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Research Article

Optimization and Development of Protocol for Detection of Foot-And-Mouth Disease Virus by Negative Staining Using Transmission Electron Microscopy

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Abstract

Using transmission electron microscopy (TEM), a specimen can be ready for examination within 10 minutes, and an experienced virologist, pathologist, or technologist can identify a pathogen morphologically. There is paucity of literature with respect to study of TEM as a tool for the detection of foot-and-mouth disease (FMD) virus in suspension in diagnostic pathology. The current study aimed to optimize and develop the process of detection of foot-and-mouth disease virus and its capsid in TEM by negative staining methods. FMD virus infected BHK-21 cell and mock infected cells were centrifuged for 5 minutes at 12000 rpm, and the supernatant was used for negative staining. The supernatant was placed on the TEM grid using either single droplet touch methods or direct application of a single drop to the TEM grid and washed three times with water. After washing, the TEM grid was stained with 1% aq. Phosphotungstic acid, PTA (negative stain), then air dried and observed under TEM for imaging. The image was captured with a Jeol 1400 Plus TEM at 120 kV and 15000X magnification. The positive cell suspension was found positive for FMD virus under TEM in which virion capsid appears to be icosahedral, whereas in mock infected cell suspension samples no FMD virus particles were observed. The current study describes the process of demonstration of FMD virion in cell suspension using a negative staining method. This method can also be used to detect FMD virus in vesicular fluid, other fluids suspected for FMD virus, and other viruses.

Keywords: Cell Suspension; Foot-and-Mouth Disease Virus; Negative Staining; Phosphotungstic Acid; Transmission Electron Microscope

Abbreviations

Transmission electron microscopy (TEM) continues to play a significant role in discovery of new organisms, particularly viruses, and helped in diagnostic pathology and research. Among these, the first electron micrograph of poxvirus and immunologic procedures of tobacco mosaic virus was published in 1938 and 1941, respectively [1]. Electron microscopy was introduced successfully in the differential diagnosis of smallpox and chickenpox infections in the late 1940s [2,3]. With the introduction of negative staining in the late 1950s [4] and the wider availability of electron microscopes, electron microscopy (as a catchall method) became essential in characterisation of many new virus isolates detected in diagnostic cell cultures and clinical samples, e.g., stool, urine, and biopsied specimens etc [5-9].

TEM has been instrumental in identification of the coronavirus that causes SARS; a new respiratory virus, NL-63, that infects children; and an antigen-positive intracytoplasmic inclusion in the ciliated bronchial epithelium of children with Kawasaki disease [10-12]. Earlier worker reported success in identification of herpesvirus and Orthopoxvirus by drop method preparation [5,13,14] of vesicle aspirates to the tune of 62% to 80%, by electron microscopy. Establishment of persistent infection with foot and mouth disease virus in BHK-Op48 cells has been established by electron microscopy assays [15].

The Foot and mouth disease (FMD) is a vesicular, contagious and infectious viral disease of cloven-hooved domestic animals as well as more than 70 wildlife species [16,17]. It is caused by FMD virus

(FMDV), an *Aphthovirus* of the family *Picornaviridae* [18]. *Aphthovirus* is positive sense single stranded, non-enveloped RNA virus. During active phase of replication FMDV is present in vesicular fluids of epithelium of tongue, mouth, feet, teat etc [19]. Confirmatory diagnosis of FMD is based on detection of FMDV or its genome using virus isolation technique or molecular tools like PCR, respectively in clinical samples particularly tissue samples collected from FMD suspected animal. Detection time of FMDV or its genome varies from 8 hrs (molecular diagnosis) to 72- 96 hrs (virus isolation).

A specimen can be ready for examination and an experienced virologist, pathologist or technologist can identify, by electron microscopy, a viral pathogen morphologically within 10 minutes of arrival in the electron microscopy laboratory [20]. There is paucity of literature with respect to study of TEM as a tool for the detection of FMD virus in suspension in diagnostic pathology. The technique can be made more specific if combined with immuno-electron microscopy using suitable monoclonal and polyclonal antibodies specific to FMDV serotypes. Considering these backgrounds, present study was taken to optimize the process of detection foot-and-mouth disease virus in TEM by negative staining methods and subsequently this process can be applied for detection of other viruses in cell suspension or vesicular fluid.

Materials and Methods

Materials

Equipments and reagents like transmission electron microscope, table top centrifuge, fume hood, TEM grade tweezer (fine point), parafilm, Whatman filter paper, petri dish, carbon coated copper grid of 400 mesh, grid storage box, FMD virus cell suspension (supernatant), micropipette, distilled water (DW), 1% aq. phosphotungstic acid (PTA) as a negative stain, and discards were required to perform negative staining.

Samples

FMD virus cell suspension of BHK-21 was used for the optimization and detection of FMD viruses by negative staining technique. Known FMDV positive cell suspension was used in this study while mock infected cell culture of BHK-21 was used as a negative control. Confirmation of positivity of cell suspension was carried out by cytopathic effect (CPE) on BHK-21 cell line, RT-mPCR [21] and RT-qPCR [22].

Nucleic acid extraction

The QIAamp total RNA mini kit (QIAGEN, Hilden, Germany) was used to extract total RNA from all tissue suspensions according to the manufacturer's instructions. The extracted RNA was purified and DNaseI-treated with the RNeasy kit before being quantified with a NanoDropTM 1000 spectrophotometer using the OD260/OD280 ratio, which was 1.8–2.0 for all samples. Total RNA preparations were stored at -80°C until needed.

Detection of FMD Viral genome

Using total RNA extracted from the above suspensions, RT-mPCR was used to confirm the FMDV serotype [21]. In brief, the RT-mPCR mix included three serotype-specific forward primers, DHP13, DHP15, and DHP 9, against FMDV serotypes O, A, and Asia 1, respectively, and one FMDV-specific reverse primer, NK 61. FMDV serotypes were classified based on amplicon size (249 bp, 376 bp and 537 bp specific for serotype O, A, and Asia 1, respectively). RT-mPCR amplified products were electrophoretically separated and stained with ethidium bromide on a 2% agarose gel.

Virus isolation

Rescue of infectious FMDV was performed by chemical transfection of the extracted total RNA from each tissue collected on BHK-21 cells as previously described [23]. In brief, 1 µg of extracted RNA and 2 µl lipofectamine 2000 (Invitrogen, Carls-bad, USA) diluted in OPTI-MEM®I (Gibco, Life Technologies, NY, USA) were mixed and kept at room temperature for 20 min. These mixtures were transferred to monolayer BHK-21cells in 24-well plates overlaid with 200 µl of GMEM and incubated for 4 h. After 4 h of incubation, 700 µl of GMEM was added and again incubated at 37°C for 48 h. Then the whole contents in the wells were harvested and stored at -80°C. 200 µl of harvest aliquot was subjected to further passage in BHK-21cells in order to amplify the virus rescued by transfection for further use.

Preparation of sample

Cell suspension was centrifuged at 12000 rpm for 5 minutes and supernatant were used for further study.

Negative staining methods

Protocol for negative staining of FMD virus cell suspension (supernatant) on TEM grids was carried out either by single droplet touch methods or single droplet on TEM grid application method (Figure 1).

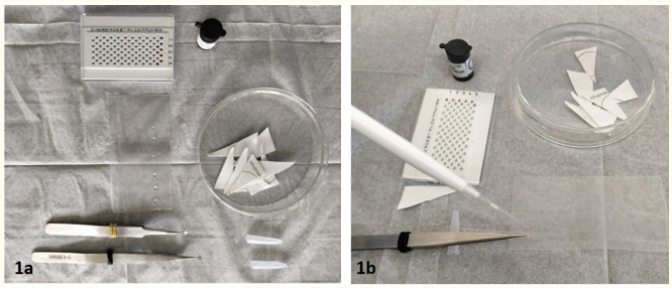

Figure 1: The procedures of negative staining technique for detection of foot-and-mouth virus in the cell suspension. a) single droplet touch method, 5-6 drops on clean parafilm sheet, among these drops, first drop- cell suspension containing test sample; next 03 drop- water drops for washing; last drop- 1% aq. phosphotungstic acid (PTA) as negative stain, b) single droplet on TEM grid application method, test sample, washing and negative stain were place directly on single TEM grid in sequential manner with appropriate time interval in following sequence like test sample, three consecutive washing with water, application of 1% aq. PTA as negative stain followed by air dried.

Single droplet touch method

In this method, 5-6 drops were used, in which first drop was FMD virus cell suspension (supernatant), 3-4 drops of distilled water, DW (number of DW drops depending upon concentration of samples) and last drop was negative stain (1% aq. PTA). All drops, as mentioned above, were place on parafilm strip in linear fashion (drop 1- sample, drops 2,3,4- DW and drop 5- 1% aq. PTA negative stain). Hold a fresh new carbon coated copper grid of 400 mesh in tweezer. After proper holding of TEM grid, carbon coated side of grid was touched to the sample drop up to 10 second (s) for adsorption of samples. After 10 s, removed liquid adhered to grid by using Whatman filter by touching the edge of grid followed by washing. Washing of grid were carried out by touching the TEM grid sample side with DW drops three time, each washing was for 10 s. After washing with DW, TEM grid was stained with 1% aq. PTA as negative stain for 10 s followed air drying at room temperature. After air drying, negatively stain TEM grid was stored in suitable grid storage box for TEM imaging and study in a TEM was carried out within 24hours.

Single droplet on TEM grid application method

In this method, single drop, 3 µl, of liquid samples was directly applied on carbon coated copper grid of 400 mesh weight for 60 second for proper adsorption. After 60 s, removed liquid adhered to grid by using Whatman filter by touching the edge of grid followed by washing. Washing of grid were carried out by application of 10 µl of DW directly on TEM grid sample side three times, each washing was for 10 s. After washing with DW, TEM grid was stained with 1% aq. PTA as negative stain for 10 second followed air drying at room temperature. After air drying, negatively stained TEM grid was stored in suitable grid storage box for TEM imaging and study in a TEM were carried out within 24hours.

TEM imaging of stained grid

Negatively stained TEM grid was screened by Jeol 1400 Plus TEM at 120 kV, using various magnifications and images were captured.

Results

Confirmation of positivity of FMDV cell suspension

Cell suspension used in the current study were confirmed for its positivity of FMD virus by showing CPE on BHK-21 cell line (Figure 2), and FMDV specific genome detection by RT-mPCR (Figure 3a) and RT-qPCR (Figure 3b).

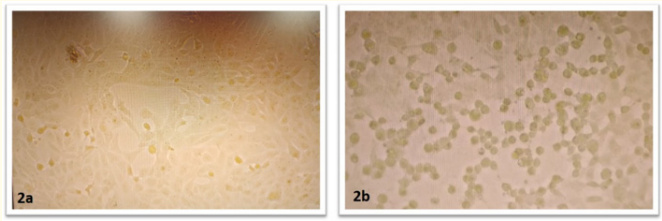

Figure 2: Photograph showing, a) normal healthy BHK-21 cell line morphology, b) FMD virus on BHK-21 cells line showed cytopathic effect (CPE) like cell swelling, rounding, and detachment of affected cells from the surface on which they cultured.

Detection of FMD virus particle in cell suspension

Positive confirmed cell suspension for FMDV by genome detection were subjected for negative staining as described in MM using 1% PTA as negative stain. Negatively stained TEM grid was screened for detection of FMDV particle by using Jeol 1400 Plus

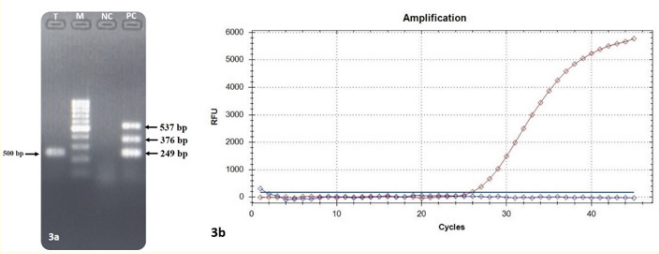

Figure 3: Confirmation of FMD virus infection BHK-21 cell suspension by multiplex PCR (RT-mPCR) and RT-qPCR molecular tool. a) agarose gel electrophoresis of RT-mPCR products showing presence of FMD serotype 'O' specific bands in test sample, 100 bp molecular weight marker, negative control and positive control in column 1 (T), column 2 (M), column 3 (NC) and column 4 (PC), respectively. Column 4 showing positive control for FMDV serotype 'O', 'A' and 'Asia 1' specific band of 249 bp, 376 bp, and 537 bp, respectively. b) Showing threshold cycle value in RT-qPCR amplification curve of foot-and-mouth disease serotype 'O' virus in cell suspension (red colour) and no template control (violet colour).

TEM, 120 kV, 100 nm scale and viral capsid appears as icosahedral shape while in negative control samples FMD virus particle were not seen (Figure 4).

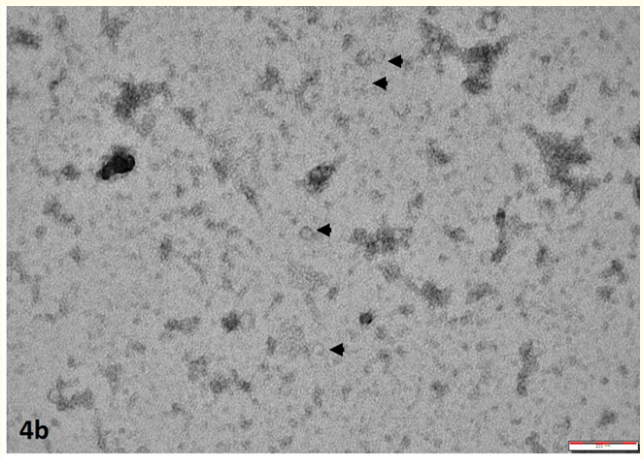

Figure 4: Showing FMD virus particle by negative staining technique using 1% aq. phosphotungstic acid (PTA) as negative stain under transmission electron microscope (TEM) in which viral capsid appears as icosahedral shape (black arrow), a) detection of FMD virus particle in BHK-21 cell suspension, b) showing detection of FMD virus, 146 s particle under TEM (black arrow). (Jeol 1400 Plus TEM, 120 kV, 100 nm scale).

Discussion

Negative staining is widely practiced technique for detection of virus isolates, virus like particle, protein molecules etc. A positive staining method for diagnosis of pathogens may vary from 4-5 days before samples was ready for examination under TEM. In contrast, negative staining technique is simple, rapid, and easy to perform for study of small particulate samples like vesicular fluid, cell suspensions etc. Use of negative staining technique in biological science is useful in diagnostic virology/pathology in which a virologist or pathologist can diagnose or discover new pathogen within 10 minutes through electron microscopy [20]. Negative staining protocol was optimized for detection of FMD virus particle in cell suspension in the present study and was performed on FMDV positive cell suspension. FMD virus particle was detected under TEM and identified on the basis of its shape and capsid morphology very quickly as reported by Gelderblom and Hazelton [20]. This icosahedral shape virion particle varies between 27- 32 nm in diameter and do not exhibit any fine structure. Present study described the

process of demonstration of FMD virus by negative staining method in cell suspension and this technique can also be used in detection of FMD virus in vesicular fluid, other fluid suspected for the presence of FMD virus. The technique has the further scope to be combined using serological methods such as immune-electron microscopy, which can further help to identify the virus serotypes of FMDV. Such an effort is in progress using serotype specific monoclonal antibodies and monospecific serum against FMDV. It is unbiased technique against nucleic acid detection because it mainly targets proteins, viral capsid or ribonucleoprotein complex [24]. Further, if the morpho diagnosis is combined and correlated with clinical findings it could be an important tool for providing provisional diagnosis and to start preventive measures and treatment without waiting the other test results.

Conclusions

Although TEM is thought be an old-fashioned technique but still it can play an important role in early detection of discovered or undiscovered viruses. For the diagnosis of FMD, a positive staining method may take 4-5 days before samples are ready for examination under TEM, while negative staining technique provides detection of FMD virus within 10 minutes through electron microscopy in laboratory. Present study described the process of demonstration of FMD virus by negative staining method in cell suspension and this technique can also be used in detection of FMD virus in vesicular fluid, other fluid suspected for the presence of FMD virus. If the morpho diagnosis is combined and correlated with clinical findings then it could be an important tool for providing provisional diagnosis and to start preventive measures and treatment without waiting the other test results**.**

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Ethical Approval

The authors declare that present work has no animal ethics issues.

Authors Contribution

Conceptualization, RR and RPS; Methology, RR and JKB; Analysis, RR; data curation, RR, JKB; writing first draft of manuscript, RR; writing-review-editing, RR, JKB, and RPS. All authors agreed on the final version of the manuscript.

Conflict of Interest Statement

Authors declare no conflict of interest.

Bibliography

1. Krüger DH., *et al.* "Sixty years ago: Helmut Ruska and the visualization of viruses". *The Lancet* 355.9216 (2000): 1713-1717.

2. Nagler FPO and Rake G. "The use of electron microscopy in diagnosis of variola, vaccinia and varicella". *Journal of Bacteriology* 55.1 (1948): 45-51.

3. Van Rooyen CE and Scott MA. "Smallpox diagnosis with special reference to electron microscopy". *Canadian Journal of Public Health* 39.12 (1948): 467-477.

4. Brenner S and Horne RW. "Negative staining method for high resolution electron microscopy of viruses". *Biochimica et Biophysica Acta* 34 (1959): 103-110.

5. Biel SS and Gelderblom HR. "Diagnostic electron microscopy is still a timely and rewarding method". *Journal of Clinical Virology* 13.1-2 (1999): 105-119.

6. Hazelton PR., *et al.* "Identification of a proposed novel agent of viral gastroenteritis". 18th Annual Meeting of the American Society for Virology; 1999 Jul 10-14; Amherst, MA. Washington: Abstract W8-8. *American Society for Microbiology* (1999): 73.

7. Kapikian AZ., *et al.* "Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious non-bacterial gastroenteritis". *Journal of Virology* 10.5 (1972): 1075-1081.

8. Madeley CR and Cosgrove BP. "Letter 28 nm particles in faeces in infantile gastroenteritis". *The Lancet* 2.7932 (1975): 451-452.

9. Plummer FA., *et al.* "An erythema infectiosum-like illness caused by human parvovirus infection". *The New England Journal of Medicine* 313 (2) (1985): 74-79.

10. Goldsmith CS., *et al*. "Ultrastructural characterization of SARS coronavirus". *Emerging Infectious Disease* 10.2 (2004): 320-326.

11. McIntish K. "Coronavirus in the limelight". *The Journal of Infectious Disease* 191.4 (2005): 489-491.

12. Rowley AH., *et al.* "Cytoplasmic inclusion bodies are detected by synthetic antibody in ciliated bronchial epithelium during acute Kawasaki disease". *The Journal of Infectious Disease* 192.10 (2005): 1757-1766.

13. Gelderblom HR., *et al.* "Negative staining in diagnostic virology". *Micron and Microscopica Acta* 22.4 (1991): 435-477.

14. Hammond GW., *et al.* "Improved detection of viruses by electron microscopy after direct ultracentrifuge preparation of specimens". *Journal of Clinical Microbiology* 14.2 (1981): 210-221.

15. Huang X., *et al.* "Establishment of persistent infection with foot-and-mouth disease virus in BHK-21 cells". *Virology Journal* 8 (2011): 169.

16. Alexandersen S., *et al.* "The pathogenesis and diagnosis of foot-and-mouth disease". *Journal of Comparative Pathology* 129.1 (2003): 1-36.

17. Biswal JK., *et al.* "Isolation and characterisation of foot-and-mouth disease virus from a captive Indian elephant (*Elephas maximus*)". *Indian Journal of Veterinary Pathology* 39.4 (2015): 376-379.

18. Grubman MJ and Baxt B. "Foot-and-mouth disease". *Clinical Microbiology Reviews* 17.2 (2004): 465-493.

19. Ranjan R., *et al.* "A Review on Foot-and-mouth disease: pathology, diagnosis and its management". *Indian Journal of Veterinary Pathology* 40.2 (2016): 105-115.

20. Gelderblom HR and Hazelton PR. "Specimen collection for electron microscopy". *Emerging Infectious Disease* 6.4 (2000): 433-434.

21. Giridharan P., *et al.* "Development and evaluation of a multiplex PCR for differentiation of foot-and-mouth disease virus strains native to India". *Journal of Virological Methods* 126.1-2 (2005): 1-11.

22. Callahan JD., *et al.* "Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus". *Journal of American Veterinary Medical Association* 220.11 (2002): 1636-1642.

23. Bisht P., *et al.* "Efficient rescue of foot-and-mouth disease virus in cultured cells transfected with RNA extracted from clinical samples". *Journal of Virological Methods* 196 (2014): 65-70.

24. King AMQ., *et al.* "Ninth Report of the International Committee on Taxonomy of Viruses". Elsevier (2012).