODS**

The present paper provides only a brief summary of standard methods for rotavirus detection and characterisation, since many of these procedures have been recently described elsewhere [49]. The high virus level during the acute phase of the disease facilitates the routine detection of rotavirus in faecal extracts and allows for a variety of methods to be applied. Negatively stained virions can be detected by electron microscopy, and virus antigen
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can be detected by enzyme immunoassay [50], passive particle agglutination tests [51] and lateral-flow immunoassays (immunochromatography) based on immunogold technology [52]. In addition, direct detection of the viral genome can be done when rotavirus nucleic acids are isolated from stool by lysis of stool extracts with guanidinium isothiocyanate (GITC), and purified with either silica beads [53], RNAID glass powder [54] or by standard phenol-chloroform extraction and ethanol precipitation, followed by either polyacrylamide gel electrophoresis (PAGE) and silver nitrate or ethidium bromide staining, or reverse transcription polymerase chain reaction (RT-PCR) [57].

Procedures based on phenol-chloroform extraction of RNA followed by purification through binding to and elution from CF-11 cellulose or hydroxypatite have been effective in removing stool inhibitors of RT-PCR [55,56]. Standard phenol-chloroform followed by ethanol precipitation procedures have often been used when the extracted RNA is not to be subjected to RT-PCR [58]. The use of GITC and silica beads or powder has major advantages over standard phenol-based procedures in that RNase is irreversibly inactivated, and stool inhibitors of RT-PCR are at least partially removed so that nucleic acid prepared by these methods is more suitable for subsequent gene amplification.

The primary goal of most rotavirus strain surveillance studies has been to determine the G types of the strains because of the importance of these antigens’ vaccine design. Historically, the most common strain characterisation method has been to start with an enzyme-linked immunosorbent assay (ELISA), using serotype-specific monoclonal antibodies (Mabs) generated in the 1980s to define the VP7 serotype directly in stool specimens [59–61]. Approximately 70%–85% of samples are typeable using one to several Mabs specific for each serotype. The method depends, however, on the presence of triple-layered particles, which are not always found (due to digestion or degradation).

In 1990, a multiplexed, hemi-nested RT-PCR-based VP7 genotyping assay, correlating well with Mab-based G-serotyping, was developed [62].

Two common sets of consensus primers designated Beg9/End9 combined with type-specific primers (G1, G2, G3, G4, G8 and G9) introduced by Gouvea et al. in 1990 [62], or 9con1/9con2 combined with type specific primers (G1, G2, G3, G4 and G9) developed by Das et al. in 1994, are currently used [63]. In addition, a modified procedure utilises the Beg9/End9 consensus primers for RT-PCR amplification and the 9con1 + G1-G9 Das primers in a nested multiplex PCR [48]. It should also be noted in this connection, that random priming can be used instead of a terminal primer for the RT-step [26,49,64]. This procedure has the advantage that the cDNA produced can be amplified with primers specific for the genomes of other enteric RNA-viruses (e.g. Noro-, Sapporo- and Astroviruses, but also Toro- and Coronaviruses).

Since vaccines may also target the rotavirus P serotype antigen, surveillance for the most common rotavirus P types has also been undertaken. Typically, these studies have been conducted by identification of VP4 genotypes using RT-PCR [54,65] or by using PCR-derived probes or VP4 serotyping by neutralisation [66]. The correlation between VP4 genotypes and serotypes is not yet fully established. Therefore, identification of VP4 types at the nucleic acid level is considered to be the best available method for assessing the diversity of gene 4 in co-circulating rotavirus strains [54]. For strains whose P serotype has been identified by neutralisation, dual designations are given, e.g. P1A [8], in which the serotype number is followed by a genotype number in square brackets [11]. For VP4 genotyping of rotavirus in faecal specimens by RT-PCR, two sets of consensus primers, Con3/Con2 and HumCom5/HumCom3, are being used with two different sets of primers specific for genotypes P[8], P[4], P[9], P[10] and P[6] [54,65]. For confirmation of PCR results or for identification of strains that cannot be typed by PCR or EIA, nucleotide sequencing either of PCR amplification products directly or after cloning into a suitable vector plasmid is increasingly being used. Sequencing also allows for discrimination of human and animal rotaviruses [67] and provides the genomic sequence of potentially novel genotypes [68,69].

Digoxigenin-labelled P and G genotype specific oligonucleotide probes [68,69], RT-PCR genotyping confirmation primers [54] and restriction length polymorphism (RFLP) analysis [70] have also been developed for the confirmation of genotyping results, whereas single-strand conformational polymorphism (SSCP) has only rarely been used [71]. Furthermore, oligonucleotide microarray hybridisation is a new and promising method for the detection and genotyping of rotaviruses combining the high sensitivity of PCR with the
selectivity of DNA–DNA hybridisation [72]. For a more complete characterisation, rotavirus strains can be cultivated in cell culture and plaque-cloned, and the VP4 (P) and VP7 (G) serotypes can be confirmed by the use of cross-neutralisation [73,74]. Recently, studies in animal models have shown that VP6 and NSP4 may play important roles [75,76] in rotavirus immunity and protection. Although candidate vaccines to these proteins are not being tested in humans yet, methods to genotype the most common VP6 and NSP4 genes have nonetheless been developed and are increasingly being utilised [77,78]. Recent studies have proved VP6 genotyping to be more precise than antigenic typing of VP6 and it should therefore be considered as a good alternative [77,79].

ALGORITHMS FOR STRAIN CHARACTERISATION

Suggested approaches for group A rotavirus strain characterisation are depicted in Figure 1. We consider ELISA to be a very user-friendly rotavirus detection method. Depending upon the laboratory capability, PAGE analysis of some specimens can be considered as an additional procedure, which can provide information on the RNA profile, such as determining whether the strain has a long or short electropherotype [58]. Since long versus short electropherotype is determined by segment 11 [80], RNA profile analysis allows the investigator to genotype segment 11. However, PAGE analysis does not allow for differentiation between serotypes/genotypes, although historically most short electropherotype strains belong to serotype 2. In a previous study, the results from PAGE and serotyping were compared [81], and isolates with the same electropherotype were generally found to belong to the same serotype [58].

The two most common strategies used recently (Figure 1) include a combination approach of serotyping and genotyping (method A) incorporating G-serotype characterisation with Mabs followed by RT-PCR genotyping of IT strains, whereas other surveillance sites have relied exclusively on RT-PCR for genotyping of all or a representative selection of specimens. We recommend the latter method since it has the advantage of requiring the set-up of one rather than two costly methods, thus requiring fewer resources, combined with the fact that many of the Mabs used for serotyping are not readily available. We reviewed the published literature on human rotavirus group A surveillance data and methods during the period 1995 to 2002, and calculated the percentage of studies exclusively relying on RT-PCR analysis for strain characterisation. A total of 181 rotavirus surveillance studies were identified, and PCR was used as the exclusive characterisation method in approximately 45% of these surveillance studies, whereas a combination of Mab- and PCR-based procedures was applied in approximately 20% of studies. Thus, RT-PCR is now the method of choice for strain typing and can be regarded as the gold standard.

Confirmation of the genotypes assigned by RT-PCR should be carried out routinely, either for the majority of products, or at least selectively, to verify accuracy and to avoid occasional misclassifications [82]. Sequence analysis is the most definitive method for confirmation of PCR results. This can be conducted either on site or in collaboration with a reference laboratory. The RT-PCR products may be sequenced directly or after cloning. Both methods have advantages and drawbacks. The advantage of direct sequencing is the speed of obtaining the sequence, while one potential drawback is that the sequence may represent several virus sequences if the specimen contains more than one serotype of rotavirus. In the case of a mixed infection, the sequence of the type-specific product obtained from the multiplex typing PCR is often required. This PCR-product can either be cut out from a low melting temperature gel and subsequently purified and prepared for sequencing, or cloned. If cloned, several clones of each mixed sample are required, as a drawback of cloning is that it is often not clear how representative the sequences of the clones are for the mixed infections or for the quasispecies. Nevertheless an advantage of cloning is that only a small amount of PCR product is required, thereby enabling sequencing of strains even when there is an insufficient product for direct sequencing. Direct sequencing of RT-PCR products can be done by either using type-specific or internal primers, or by designing the PCR primers with non rotaviral (e.g. M13) sequences at the 5’ end. For laboratories that do not have sequencing facilities, probe analysis with digoxigenin-labelled oligonucleotides or RFLP are viable options for confirmation.

Additional analyses, such as electron microscopy, oligonucleotide probe hybridisation and VP4 neutralisation assays, can be considered as
optional procedures for further in-depth analysis of rotavirus strains. Electron microscopy has the advantage of being a cheap and rapid way to ascertain if rotavirus particles are present and whether or not they are degraded. However, the maintenance of an electron microscope and the professional training required in order to operate it are considerable drawbacks of this method. Oligonucleotide probe hybridisation is a comprehensive but reliable method, and is particularly useful for confirmation of uncommon PCR-results in settings with no access to sequencing. VP4 typing assays with Mabs are available but are too cross-reactive for use in routine P serotyping. In the case of

Figure 1. Rotavirus strain characterisation flow chart. The flow chart is thought of as a step-wise guideline. If results are obtained at a present step, the further steps are optional. (Step A is an alternative to step B.)

Published in 2004 by John Wiley & Sons, Ltd. Rev. Med. Virol. 2004; 14: 71–82.
difficult-to-type strains, the use of primers specific for animal rotaviruses in PCR, design of degenerate primers, cell cultivation plaque-cloning and/or cross-neutralisation, can be considered as optional procedures for further in-depth analysis.

CHARACTERISATION OF INCOMPLETELY TYPED (IT) STRAINS

Most rotavirus strain surveillance studies initially observe a substantial percentage of strains that cannot be serotyped or genotyped for one or both types (G or P) by standard methods such as EIA serotyping with Mabs and RT-PCR genotyping.

Characterisation of IT strains in individual collections has often led to identification of uncommon strains. Characterisation of IT strains, whether partially typed (PT) (with either a G or a P type identified) or non-typeable (NT) (with neither a G nor a P type identified), usually requires a variety of methods, first to characterise the potential unusual strains and then to develop routine methods for their detection in ongoing and subsequent studies. A suggested approach to identify IT strains is depicted in Figure 2. The flow charts can be followed step by step. G-typing is described on the left side of the figure, and P-typing on the right. Since some IT strains turn out to be stool specimens that have low amounts of virus present or have degraded virus particles, it is valuable to identify such samples before additional efforts and expense are

Figure 2. Characterisation of G and P incompletely typed rotavirus strains from stool. The flow chart is thought of as a step-wise guideline. If results are obtained at a present step, the further steps are optional.
invested in attempts to characterise them. Thus, assaying the original RNA extract for the presence of viral RNA by PAGE with silver staining [83], if this was not already done, should be considered. If RNA is not present, repeating the extraction of RNA followed by an antigen test may be considered as a next step. If a large amount of antigen is present but RNA is still not detected, it is likely that degraded virus-particles are present, and consequently these specimens are unsuitable for further analyses. If bands are revealed after PAGE, these specimens may warrant further analyses, including a repeated attempt to type them with standard or modified methods. These extra steps are laborious, especially if the number of IT strains is large, but may be essential to characterise them.

For the ITs, nucleotide sequencing of primary amplification products is becoming increasingly common because of the availability of automated instruments. Using either the normal consensus primers [54,62,84], degenerate VP7 and VP4 consensus primers [48,64,85], or random priming [26,49,64] for the first-round RT-PCR step of G or P typing, IT samples are amplified by RT-PCR in an attempt to prepare enough PCR amplicon for direct sequencing or cloning and sequencing experiments. Whereas the normal consensus primers are based upon sequences of the most common G and P genotypes worldwide, the degenerate primers can be designed based on the sequences from individual IT samples, in case the sequence differs from already published degenerate primer sequences. In some cases, the substitution of commercially available RT-PCR kits with modified RT and Taq enzyme components has resulted in enhanced efficiency of amplicon preparation or in the production of amplicons when the use of standard reagents has failed to produce detectable products for sequencing [86]. For characterisation of the VP7 gene by sequence analysis, one approach has been to use the primer pair homologous to the 5’ and 3’ ends of the VP7 genes of strains Wa (G1) and SA11 (G3), designated Beg9/End9 or a mixture of degenerate derivates of this primer pair [62]. Otherwise, modified degenerate Beg9/End9 primer pairs, prepared by mixing individual primers to the VP7 genes of serotypes G1 and G2 (Beg9) and G1 to G4 [62, 68,69,86], may be used. The consensus primer pair for a fragment of the VP4 gene, designated Con2/Con3, has proved to be useful for determining the P genotypes of P IT strains [48]. Alternatively, consensus primer pairs for shorter fragments of the VP4 and VP7 genes have been useful to characterise P and G types, including a pair to sequence across the antigenic regions of VP7 [87] and another for a region flanking the protease cleavage site of VP4 (JR Gentsch, unpublished data). Once the sequence of the VP7 or VP4 gene fragments has been determined, the genotype and probable serotype of the strain can be accurately assessed by comparison with sequences of genotype reference strains in the GenBank database [88]. Sequence characterisation of ITs is not only important in genotyping strains but also valuable in discovering genetic variation in primer binding regions, which in some cases explains the inability of standard PCR to amplify particular strains. Several variants of rotavirus strains have been identified by sequence analysis [12,69,86,87,89], and alternative primers to detect certain strains (e.g. P[8], G1 and G8) have been designed for use in routine genotyping of these variants [15,26,64]. Such results suggest that the sequence variation that occurs over time in all strains or in the same year for strains isolated in different localities, and that these changes will necessitate the periodic redesign of genotyping primers.

Other less commonly used methods for identifying IT strains do not utilise sequencing. For example, digoxigenin-labelled oligonucleotides specific for individual G or P types have been used to detect PCR genotyping products that were too faint to be seen by ethidium bromide staining. The same methods have been used to confirm genotyping results [68,69]. Another non-sequencing approach was based on the hypothesis that under some circumstances, multiplexed PCR primers may suppress one or most others of the genotyping primers in the mixture (i.e. ‘inter-primer-suppression’), resulting in an apparent IT strain. To test this hypothesis, the individual genotyping primers were used one at a time with the consensus primer in single locus PCR assays, to attempt to genotype IT strains. The method was found to be successful in many cases, especially when multiple rotavirus infections were present, suggesting that inter-primer suppression or competition is more pronounced when homologous templates for more than one primer are present [48]. Sequencing of the individual amplicons confirmed that true mixed infections were present. Thus, this confirmation method may be particularly useful.
in regions where large numbers of mixed infections are observed [48,90].

When application of all of these conventional methods fails to identify the genotype or serotype of a rotavirus-positive specimen, cultivation of the rotavirus strain can be attempted [91], either in primary rhesus monkey kidney cells or MA104 cells with trypsin-containing medium [92], followed by sero- and/or geno-typing of the culture-adapted strain. A systematic approach for work with culture-adapted IT strains is depicted in Figure 3. This procedure is comprehensive but resolves many of the problems with the IT strains. Such strains often prove to be standard strains that for some reason, e.g. a low number of intact viral particles or the presence of RT-PCR stool inhibitors, cannot be characterised by conventional methods. Therefore, after cultivation, retyping the strains by standard P and G genotyping RT-PCR or EIA is straightforward in many cases. If the cultivated strains still cannot be serotyped or genotyped, the nucleotide sequencing approach described above (Figure 2) can be repeated. Cloning of dsRNA of unknown sequence is potentially an option [93,94]. As a last resort should sequencing fail, or as an alternative, if a collaboration with a reference laboratory has been set up, the strain serotypes can be determined by cross-neutralisation studies with panels of antisera to the known G and P serotypes [73,74]. Although laborious, these detailed procedures have sometimes resulted in the identification and characterisation of rotavirus strains previously unrecognised in humans, and some of these strains are now known to be important causes of diarrhoea in children. When novel strains are identified, additional methods may be applied to more fully characterise them. RNA–RNA hybridisation in solution and northern hybridisation have been used to identify the origin of each gene in the strain [95–97]. These methods, most notably RNA–RNA hybridisation in solution, have increasingly helped identify

Figure 3. Characterisation of incompletely typed rotavirus strains from cell cultivation. The flow chart is thought of as a step-wise guideline. If results are obtained at a present step, the further steps are optional.
human rotavirus strains that have shared some, or in rare cases all, of their genes with animal rotaviruses [98]. Complete sequencing of genes important for virulence and protection (e.g. VP4, VP7, NSP4) and G and P serotype determination by cross-neutralisation with antisera to the known serotypes has been used to determine the detailed relationships of the novel strains to known rotaviruses [45,99,100].

The steady advances in modern molecular techniques challenge epidemiologists to identify links between severity of disease and sequential point mutations (‘drift’), genomic reassortment—the exchange of RNA segments within genotypes (‘shift’) or genomic rearrangement and protection against re-infection. These possible associations are of particular interest in future vaccine trial settings, where detailed pre- and post-vaccination strain surveillance should be conducted, and efforts should be made to evaluate the extent of homotypic compared with heterotypic protection provided by rotavirus vaccines [101]. Characterisation of new strains as well as explanations for the failure of conventional methods to identify ordinary strains (like alterations in primer binding sites) can be revealed by nucleotide sequence analysis.

As animal strains apparently contribute to the gene pool of strains circulating in man, comprehensive surveillance at various stages of testing of rotavirus vaccine candidates should also include screening for animal strains [47]. A parallel to human and animal rotavirus epidemiological features is that seen for the influenza viruses, where surveillance of animal strains is essential for vaccine development [47].

Improved strain surveillance and in-depth characterisation of IT strains will provide an essential key in the prediction and analysis of future rotavirus vaccine trials.

ACKNOWLEDGEMENTS

Thea Fischer was funded through a doctoral research programme by the University of Bergen. We thank Halvor Sommerfelt for useful comments.

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