Control Experiments

Something Modern Virologists are allergic to-

The Scientific Method and the Reason for Controls

1. Observe a phenomenon
2. Alternate hypothesis
    - Independent variable (the presumed ***cause***)
    - Dependent variable (the observed ***effect***)
    - Control variables
3. Null hypothesis
4. Test/experiment
5. Analyze the observation/data
6. Validate/invalidate hypothesis

"A study with control(s) is designed to ensure that the effects are due to the independent variables in the experiment. The use of controls allows to study one variable or factor at a time. **It is, however, important that both the control and other (experimental) group(s) are exposed to the same conditions apart from the one variable under study.** Doing so will help draw conclusions that are more accurate and reliable."

History of the cell-culture “isolation” method

John Franklin Enders and his “discovery” of the “measles virus” in the 1950s

Materials and methods. Collection of specimens. **Throat washings**, **venous blood** and **feces** were obtained from 7 patients as early as possible after a clinical diagnosis of measles was established. In 5 instances the time at which specimens were collected in relation to the onset of exanthem is given in the case histories described below or in Table I. When capable, patients were asked to gargle with 10-15 ml of sterile neutralized fat-free milk. Certain specimens from the throats of younger children were obtained by cotton swab previously moistened in milk. After swabbing **the throat the swab was immersed in 2 ml of milk**. **Penicillin, 100 u/ml, and streptomycin, 50 mg/ml** were added to all throat specimens which were then centrifuged at 5450 rpm for about one hour. Supernatant fluid and sediment resuspended in a **small volume of milk** were used as separate inocula in different experiments in amounts varying from 0.5 ml to 3.0 ml. About 10 ml of blood immediately after withdrawal were placed in tubes containing 2 ml of 0.05% solution of **heparin**. As inocula for tissue cultures amounts varying from 0.5 ml to 2.0 ml of the whole blood were employed. After addition of **antibiotics as described above** 10% fecal suspensions were prepared by grinding the material in **bovine amniotic fluid medium**. The suspensions were then centrifuged at 5450 rpm for about one hour and the supernatant fluids used as inocula, in amounts varying from 0.1 ml to 3 ml. All specimens were refrigerated in water and ice or maintained in the cold at about 5°C from the time of collection until they were added to the cultures. The maximum time that lapsed between collection of specimens and inoculation was 3 5 hours.

Tissue culture technics. In the initial isolation attempts roller tube cultures(1112) of human kidney, human embryonic lung, human embryonic intestine, human uterus and rhesus monkey testis were employed. Subsequent passages of the agents isolated were later at- tempted in human kidney, human embryonic skin and muscle, human foreskin, human uterus, rhesus monkey kidney and embryonic chick tissue. Stationary cultures prepared according to the technic of Youngner(13) with **trypsinized** human and rhesus monkey kidney were later employed for isolation of agents and their passage. The culture medium consisted of **bovine amniotic fluid (go%), beef embryo extract (50/0), horse serum *(5%)*, antibiotics, and phenol red** as an indicator of cell metabolism(12). **Soybean trypsin** inhibitor was added to this medium unless it was used for the cultivation of human and monkey kidney

(11). Fluids were usually changed at intervals of 4-5 days. For histological examination the cell growth after fixation in 10% **formalin** was embedded in collodion, dehydrated and stained with **hematoxylin** and **eosin**.

History of the cell-culture “isolation” method

John Franklin Enders and his “discovery” of the “measles virus” in the 1950s

Throat, Blood and Poop Samples,

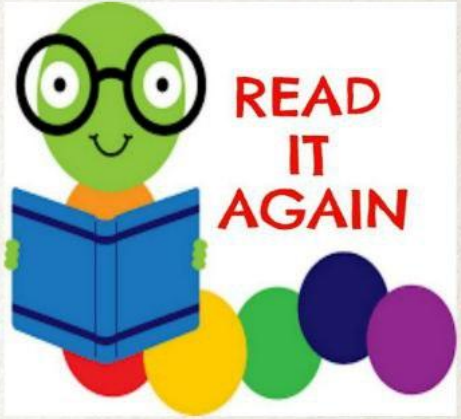
Milk,
Streptomycin
Penicillin
Bovine Amniotic Fluid
Beef Embryo Extract
Horse Serum
Antibiotics
Formaldehyde
Hematoxylin
Eosin
Soybean
Trypsin
Phenol Red
On a monkey kidney cell
CPE occurred
Fragments were called “viruses”

“The cytopathic changes it induced in the unstained preparations could not be distinguished with confidence from the viruses isolated from measles.”
-Enders

A second agent was obtained from an uninoculated culture of monkey kidney cells. The cytopathic changes it induced in the unstained preparations could not be distinguished with confidence from the viruses isolated from measles. But, when the cells from infected cultures were fixed and stained, their effect could be easily distinguished since the internuclear changes typical of the measles agents

measles. But, when the cells from infected cultures were fixed and stained, their effect could be easily distinguished since the internuclear changes typical of the measles agents were not observed. Moreover, as we have already indicated, fluids from cultures infected with the agent failed to fix complement in the presence of convalescent measles serum. Obviously the possibility of encountering such agents in studies with measles should be constantly kept in mind.

Discussion. Of the numerous experiments that have been reported in the past describing the successful isolation of the etiologic agent of measles only those in which monkeys were employed as the experimental animal have been consistently confirmed by other workers. Great caution should therefore be exercised in the interpretation of any new claims that the virus has been propagated in other hosts or systems. Accordingly, the results that are summarized here must be subjected to the most critical analysis.



may, therefore, be applied to the study of these agents in the same manner as cultures of human kidney. In so doing, however, it must be borne in mind that cytopathic effects which superficially resemble those resulting from infection by the measles agents may possibly be induced by other viral agents present in the monkey kidney tissue (*cf.* last paragraph under G) or by unknown factors. In a few cultures of human prepucial tissue inoculated with one of the measles agents

Infection of Monkey Kidney Tissue Cultures with Virus-Like Agents.* (21478)

ROBERT RUSTIGIAN, PAUL JOHNSTON, AND HELEN REIHART.
(Introduced by S. A. Koser.)
From the Department of Microbiology, University of Chicago.

Summary. During attempts to adapt dengue virus to rhesus monkey kidney cultures, an unidentified agent which causes formation of syncytial masses and vacuolation in such cultures was encountered. Subsequently, 3 additional agents with similar cytopathogenic effects were passed and maintained in HeLa cell cultures from uninoculated monkey kidney cultures. Renal tissue and not the medium constituents is the source of the agent. Bacteriological studies with one agent were negative. The same agent passed through a Selas filter. Accordingly it is considered to be virus-like in nature. Similar experiments were not done with 3 other agents but because of certain common characteristics are believed to be of the same nature.

Recovery of agents MK1, MK3, and MK4 from uninoculated monkey kidney cultures. Shortly after encountering agent MK-D in attempts to adapt dengue virus to monkey kidney cultures, **syncytial masses and vacuolatinn were again observed in an uninoculated roller tube culture 12 days after its preparation.**

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Fluorescent Antibody and Complement-Fixation Tests of Agents Isolated In Tissue Culture from Measles Patients.*

(21957)

SOPHIA M. COHEN, IRVING GORDON, + FRED RAPP, JOHN C. MACAULAY, + AND SONJA M. BUCKLEY.

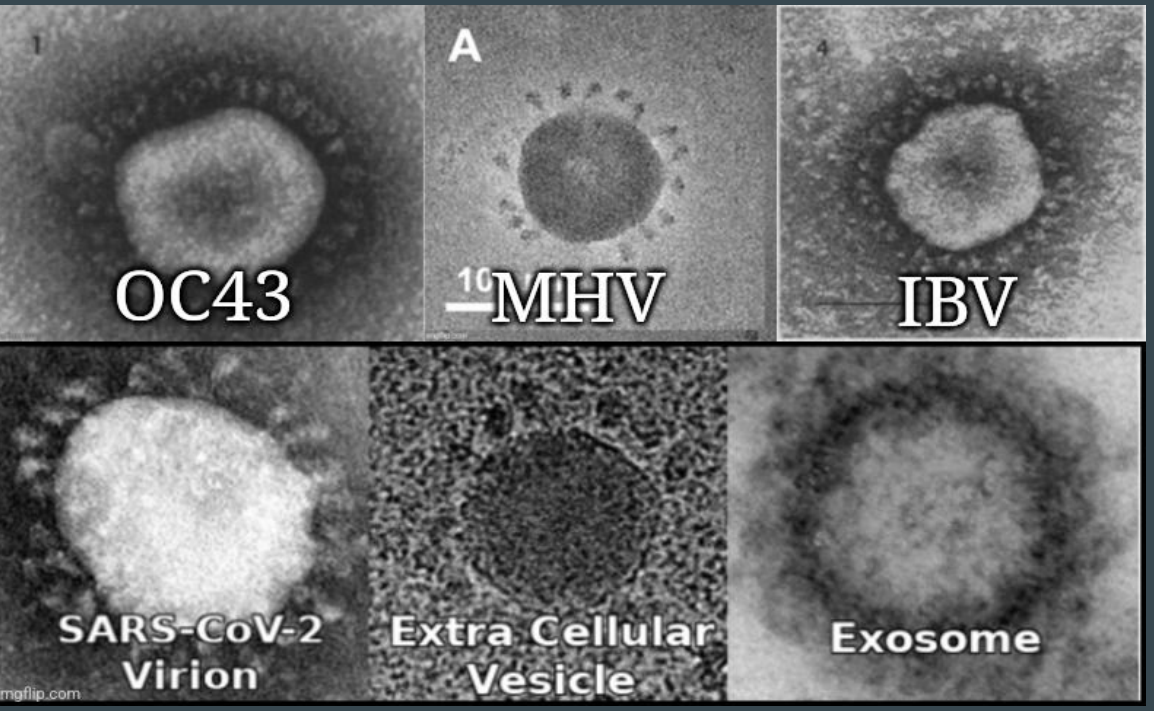
From the Division of Laboratories and Research, N. Y. State Department of Health, Albany, and Department of Pediatrics, Albany Medical College.

Enders and Peebles (1) and Rustigian et al. (10) encountered latent virus-like agents that induce marked vacuolization and syncytial masses in monkey kidney tissue cultures. The cellular degeneration characteristic of these "monkey-kidney agents" frequently appeared in our cultures, both in those inoculated with specimens from measles patients and in controls; hence cytologic criteria for recognition of measles agents were difficult to apply. In some tissue culture series the "monkey-kidney agents" destroyed the cell sheets in 10 to 14 days.

› Acta Pathol Microbiol Scand. 1958;42(1):75-85.
Studies on measles virus in monkey kidney tissue cultures. 1. Isolation of virus from 5 patients with measles
V BECH, P VON MAGNUS
PMID: 13508250
As described by Enders & Peebles (6), and later by Rustigian et al. (13) and by Cohen et al. (3) cytopathic changes similar to those caused by measles virus may be observed also in uninoculated cultures of monkey kidney tissue (Figs. 4-5). These changes are probably caused by virus-like agents, so called "foamy agents", which seem to he frequently present in kidney cells from apparently healthy monkeys.

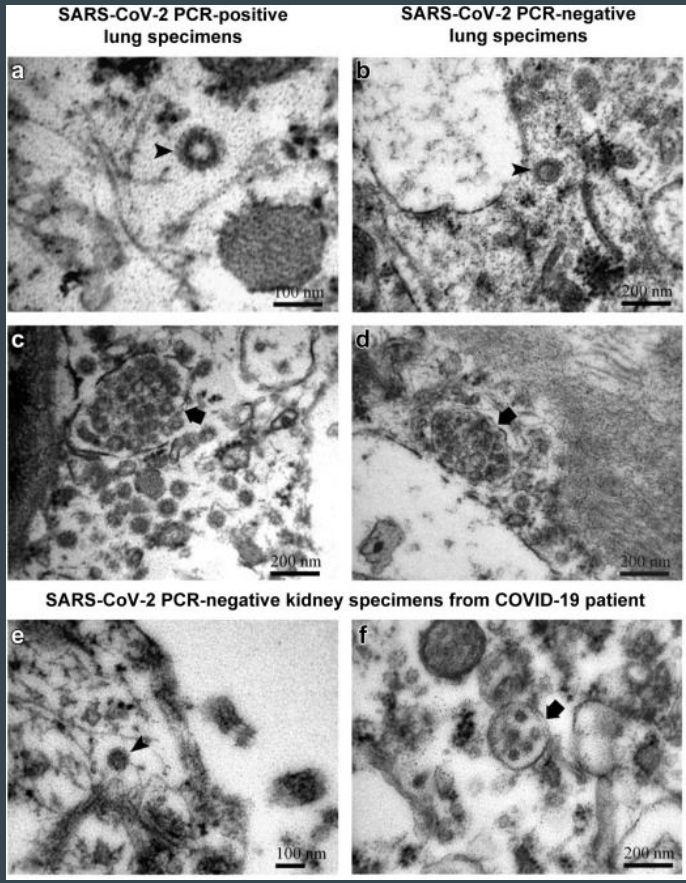
However, monkey kidney viruses or "foamy agents" may give rise to cellular degenerations which microscopically are indistinguishable from those caused by measles virus. For this reason the cytologic manifestations are of limited value in the study of measles and additional criteria are required to establish the identity of the cultivated agents.

The ol’ “Point and Declare” method



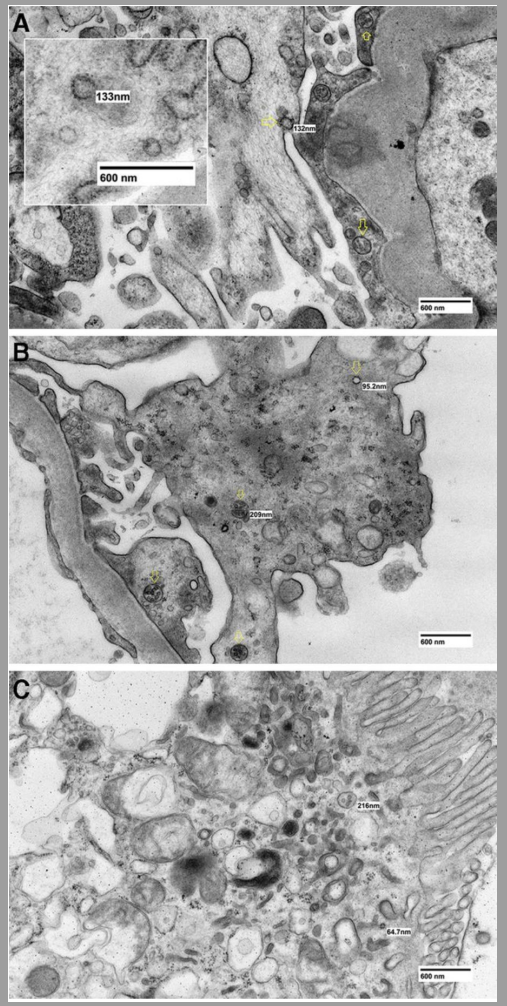
SARS-CoV-2 Virions or Ubiquitous Cell Structures? Actual Dilemma in COVID-19 Era

"Figure 1 Individual vesicle with electron-dense coat (arrowhead) located freely in the cytosol of endothelial cell in lung with positive reverse-transcriptase polymerase chain reaction (RT-PCR) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA (a) and in lung with negative RT-PCR for SARS-CoV-2 RNA (b). **Note similar morphology of the 2 structures in images (a) and (b), which could be virus or coated vesicle.** In view of the RT-PCR results, the observed structures **might be virus in image (a)** but not in image (b). Vacuole with many small vesicles inside the limiting membrane (arrow) in the cytosol of endothelial cell in lung with positive RT-PCR for SARS-CoV-2 RNA (c) and in lung with negative RT-PCR for SARS-CoV-2 RNA (d). Note again similar morphology of the 2 structures in images (c) and (d), **which could be a cluster of viral particles or multivesicular bodies (MVBs) with intralumenal vesicles inside.** In view of the RT-PCR results, the observed structures **might be a cluster of viral particles in (c)** but not in (d). (e,f) Structures resembling virions, coated vesicles or MVBs were observed in the cytosol of kidney podocytes in a SARS-CoV-2–positive patient but with negative RT-PCR for SARS-CoV-2 RNA. In view of the RT-PCR results, **the presented structures are not viruses but ubiquitous coated vesicles and MVBs."**



Appearances Can Be Deceiving…

“we have observed morphologically indistinguishable inclusions within podocytes and tubular epithelial cells both in patients negative for coronavirus disease 2019 (COVID-19) as well as in renal biopsies from the pre–COVID-19 era.”

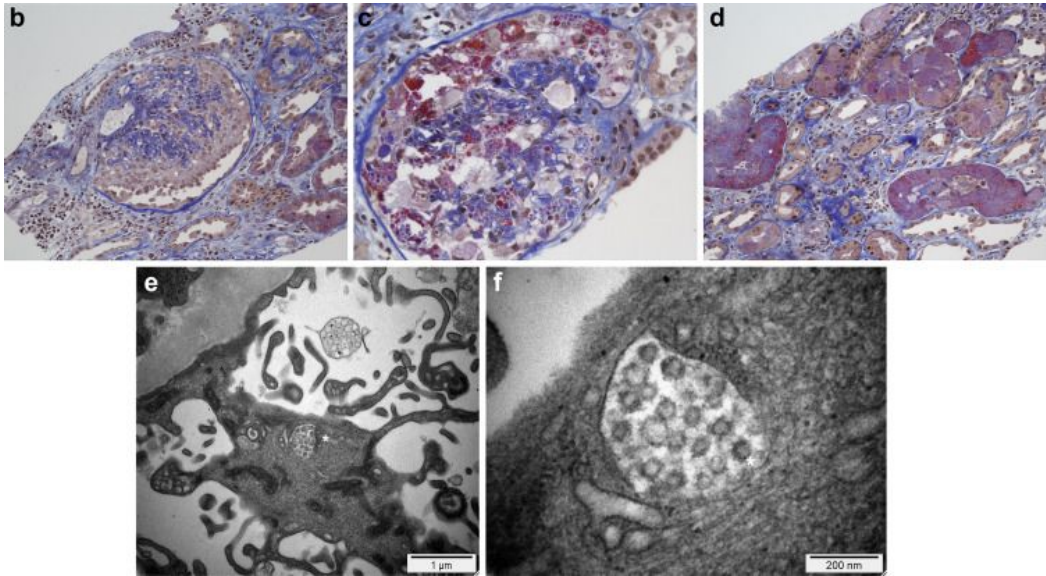


Source: (Appearances Can Be Deceiving-Viral-like Inclusions in COVID-19 Negative Renal Biopsies by Electron Microscopy. Kidney360. https://kidney360.asnjournals.org/content/1/8/824)

Electron microscopy of SARS-CoV-2: a challenging task

“We read with interest the Correspondence by Zsuzsanna Varga and colleagues on the possible infection of endothelial cells by SARS-CoV-2 using electron microscopic (EM) images as evidence. However, we believe the EM images in the Correspondence do not show coronavirus particles but instead show cross-sections of the rough endoplasmic reticulum (RER).

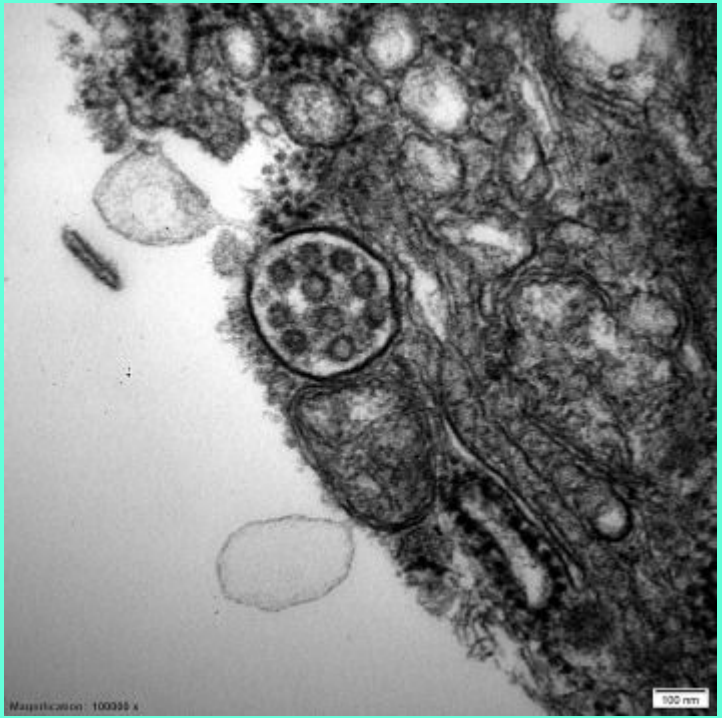
Just recently, there have been two additional reports, in which structures that can normally be found in the cytoplasm of a cell have been misinterpreted as viral particles. EM can be a powerful tool to show evidence of infection by a virus, but care must be taken when interpreting cytoplasmic structures to correctly identify virus particles.”



Source:
(Electron microscopy of SARS-CoV-2: a challenging task - The Lancet)

Multivesicular Bodies Mimicking SARS-CoV-2 in Patients Without COVID-19

"Recent publications in *Kidney International* used electron microscopy (EM) to detect the virus in autopsy or biopsy specimens of the kidney. Most of the published images depicting the suspected virus are very similar, if not identical, to multivesicular bodies (MVBs). MVBs have been well-known since the 1960s and their appearance and occurrence is detailed in the classic monograph of Feroze Ghadially; however, their exact significance and function is unclear. We suspect that the EM images of SARS-CoV-2 published to date are in fact MVBs."



Source: (Multivesicular bodies mimicking SARS-CoV-2 in patients without COVID-19 - Kidney International. kidney-international.org)

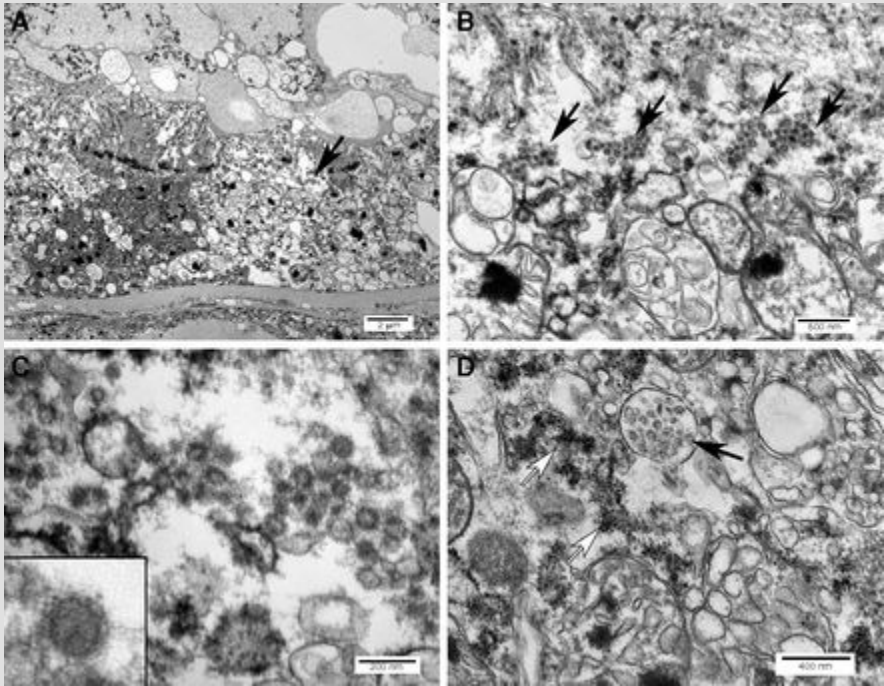
Caution in Identifying Coronaviruses by Electron Microscopy

"The evidence provided in the article by Farkash *et al.*[8] in *JASN* likewise does not confirm the presence of SARS-CoV-2 in kidney tissue.

In the article by Farkash *et al.*, the electron microscopic images in their Figure 3, A–C do not demonstrate coronaviruses. Rather, the structures described as virus are clathrin-coated vesicles (CCVs), normal subcellular organelles involved in intracellular transport.

Additionally, Farkash *et al.* document their findings by referring to an article by Su *et al.* that purports to have identified coronavirus in kidney. Likewise, that article shows only normal cell structures that, to the non-electron microscopist virologist, may resemble coronavirus. Their interpretation has been refuted in Letters to the Editor of *Kidney International*.

Identification of viruses is not always straightforward. Consideration should be given to the mechanism of virus production, including the location inside of cells, as well as the appearance (size, shape, internal pattern of the nucleocapsid, and surface spikes). Care should be taken to prevent mistaking cell organelles for viral particles."



Source: (Caution in Identifying Coronaviruses by Electron Microscopy | American Society of Nephrology. asnjournals.org)

Why misinterpretation of electron micrographs in SARS-CoV-2-infected tissue goes viral

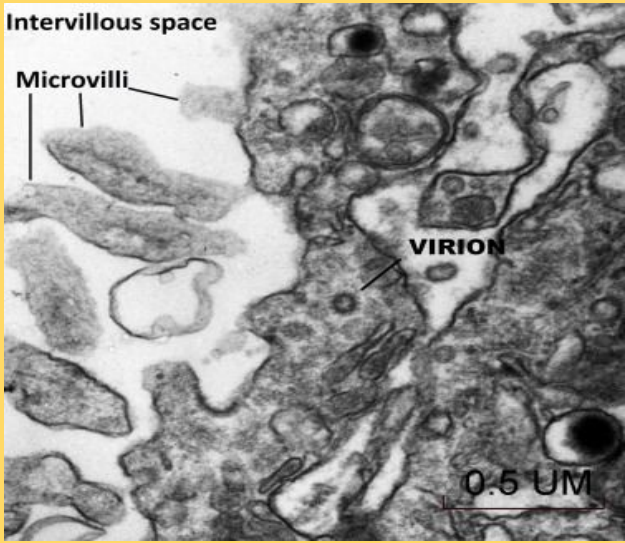
“Nevertheless, ultrastructural details in autopsy tissues have been misinterpreted as coronavirus particles in recent papers. Bradley and colleagues described ‘coronavirus-like particles’ in autopsy specimens of the ‘respiratory system, kidney, and gastrointestinal tract’, and in a case report Dolhnikoff and colleagues described ‘viral particles’ in ‘different cell types of cardiac tissue’ of a deceased child. However, the images in these publications show putative virus particles that lack sufficient ultrastructure for an unambiguous identification as virus. Some of these particles definitely represent other cellular structures, such as rough endoplasmic reticulum (eg, Dolhnikoff and colleagues,4 figure 3B), multivesicular bodies (Bradley and colleagues,3 figure 5C) and coated vesicles (Bradley and colleagues,3 figure 5B, G). Moreover, it is remarkable that Dolhnikoff and colleagues referred to findings, described by Tavazzi and colleagues, of ‘viral particles’ in interstitial cells, which are clearly non-viral structures, such as coated vesicles. Furthermore, Bradley and colleagues quoted publications as a reference for their virus particle identification, which, in our opinion, both identified non-coronavirus structures as coronavirus particles, as already discussed by Goldsmith and colleagues and by Miller and Brealey.

As diagnostic EM requires both specialised staff and expensive equipment, and has been replaced by other methods (eg, immunohistochemistry) in several fields of application, its use has been in decline in the past decades, resulting in irreversible loss of expertise that now becomes dramatically overt during the SARS-CoV-2 pandemic. This dilemma of diagnostic EM should alarm us all, as misleading information on the presence of SARS-CoV-2 in tissue has already made its way into the scientific literature and seems to be perpetuated.”

Source: (Why misinterpretation of electron micrographs in SARS-CoV-2-infected tissue goes viral - The Lancet)

Alternative interpretation to the findings reported in visualization of SARS-CoV-2 invading the human placenta using electron microscopy

"The report of virus-like inclusions in syncytiotrophoblast is intriguing and thought-provoking. However, I respectfully offer an alternative interpretation of the data. The structures identified as SARS-CoV-2 virions look exactly like clathrin-coated pits or vesicles.
Clathrin-coated vesicles are spherical structures employed by trophoblasts and other cell types to internalize cargos from the extracellular space. Coated vesicles and coated pits derive their name from
the external scaffold coat composed of clathrin triskelions that decorate
the surface of the structure. In transmission electron micrographs in which tissue-thin sections are stained with uranyl acetate and lead citrate,
coated vesicles have an electron-dense studded surface that appears identical to the "corona" comprising the spike protein that decorates all
coronaviruses, including SARS-CoV-2 virions. It is this studded surface or
corona that gives the genus *Betacoronaviridae* its characteristic morphology and name."



Source: (Alternative interpretation to the findings reported in visualization of severe acute respiratory syndrome coronavirus 2 invading the human placenta using electron microscopy - American Journal of Obstetrics & Gynecology. ajog.org)

Stefan Lanka's Control Experiments

Measles control experiment - CPE

- Vero cell lines(CCL-81 and hS-LAM) with various agents and concentrations of antibiotics in the combination with penicillin/streptomycin
- Added throat swab from a male cat and throat fluid from a person with a measles infection
- Both tissue cultures contained only 1% FBS

Result: The head of one of the labs said: The CPE that were found were microscopically identical to the syncytia formation described as the measles virus

Control experiment 1 - CPE

- Used commercial human primary epithelial(tissue that forms the covering on all internal and external surfaces of your body) cells
- Used various levels of antibiotics(1-3x) and nutrient levels with DMEM(for supporting the growth of cells)
- 1-10% FBS(Fetal Bovine Serum) and took pictures daily.

Result: CPE was observed with and without the yeast RNA where the added yeast RNA intensified the CPE

Control experiment 2 - SARS-CoV-2 genome

- Took the nucleic acids from a healthy human sample
- 12 cycles with PCR using strict protocol he got 20% of the SARS-CoV-2 genome
- 30 cycles with the strict protocol he got 98.5%
- With the same error rate as the "virologists" are using then he got 100% of the genome
- "Virologists" are using up to 40-45 cycles.
- Mind you that "virologists" also added synthesized small sequences in such a high amount that it covers later 20% of the alleged genome

Result: The SARS-CoV-2 genome could be completely assembled out of a healthy human sample

Control experiment 3 - Reference assembly of other genomes

- The original SARS-CoV-2 sequence data was used for comparison
- Evidence is lacking that only viral nucleic acids were used to construct the claimed viral genome for SARS-CoV-2
- With respect to the construction of the claimed viral genome strand, no results of possible control experiments have been published
- Looked for the structural similary of other alleged genomes SARS, HIV, hepatitis delta, measles, Zika, Ebola or Marburg

Result:
- The original SARS-CoV-2 genome published by Fan Wu, et al could not be reproduced by the methodology described in their own paper
- Hypothesis of a possible unintentional amplification of sequence reads not associated with SARS-CoV-2
- Possibility of accidental generation of sequences that were not present in the initial sample but were generated only by the PCR conditions
- The PCR protocols are calibrated to sequences of unconfirmed origin that are clearly found in many humans and apparently other things as well
- Fan Wu, et al could have found better matches for “HIV” and “Hepatitis D virus” than “a new coronavirus”