Research & Reviews: Research Journal of Biology e-ISSN:2322-0066

Techniques Linked to Rotavirus Characterization and Simple Research

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Review Article

Received date: 11/05/2018
Accepted date: 30/05/2018
Published date: 06/06/2018

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Keywords: Detection, Genotyping, Research, Rotavirus, Techniques

ABSTRACT

Despite Rotavirus (RV) vaccination that initiates a reasonable immunity, RV can still infect and replicate in a host; due to the endless virus evolution. Numerous RV G/P combinations characterized in both animal and human. The continuous evolution of unusual strains occurred as a result of the segmented nature of RV genome. Uncommon RV strains developed accidentally either by human-human/human-animal strains reassortment during mixed RV infection or via a direct interspecies transmission. RV genome modifiability creates an insistence for modifying the virus typing techniques for exact virus characterization; besides, precise preparation of the appropriate vaccine. Among RV detecting and classifying methods, each technique has its characteristic benefits and weak points over the others. More techniques applied in RV research to reveal the undiscovered virus secrets.

Highlights

- Rotavirus (RV) genomic material consists of 11 ds-RNA segments of varying size.
- Segmented RV genome nature leading to the continuous evolution of unusual strains.
- Genomic modifiability creates insistence for efficient RV identification and typing.
- Variant techniques applied in exact RV characterization with variable preference.
- More techniques are applied in research to reveal the undiscovered RV secrets.

INTRODUCTION

Rotavirus (RV) genomic material comprises 11 ds-RNA segments ranging in size from 667 bp (segment 11) to 3302 bp (segment 1). Each of these segments encodes for specific RV proteins. RV proteins divided into structural and nonstructural proteins. The structural viral proteins termed VP1, VP2, VP3, VP4, VP6, and VP7; however, the nonstructural proteins named from NSP1 to NSP5 [1-3]. A sixth NSP exists in some strains [4]. VP6, VP4, and VP7 proteins involved in virus classification into variant groups, P-and G-types, respectively [1]. Rotavirus Classification Working Group (RCWG) differently characterize about 27 VP7-and 37 VP4-specific types [5,6].

Unusual RV combinations are common worldwide owing to the segmented nature of RV genomic material [7,8]. Development of novel RV strains caused by point mutations (genetic drift), genetic reassortment, direct animal strains transmission into a human and accidental genetic deletions/duplications/insertions [9]. The most antigenic variations occur via genetic drift and reassortment events [9].

Owing to the high possibility of new strains evolution, there is an urgent need for efficient RV characterization and typing. RV strains typing and tracking have impact on (i) estimating RV strains/RV-diarrheal illness prevalence and disease mortality [10-13]; (ii)

novel strains discovery [14]; (iii) retrieving interspecies genetic transmission (animal-human/human-human) [7,8]; (iv) coming across species-specific epitopes (VP4/VP7 in human/pig/chicken RVs) [15]; (v) performance of phylogenetic analysis and interspecies identities [14-17]; (vi) designing appropriate vaccine by selecting VP4/VP7 epitopes of high epidemiological importance [12]; (vii) detecting the impact of RV vaccine introduction into immunization programs [18-21]; (viii) evaluating RV vaccines effectiveness/safety [22-24] and compatibility with variant infantile vaccines, besides; comparing immunogenicity of variant vaccines [25-27]; (ix) observing vaccine strains modifiability across the vaccination program [28]; and (x) post-vaccination analyzing for genotypes variation [29]; so realizing the settled RV epitopes that uncovered or developed by current vaccines [30].

Further sophisticated RV research will answer many questions about (i) RV innate and adaptive immunity [31-34]; (ii) virus assembly [35-38]; (iii) virus replication and transcription processes [39-42]; (iv) RV pathogenicity mechanisms [35]; and (v) link between RV infection and other health/nutrition problems [43-45]. Dependently, research will help for better understanding of RV puzzles, besides; the decision-making process on new vaccination approaches [46-48] and finding novel antiviral therapies [49,50].

The review article aimed at declaring of the variant conventional typing/research techniques or methodologies to understand the differences and benefits among them, and highlighting their role in RV scope.

RV CLASSIFICATION AND VARIANT CHARACTERIZATION/RESEARCH APPROACHES

Classification and Nomenclature System

Classification of RV

Preliminary issue: RV classified into groups, subgroups, serotypes, and genotypes. The grouping depends on RV VP6 protein reactivity. Seven distinctive groups (A–E) established previously, plus other recent groups (F and G). The group classified into subgroups, for example, group A subdivided into I, II, I+II, non-I, and non-II subgroups. Afterward, a dual taxonomic system exists depending on the outer-capsid neutralizable proteins (VP4 and VP7). G/P-serotyping or genotyping is reliant on VP7 and VP4 proteins, respectively. The VP4/VP7 categorization depending on reactivity with particular monoclonal antibodies reveals the serotypes and VP4/VP7 genomic characterization identify genotypes [2].

Up-to-date issue: In RCWG genotyping system, the RV whole genome of a certain genotype designed in Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx formula. The designed formula refers to viral proteins in order of VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6. It exhibits the entire RV genome sequence for each strain and reveals an explicit genotype for each genome segment (11 segments) [5,6].

RV characterization depends on the VP6, VP7, and VP4 antigenicity (referring to subgroups, G-serotypes, and P-serotypes); RNA migration pattern (referring to long, short, super-short or atypical electropherotypes); hybridization pattern (referring to genogroups); and genome nucleotide sequencing (referring to genotypes). Moreover, RV strains distinguished into wild-type (-wt), tissue-culture adapted (-tc), and laboratory-engineered (-lab) strains. For extensive description, the reassortant strains differentiated into *in vitro* or *in vivo* generated strain. In industry, strains labeled as “vaccine” (strains used in actual vaccine production) or “pre-vaccine” (strains generated in laboratories during the pre-vaccine industry stage) [5,6].

RV nomenclature system

Preliminary issue: Regarding nomenclature, the G-serotypes and genotypes are equivalent and have the same nomenclature. P-serotypes and genotypes nomenclature are atypical and named by absolute ordered numbers and a numerical order in brackets, respectively [2].

Up-to-date issue: Recently, RCWG recommends a uniform nomenclature system for each stain including RV group, species, identification country, common name, identification year, and strain G-/P-type [5,6].

RV Visualizing and Typing Approaches

Electron microscopy (EM): EM is a useful approach that detects RV morphology after staining process, whatever the virus group [2,51]. RV electron-microscopic assessment disregards the low stool quality circumstance [52]. Immune EM (IEM) including solid-phase immune EM (SPIEM) is an advanced EM technique, aimed for sensitivity doubling and differentiation of morphologically identical RV particles [2]. SPIEM is more specific and 2-16 times more sensitive in RV serotyping, rather than the enzyme-linked immunosorbent assay (ELISA). Besides that, SPIEM could beneficially neglect the ELISA cross-reactivity [52].

However, EM/IEM technique is highly costing, burdensome, and arduous; besides, needing a skillful expert [51,52]. Accordingly, EM approach is not applicable for routine RV-estimation in huge samples size [51].

Passive particle agglutination tests (PPAT): PPAT facilitates RV-particles detection within stool specimens using latex particles or red blood cells that specifically coated with RV-antibodies and agglutinated in RV presence yielding noticeable aggregates [2]. PPAT, especially latex agglutination test (LAT), is rapid, easy for on-spot diagnosis with no need of skilled experts, having extended reagents shelf lives, and lacking expensive instruments. It requires a low financial requirements [2,51,53]. Accordingly, it is applicable in clinical laboratories or discrete areas for quick RV screening [53]. Despite being less sensitive and nonspecific [2,51],

specific kits with high sensitivity are currently available [53].

ELISA (Serological method): RV identification depends on using reactive monoclonal antibodies (MAbs) against the unvarying VP6 protein. However, precise serotyping accomplished using VP7/VP4-specific, reactive MAbs. The efficient G/P serotyping necessitates intact doubled-layered virus presence [2].

ELISA technique obstacles include: (I) Difficulty in serotype-specific MAbs production owing to a) the outer capsid layer fragility so unavailability of intact triple-layered virus (TLV), b) the technical reasons of hybridoma and the proper clones selection, c) the time exhausted in TLV collection process, d) long-winded and problematic stages of mice immunization and most-fitting clones picking by RV neutralization or haemagglutination inhibition (HAI) assays, and e) the frequent expression of the VP3/VP7 dual neutralizing antigens [54]; (II) The devoid of specific hyperimmune sera for new, uncharacterized strains to ensure specificity and sensitivity [52,55-58]; (III) Insistent need for continuous MAbs production against upcoming strains [59-62]; (IV) Cross-reactivity due to antigenic regions homology and high ELISA background [55-58]; (V) Estimating crude RV-antigen only by applying of non-specific reagents in serological assays [55,57]; (VI) RV strains *in vitro* cultivation challenge that preceding ELISA technique for virus titer elevation [57]; (VII) Unsuccessful ELIZA typing as result of low virus titer and loss of outer shell layer in low-quality samples [52-57,59-61] (VIII) Time-consumption [59]; and (IX) The need of spectrophotometer for result recording [58].

Despite limitations, MAbs-serotyping technique is: (i) straightforward and instant method for RV characterization [62]; (ii) advance the polymerase chain reaction (PCR) technique in diminishing the cost, skill-proficiency, technical effort, and the need for more complicated instrumentation or reagents [55] and (iii) suitable for application on sample of large size [51].

ELISA sensitivity improved by (i) MAbs purification [63] (ii) Skim milk powder incorporation [63]; (iii) Applying of sandwich ELISA approach using capture and detection antibodies [63] (iv) Applying of homologous antigenic-coating layer; as G-type harmonization in MAbs and typed RV-strains maximize the specificity [56,63] (v) Fecal solution concentration by three folds [63] and (vi) Development of specific MAbs [56,62-65].

Hybridization technique: Hybridization analysis using oligonucleotides is a substitute technique for RV serotyping and even more sensitive [65]. Genomic RNA-RNA hybridization is advantageous in: (i) Being easy and specific [66]; (ii) Providing instant RV diagnosis [57]; (iii) Remedying the existed epidemiological and evolutionary questions [57] as the possessing of strongly related genetic pattern, of utmost of the 11 gene segments by variant RV strains isolated from diverse animal species, supports the interspecies transmission fact [66]; (iv) Being a valuable tool for investigating the animal-animal/animal-human interspecies transmission, expecting novel RV strains origin, characterizing unusual strains, and finding the genetic correlations among variant strains using [32p]-labeled probes [66]; (v) Inspecting up to 500 RV strain with about 8 pmol only of every single oligonucleotide [67]; and (vi) Ability of oligonucleotides drying and shipping in exclusive of refrigeration [67].

However, hybridization limitations include: (i) Dealing with radioisotopes that represent a problematic concern during handling, distribution or shipping and resulting in urgent need of qualified experts or skilled operators [67]; (ii) Inaccessibility of DNA synthesizer or radioactive substance fabricator in developing countries [67]; and (iii) Necessity of new oligonucleotide construction towards novel arising strains [67].

Electrophoretyping [Polyacrylamide gel electrophoresis (PAGE) technique]: Electrophoretyping expresses the patterns of RV RNA genome directly and classifies RV as Ia, IIa, IIId, IVc or short and long electrophoretypes. RV classification into long or short electrophoretypes relies on migration pattern of the 10th and 11th genes. Further variances in whole segments mobility noticed among strains [51,57,68,69]. Electrophoretyping does not support any data about strains subgroup/type. It only permits RV grouping into group A, B, and C depending on the distinct genes distribution pattern [51,69].

Electrophore typing approach has the characteristics of (i) Assuming the isolates similarities among different regions, while following standard preparation methods and gel condition [57]; (ii) Being an indicative tool for virus detection, group classification, and genomic distribution-pattern verification [57,69]; (iii) Having an advanced sensitivity and specificity to identify even the non-group A RVs [69]; (iv) Discriminating between type-2/nontype-2 RV strains during mixed infection by investigation the migration pattern of 10th and 11th genome segments [58]; and (v) Absolute RV group identification in large epidemiological studies [51,69].

PAGE has the drawbacks of (i) Having confusing, non-indicative results in determining the RV antigenic specificity [69,57]; (ii) Having a similar electrophoretyping pattern for variant RV strains (excluding type-2/nontype-2) [58]; (iii) Restricted importance in typing within epidemiological studies [57]; (iv) Being challenging and problematic in multi-RV strains infection [58]; (v) Needing of qualified worker and time due to its relevant difficulty [51]; and (vi) Dependence on ds-RNA quality that determined by techniques applied in extraction and purification [70].

RT-PCR: The PCR-amplification reaction applied for VP7/VP4 typing using selective oligonucleotide primers [70]. Owing to the simplicity of G/P-genotyping system, It is widely involved in RV strains typing [15]. A definite association exists between the molecular typing (RT-PCR method) and serologic (enzyme immunoassay with monoclonal antibodies) typing techniques [59,71,72].

G/P Genotyping Advantages

RT-PCR technique characterized by: (i) Presence of a definite association across RT-PCR and serological ELISA techniques

for VP7/VP4-typing [59,71,72]; (ii) Direct RV estimation with a simplified technique [71]; (iii) Typing capability of some ELISA-uncharacterized or multi-reactive strains [59,70-73]; (iv) Being rapid method [71]; (v) Ability to detect even 10-1,000 virus particles in diluted or low-quality samples; so it beneficially type RV in fecal samples of particles less than 10^8/ml [51,59,70]; (vi) The high sensitivity and efficiency, where the RT-PCR sensitivity is up to 500-50,000 and 200-20,000 fold of ELISA (Rotaclone kit) and hybridization sensitivity, respectively [59,71]; (vii) Providing of complementary DNA that applied in genetic techniques as sequencing and cloning [71]; (viii) Applicability in RV-epidemiological surveys [71]; and (ix) No need for virus-purification or tissue-culturing approach [59,71].

G/P Genotyping Limitation

On spot considerations of RT-PCR approach include: (i) High cost of enzymes/reagents/kits involved in the reaction [51,72]; (ii) Testing of fewer samples together and restriction during routine surveillance studies due to limited space in thermocycler [51,72]; (iii) Results variability by variant circumstances including d-NTP content, $MgCl_2$/enzymes/primers/template quality or quantity, PCR stool-inhibitors/DMSO, PCR-programing, and primers-designing [70, 51], where the PCR programs optimization and meticulous primer selection are challenging factors in RV identification and characterization [59,71,72]; and (iv) The possibility of accidental cross-contamination, while variant stool-specimens handling and management [59].

The PCR-typing failure caused by: (i) Failure of PCR process due to primers/PCR-program defects, RNA degradation, or PCR-inhibitors availability [59,71,72]; and (ii) Novel strains presence that baring of primers/nucleotides mismatches [51,73].

Sequencing: Nucleotide sequencing performed with or without prior cloning of RT-PCR untypeable strains that having nucleotides mismatches [73]. Recently, the automated fluorescence-based sequencing replaces the conventional sequencing using 35S-labeled dideoxynucleotide chain terminators. Sequencing vital steps are the proper template concentration, purification, and removal of free molecules as primers to avoid further interference with the sequencing reaction [2].

Nucleotides sequencing is beneficial in (i) Facilitating the phylogenetic analysis across variant strains via investigating RV strains phylogenetic pattern and exploring the developed transformations across genotypes; (ii) Genotyping of PCR-untypeable strains [2,73]; and (iii) Characterizing RV species depending on a novel sequence-based classification approach [17].

Tissue-culturing: The tissue-culture technique detects RV in high virus titer only [70].

Complement fixation test (CF): CF test has a clearly observed result and a comparable sensitivity in serotyping, as ELISA [58].

Neutralization assay: Traditional neutralization assays (plaque reduction/fluorescence/ELISA-based neutralization assay) are not commonly used for RV detection/typing and incorporated in virus quantification [74-76]. The developed neutralization reverse passive hemagglutination (RPHA) test measures the viral antigen quantity by the RPHA test [77].

Advanced RV research approaches

Approaches applied for RV-research include (i) X-ray crystallographic technique; (ii) Computer image-processing procedures; (iii) Electron cryo-microscopy (cryo-EM); and (iv) Combined application of crystallographic and cryo-EM approach [2]. These techniques are beneficial for (i) providing the virus 3-D structure and more details about virus scaffolding with close examination of surface features, central cross sections, and aqueous channels [35,36,78,79]; (ii) investigating the trypsin-enhanced infectivity, VP4 surface spikes, and VP4 internal structure [80-82]; (iii) assessment of VP6 interactive regions [83,84]; and (iv) describing the transcriptional pathways and replication mechanisms [41,42].

The challenging of X-ray crystallography is the specimen crystallization process. However, cryo-EM and image processing tools are advantageous in (i) lack the need of crystals production and (ii) permitting the performance of experiments on RV during diverse physiological conditions; so that finding more detailed evidence on associations between RV assembly and proteins responsibility [2].

X-ray crystallographic approach: It inspects discrete viral proteins (VPs); reveals the intact RV atomic resolution structure by X-ray diffraction; and explores/analyzes the RV structure [36,37,83,84].

Cryo-EM: It is advanced approach preserving the fragile RV components to explore the virus 3-D structure assembly details, entry, pathogenesis, and immunization machinery [35-38,41-47,78-82]. It could be associated with cryo-electron tomography (cryo-ET), as well [81]. Cryo-EM resolution improved by: (i) field emission guns introduction; (ii) column and cold stage stability perfection; (iii) ice contamination reduction via vacuum and cryo-shielding development; (iv) detector performance improvement; and (v) accelerating voltage enhancement [85]. The high cryo-EM running costs are a challenging factor that limiting its accessibility. Dependently, inexpensive and low voltage cryo-microscopes with an advanced brightness field emission gun are advantageously introduced [85].

Computer image processing: It has importance in analyzing of RV 3-D configuration. Recently, "localized reconstruction" applied to explain symmetry-mismatched/flexible structures of RV macromolecular complexes as a single entity, instead of the "vertex reconstruction" method [86]. More advanced computational programs and algorithms, which used in RV identification, are considered to be reliable and computationally less expensive [36,46, 87,88].

EM, PAGE and RT-PCR techniques are used to identify RV in stool specimens with low virus titer or quality, while, ELISA is applied in case of reasonable virus titer. Tissue culture technique is helpful to increase virus titer prior ELISA technique. Hybridiza-

tion is equivalent to ELISA serotyping technique, besides, molecular typing using RT-PCR approach is equivalent. RT-PCR is used in further typing of ELISA untypeable strains and sequencing is applied for RT-PCR.

DISCUSSION

RV genetic reassortants and novel/unusual combinations are common [7,8]. Dependently, there is an urgent need for continuous virus detection and typing. Highly specific and sensitive technical approaches reveal different VP4/VP7 epitopes in human/animal RVs, disclose virus secrets and signify the current scientific updates.

Regarding virus characterization, several techniques applied in RV identification and typing. These techniques estimate virus morphology (EM), virus antigens (ELISA and PPAT), or viral genetic material (PAGE and RT-PCR). Reagents toward VP6 protein mostly involved in virus detection; however, VP4/VP7 type-specific reagents used for further serotyping/genotyping process [2]. EM, ELISA, PPAT, PAGE, tissue-culturing, and RT-PCR approaches are widely applicable for virus identification [2,51,53,69,70]. On the other hand, IEM, ELISA, Hybridization, RT-PCR, and sequencing are helpful in typing process [52,63,70-73,89,90].

Despite the variety of RV characterizing techniques, each technique preferred in definite situations. For example, PPAT (e.g. LAT) is a valuable tool for RV diagnosing in small-scale clinical laboratories or far animal farming systems, while the absence of any instrumentation [53]. ELISA and PAGE commonly used for crude RV identification in large epidemiological surveys. PAGE desired for distinct genes mobility appraisal and grouping into group A, B, and C without further typing. EM and RT-PCR are applicable with low quality/virus titer stool samples. Hybridization inspired for investigating the interspecies transmission and genetic correlations among variant strains [66]. Besides that, sequencing is optimistic in exploring phylogenetic analysis across variant strains and genotyping of PCR-untypeable strains [73]. Multiplex RT-PCR, hybridization, and nucleotide sequencing appreciated in RV typing for recognizing of new arising or ELISA multi-reactive strains. RT-PCR applied in genotyping when the complementary DNA is requested for further sequencing and cloning [71].

There is a confident link between the molecular typing and serologic typing techniques [59,71]. The molecular VP7/VP4 typing approaches are a convinced substitute aspect of representing RV serotypes [15,59,71]. The simplicity and applicability of G/P-genotyping system potentiate its wide inclusion in RV strains typing [15]. Besides, RT-PCR genotyping appraised to identify about 42 discrete strains of well-defined G/P epitopes [90]. It could be precisely and specifically used to type existing and novel upcoming strains, rather than ELISA technique that detects pre-identified well-known strains only [70]. Comparing RT-PCR and ELIZA techniques in RV typing, the sensitivity is 91% and 86%, respectively [57]. In addition, PAGE, self-made ELISA, and ELISA kit sensitivity in relation to RT-PCR are 81.8%, 98.4%, and 97.1%, in turn. However, the compared specificity of these techniques in that order recorded as 100%, 100% and 98.3% [70].

For most cases, ELISA and RT-PCR applied in virus identification and typing in epidemiological surveys owing to their relative simplicity, reagents availability, reasonable cost, fair sensitivity, and specificity [51]. However, RT-PCR implicated in genotyping rather than absolute identification; due to its elevated cost and limited sample number that could be tested once, in comparison with ELISA [51,72]. Dependently, ELIZA initiates the virus detection followed by RT-PCR for typing of positive specimens, in most cases. For RT-PCR untypeable strains, sequencing involved, besides, RNA-RNA hybridization to determine genetic correlations across the novel and existing strains [91]. The techniques applicability simplified in a flow chart **(Figure 1).**

A special concern should be provided for the selection and designing of the typing primer pairs to prevent variant primers interference during the PCR amplification stages. For each RV-strain, specific primer pairs involved, while, the annealing temperature of all primer pairs is similar to have a fruitfully multiplex-PCR process. The further innovative primer mix is hopeful for upcoming orphan strains identification and precise genotyping [92].

Regarding research, recent approaches of electron cryo-EM and X-ray crystallography, besides other assistant techniques, play a critical role in sophisticated virus research by providing more details about virus assembly, architecture, and scaffolding [35-38]; genomic material [78]; structural/nonstructural viral proteins and their importance or interactions across virus layers [37,39,83]; virus replication, transcription processes, and molecular mechanisms [39-42]; virus entry [80-82]; uncoating and pathogenicity mechanisms [35]; RV receptors [84]; and virus antigenicity or ways to develop novel vaccines/immunization machinery [46,47].

Other assistant techniques involved in virus research including spectroscopy [93,94]; spectrophotometry [93]; mass spectrometry [46,93]; analytical ultracentrifugation [93]; gel filtration chromatography and high performance liquid chromatography (HPLC) [43,93]; western blotting [39-49,93-97]; flow cytometry [33,43]; immunofluorescence and immunofluorescence microscopy [39-44,95–100]; confocal microscopy [96-101]; cloning [98]; NMR spectroscopy [84]; real-time RT-PCR [32-44,97-100]; ELISA [33,44] and SDS-PAGE [40,49,94]. Besides, immunoprecipitation [95,98,99], transfection [32,96] and techniques for determination of viral infectivity/titer [40,97].

Continuous Virus research intended for discovery/establishment of unique classification way [17]; novel strains [17,92]; innovative antiviral drugs and therapies [49,50,101-104]; unknown viral property (e.g. binding [84], autophagy [96,104], viral release [94] or immune evasion [99] mechanisms); exclusive viral inhibitory mechanisms [95,97,100] or vaccine [47]; and exceptional strategy for recombinant virus generation as the plasmid-based reverse genetics approach that used in RV replication and pathogenesis research, besides, it is application to replicate virus as a vaccine vector from cloned cDNA [106]. In addition, computer science research could find

advanced computational algorithms and new software for accurate data analysis.

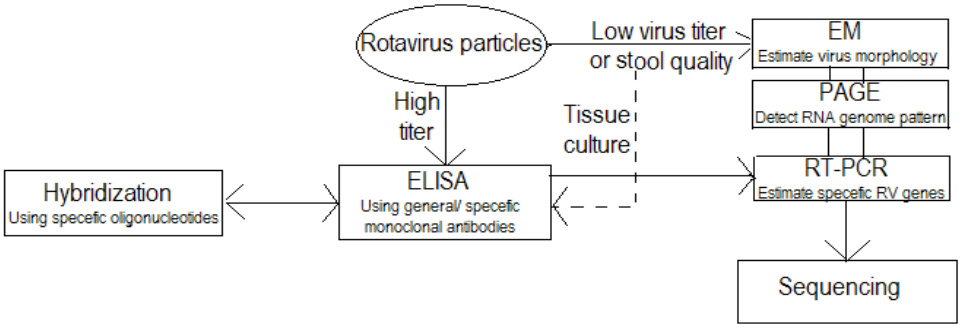

Figure 1. Applicability of variant approaches in RV estimation.

CONCLUSION

Variant techniques involved widely in RV detection, classification, and virus research. Detection techniques involve ELISA, EM, PPAT, PAGE, tissue-culturing, and RT-PCR; however, the typing techniques include IEM (e.g. SPIEM), ELISA (using type-specific MAbs), Hybridization (using specific oligonucleotides), RT-PCR (using type-specific primers), and sequencing. For virus research, variant techniques united to solve RV puzzles. X-ray crystallographic technique, computer image-processing, and cryo-EM involved in discovering more about virus assembly, replication, infection, and pathogenicity mechanisms with the assistance of other techniques.

CONFLICT OF INTEREST

The author confirmed that no conflict of interest exists.

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