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Chapter 14

Veterinary Diagnostic using Transmission Electron Microscopy

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Additional information is available at the end of the chapter

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Abstract

Transmission electron microscopy has been an excellent tool, essential for the diagnosis of bacterial and viral animal diseases. Four basic techniques have been widely used: negative staining (rapid preparation), immunoelectron microscopy, immunolabeling with colloidal gold particles and resin embedding. The negative staining technique (rapid preparation) is the most applied, due to its speed, simplicity and specificity and can be used in various clinical specimens – such as feces, semen, urine, serum, organ fragments, crusts, body fluids, cell culture suspension, oral, ocular and fecal swabs, among others –, in which the agents can be directly viewed in large numbers in the samples. The immunoelectron microscopy technique using a specific primary antibody promotes the clumping of particles, also allowing the serotyping of the agents. In the immunolabeling with colloidal gold technique antigen-antibody reaction is enhanced by marking the antigen colloidal gold particles associated with protein A. The method of resin embedding, followed by ultrathin sections of cells or infected tissues can monitor the different stages of maturation viruses or bacteria and their behavior inside of host cells by determining not only the infection, but also the course of the disease in farms. The techniques can be applied to all animal species, either large or small, including aquatic and wild animals. Its implementation allows rapid diagnosis, providing subsidies for the immediate institution of prophylactic measures, and control and prevention of bacterial and viral animal diseases.

Keywords: Veterinary diagnostic, Negative staining, Immunoelectron microscopy, Immunolabeling with colloidal gold particles, Resin embedding, Transmission electron microscopy

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1. Introduction

In recent decades there has been a major advance in the diagnosis field, allowing the development mainly of sophisticated techniques at the molecular level, facilitating increasingly improved detection of diseases of viral and bacterial origin.

Despite this progress, electron microscopy remains an excellent and sensitive tool in the application of such techniques, and they are essential for the rapid diagnosis of these agents [1,2,3].

Four basic techniques have been widely used: negatively stained (rapid preparation), immunoelectron microscopy, immunolabeling with colloidal gold particles and resin embedding [4].

2. Negative staining (Rapid preparation)

The negative staining technique is the main method of electron microscopy, as well as the most used, due to its ease in preparation and speed in obtaining results, which are achieved in 5-10 min [1,5,6]. It was developed by Brenner & Horne in 1959 [7], when the viral particles could be viewed at the ultrastructural level [8].

The technique utilizes an electron opaque substance to surround the virus or another biological structure, showing contrast between the electron lucent biological material and the background against which it is viewed. The image formation is the result of electron being absorbed or deflected by the stain [9,10].

To develop the samples, copper or nickel grids measuring from 200-400 meshs are used and require plastic film pretreatment before being covered with carbon. Several types of plastic films are used for this purpose, with Formvar, Parlodion and Pioloform being the most common ones [11,12]. In our laboratory we use the Parlodium 0.5% in amyl acetate, followed by deposition of thin carbon under a metallizer.

Various types of contrasting (heavy metals salts) are used to provide better contrast to agents. The most used are the phosphotunstic acid (PTA), uranyl acetate, sodium silicotungstate, methylamine tungstate and ammonium molybdate 11,12].

We chose the 2% ammonium molybdate and pH5.0 as standard contrasting in our laboratory, in fact, in order to provide a soft contrast in all types of samples, clearly showing ultrastructural detail, such as the envelope covered with spikes and tape nucleocapsid shaped "Hering-bone", found in paramyxovirus [4,13,14].

This heavy metal has been used to contrast negatively many virus species [15].

The technique can be applied in various types of samples, such as feces; urine; serum; organ fragments; crusts; vesicular and peritoneal fluid; semen; ocular; nasal and fecal swabs; epithelium; cell culture supernatant; and others [16,17,1,18,19,20,21,22,6].

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Small amounts of samples (1 drop of 40 μl) are necessary for the technical processing [6].

Samples must be collected and maintained immediately under refrigeration (4º C) before being transported and sent to the electron microscopy laboratory to ensure preservation of the morphology of viral particles [19].

In this technique the samples are suspended and drops collected from the surface of the suspension are transferred to metallic grids, which are then negatively contrasted [7,23,24,25, 26,27,28,29,20,15,30,19,6].

The rapid diagnosis of viral agents is accomplished through the comparison between the dimensions and specific morphology of the visualized particles and the other taxonomically combined viral families. The viruses can be morphologically classified according to their symmetry, which can be helical, cubic, icosahedral or complex [31]. The more commonly used criteria for the taxonomic classification are the following: dimension, shape and structure; shape of the viral capsid; presence or absence of the envelope; and surface projections. Complete and incomplete particles, as well as empty capsids, can also be distinguished [4,2].

The most commonly found viruses in cases of diarrhea outbreaks in several animal species are the coronavirus (fig. 1), rotavirus (fig. 2), adenovirus (fig. 3), astrovirus, and parvovirus, which can be seen in large numbers in feces, fecal swabs or intestine fragments [1,4,13,6].

Viruses that cause respiratory diseases such as influenza virus (fig. 4), coronavirus, and paramyxovirus (fig. 5), can also be easily detected in lung fragments, lung washes and nasal discharge [1,13].

Other viruses responsible for important reproductive diseases such as herpesvirus (infectious bovine rhinotracheitis), flavivirus (bovine viral diarrhea virus) (fig. 6), parvovirus (porcine parvovirus), herpesvirus (Aujeszky's disease), and circovirus (circovirosis swine) can be widely found in organ fragments [13].

Negative staining also plays an important role in the rapid investigation of viral diseases that cause skin lesions caused by herpes virus (fig. 7), poxvirus (figs. 8, 9) and papillomavirus (fig. 10) in fragments of lesions, crusts, epithelium and vesicular fluid [1,19,6,12,20,32,33,34].

The large brick-shaped orthopoxvirus can readily be distinguished from the parapoxvirus and the smaller icosahedral herpesvirus [35], being one of the most recommended techniques by the OIE for the accomplishment of the laboratorial diagnosis of the virus in skin lesion [36].

This technique also allows the detection of different viral particles in the same sample [13,6], especially in cases of diarrhea caused by the simultaneous presence of rotavirus and coronavirus (fig.11).

The negative staining can also be applied to identify the presence of a few types of bacteria of easy morphological identification, such as Leptospira (fig. 12), mycoplasma (fig. 13) and other bacteria types found in specific cell cultures.

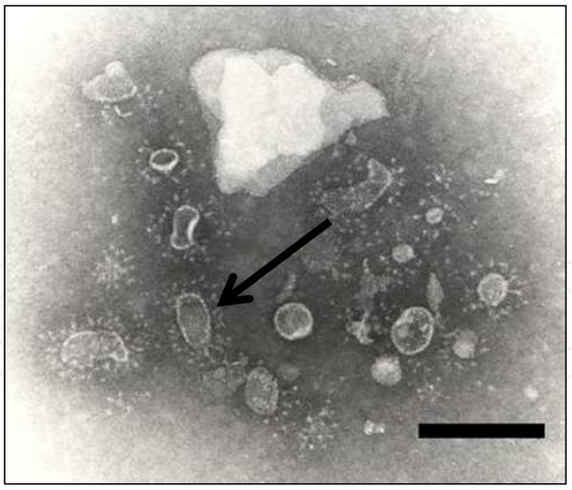


Figure 1. Negatively stained coronavirus particles showing characteristic radial projections forming a corona, in feces of *Mazama gouazoubira* (arrow). Bar: 200 nm.

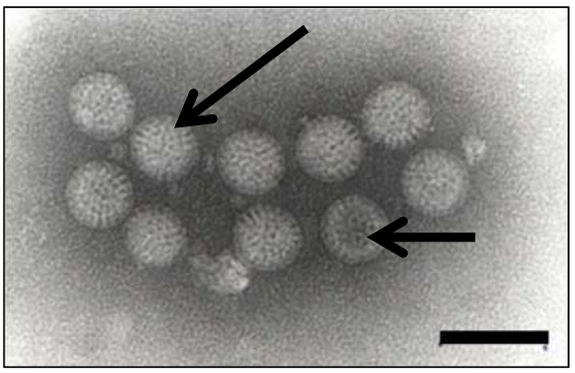


Figure 2. Negatively stained rotavirus, showing complete" (big arrow) and "empty" (minor arrow) particles in feces of swine. Bar: 110 nm.

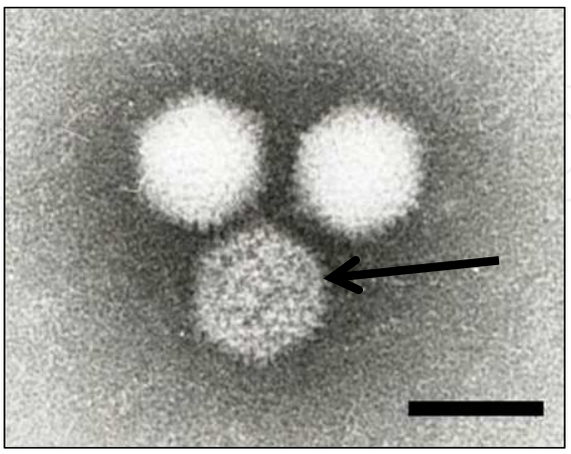


Figure 3. Negatively stained adenovirus particles, exhibiting the hexagonal outline formed by the distinct capsomers (arrow) in feces of pigeon. Bar: 70 nm.

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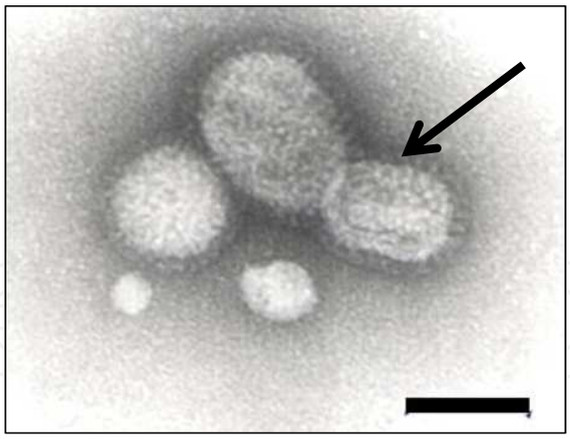


Figure 4. Influenza virus in feces of ferret, showing well defined spikes (arrow). Bar: 80 nm.

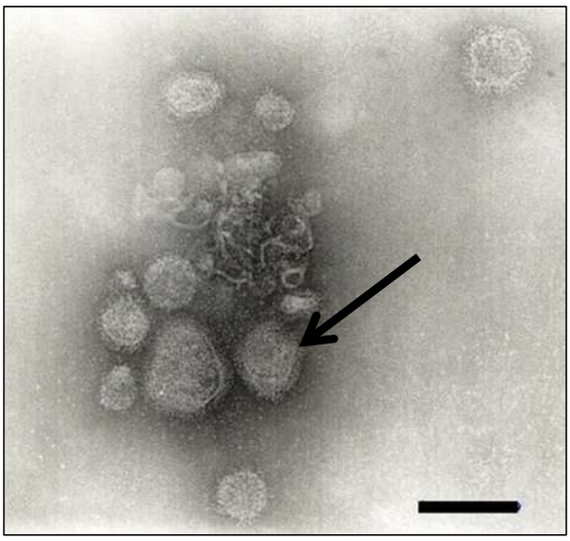


Figure 5. Negatively stained paramyxovirus particles, pleomorphic,roughly spherical, containing an envelope covered by spikes (arrow) in feces of parrot. Bar: 140 nm.

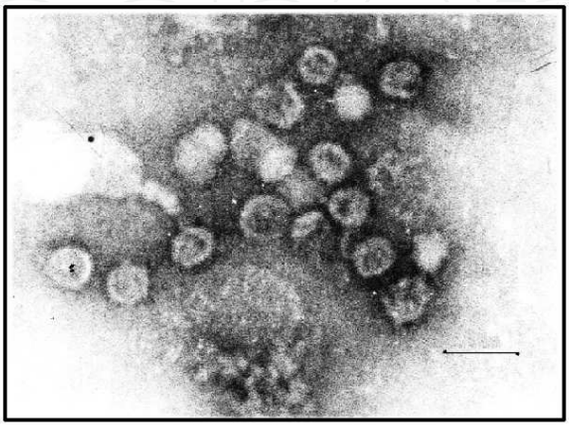


Figure 6. Flavivirus isometric particles in bovine intestine. Bar: 70 nm.

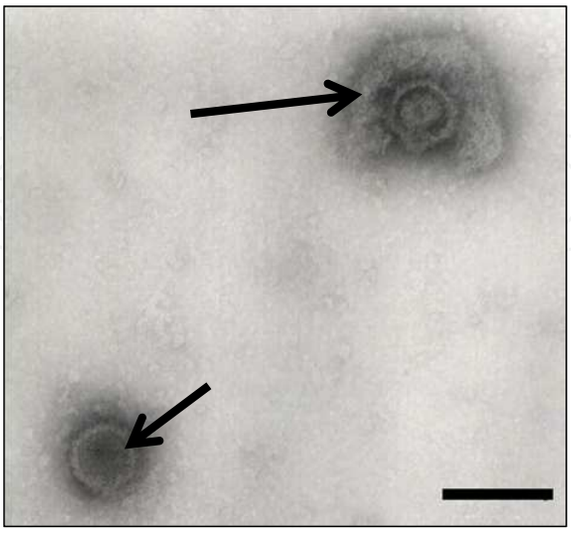


Figure 7. Herpesvirus in skin lesion of parrot, showing enveloped (big arrow) and non-enveloped particles (minor arrow). Bar: 100 nm.

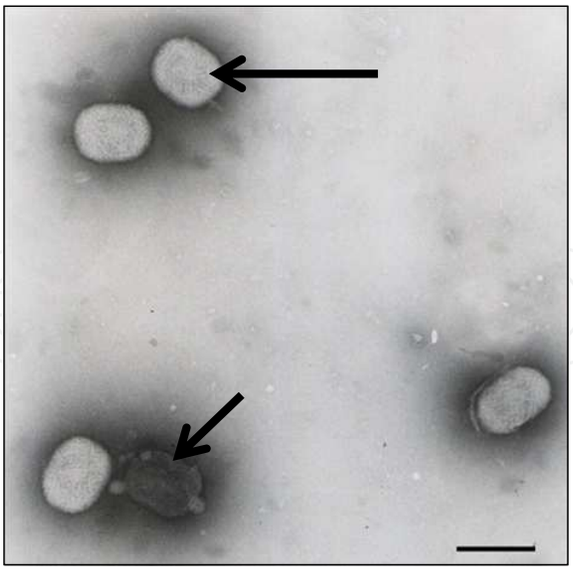


Figure 8. Negatively stained poxvirus showing regular spaced thread-like ridges comprising the exposed surface (big arrow) and enveloped particle (minor arrow). Bar: 320 nm.

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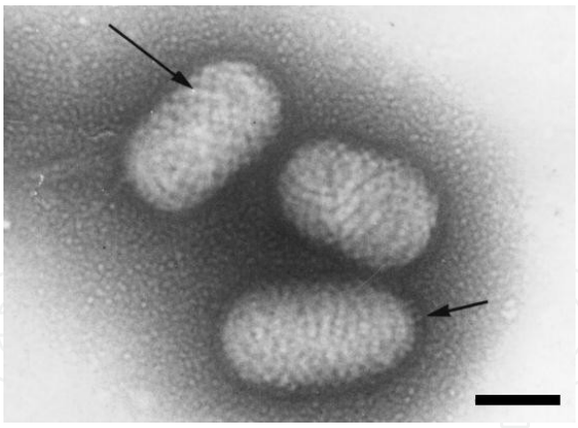


Figure 9. Parapoxvirus particles with a distinctive criss-cross filament pattern derived from the superimposition of upper and lower virion surfaces (big arrow) and projections surface (minor arrow). Bar: 110 nm.

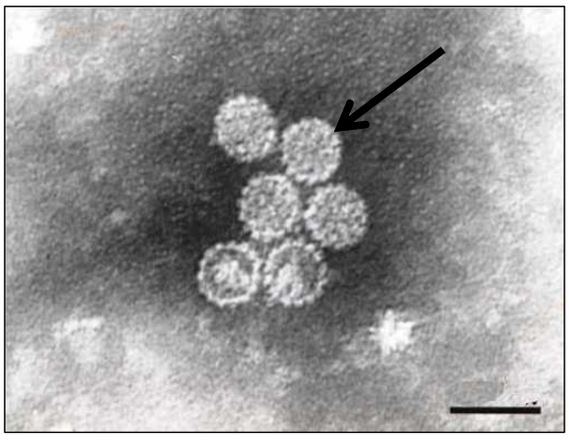


Figure 10. Negatively stained papillomavirus particles exhibit distinct, isolated capsomers (arrow). Bar: 70 nm.

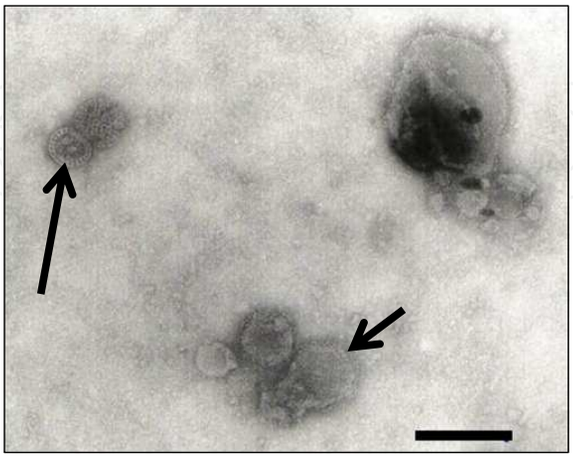


Figure 11. Simultaneous presence of particles of rotavirus (big arrow) and coronavirus (minor arrow) in feces of cattle. Bar: 150 nm.

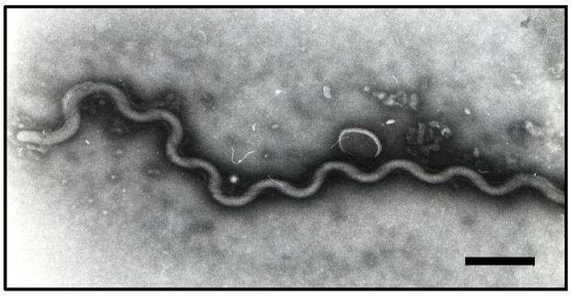


Figure 12. Negatively stained *Leptospira interrogans*. Bar: 1000 nm.

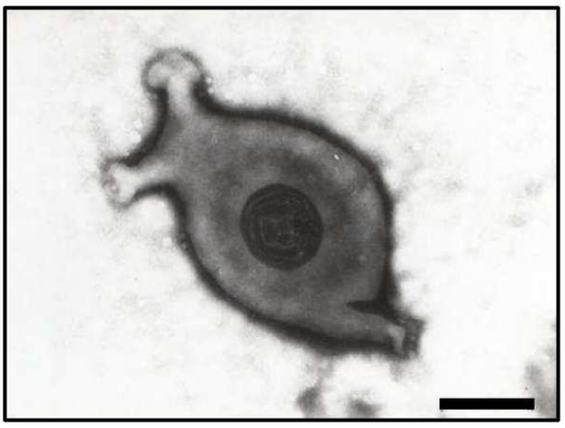


Figure 13. Negatively stained *Mycoplasma gallisepticum*. Bar: 140 nm.

2.1. Immunoelectron microscopy technique

The immunoelectron microscopy technique is an antigen-antibody reaction which promotes increased sensitivity in 100-fold when using a specific antibody connected to a known antigen [35,37,11].

It was first developed for quantifying plant virus by Derrick (19730 [38] being subsequently used in various types of clinical specimens [39,40,41,42].

Used when the number of agent particles in a sample is very low, it allows identification of the agent for specific antigen-antibody reaction - such identification can be achieved by its morphology.

The technique does not require purification of antibodies and detects contaminating antigens and/or antibodies [37]. It is also used to serotype morphologically similar (but antigenically distinct) particles [43,9].

Three variations of the method have been described, immune clumping or direct immunoelectron microscopy [4,44,45] solid phase immune electron microscopy (SPIEM) [38], and decoration [46].

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In the immune clumping method, an antigen suspension is blended with an equal volume of specific antibody against the said antigen; the suspension is incubated for 1 h at 37º C and negatively contrasted to allow the execution of the antigen-antibody reaction. The particles of the agent create aggregation through the reaction with its homologous antibody [4,9].

This procedure has been used to facilitate the detection and distinction of small viral particles from the background debris present in clinical specimens [47].

The sensitivity of immunoelectron microscopy can be increased by applying the most used SPIEM method in our laboratory.

In this technique, a grid is previously prepared with colodium film and sensitized with the antibody, cleaned with PBS, incubated with the antigen and negatively contrasted. As a result of the antigen-antibody reaction, the particles of the agent are agglutinated, creating groups on the grid surface (fig. 14) [4,9].

Several types of viruses can be added by this technique, such as flaviviruses, rotavirus, poxvirus, paramyxovirus, and parvovirus, among others.

Immunoelectron microscopy was used to trap particles of equine herpesvirus in organ fragments in cases of abortion [48].

The technique can also be applied to trap some types of bacteria and mycoplasma.

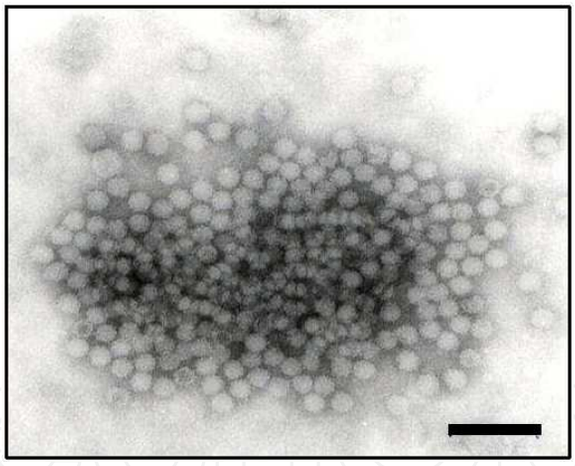


Figure 14. Immunoelectron microscopy of parvovirus particles aggregated by antigen-antibody interaction in suspension of swine liver. Bar: 120 nm.

2.2. Decoration technique

In the decoration or antigen-coated grid technique, the antibodies are used to "decorate" or coat viruses [37,46].

Carbon-coated electron microscope grids are incubated with a specific antibody that is used to trap antigen particles found in a suspension. After the trapping, the treated grids are washed and negatively stained. The antigen particles covered in a dense and relatively continuous

antibody coat, which bypasses the said particles, result in a “decoration”. Such a feature indicates a positive reaction [49,50].

This technique can be used to detect very small viruses or to serotype virus [15].

Some types of bacteria such as Leptospira can be perfectly "decorated" by this technique (fig. 15).

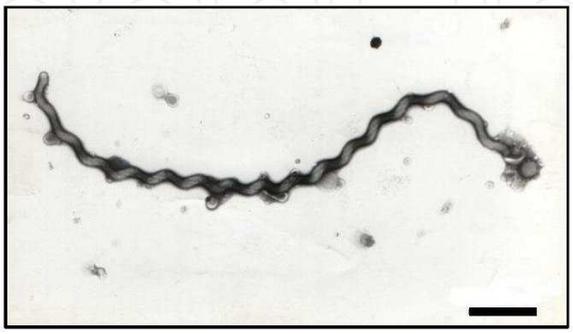


Figure 15. *Leptospira interrogans* “decorated” by decoration technique. Bar: 1000 nm.

2.3. Immunolabeling with colloidal gold particles by negative staining technique

Immunocytochemical techniques are powerful tools for the localization of special antigens utilizing a specific marker 51].

Tracers or markers are exogenously administered substances that, when visualized in electron microscopy preparations, provide valuable information about cell compartments, junctional elements, and cells surfaces [24].

The antigen marking plays a fundamental role in the identification of difficult viruses to be visualized or those with a low titer [23].

Two types of markers have been used to detect virus and virus-antibody interaction in liquid preparations, ferritin and colloidal gold. Ferritin is a protein enclosing an iron core and colloidal gold is formed by the reduction of chloroauric acid with sodium citrate [9].

Ferritin conjugated to antibody combined with negative staining methods has been utilized to show the attachment of IgG on influenza virus and on hepatitis B core antigen. The method has also been used to detect rotavirus, adenovirus, and enterovirus [52,53,54,55,56].

The colloidal gold has been the most widely used marker in liquid preparations.

This gold-labeled antibody decoration technique antigen differs by the amount of gold label attached to the antigens [9].

The advantage of colloidal gold compared to other markers can be attributed to the particulate nature of the probe and the availability of different sizes and densities of the electron beam [1,57].

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In this technique the antigen-antibody reaction is enhanced by determining the protein-A associated antigen through colloidal gold particles which, when linked to the antigen, allow the latter to be easily visualized through an electron microscope [11].

In our laboratory, we use a method originally developed by Knutton (1995) [51] to identify and characterize proteinaceous filamentous bacterial surface structures, such as fimbriae or pili, which function as adhesins and can also be applied to mark viral particles.

In this method, the grids are treated with drops of viral or bacterial suspension, incubated with the primary antibody diluted to 1/80, post-incubated in protein A drops in association with gold particles (secondary antibody) and negatively stained. The antigen-antibody interaction is strongly enhanced by the dense colloidal gold particles on viruses, indicating a positive reaction (fig. 16).

The method also allows detection and identification of antigen structures induced by the virus and its localization in infected cells, serotype viral strains [58], and determines antigenic variants in isolated strains [59].

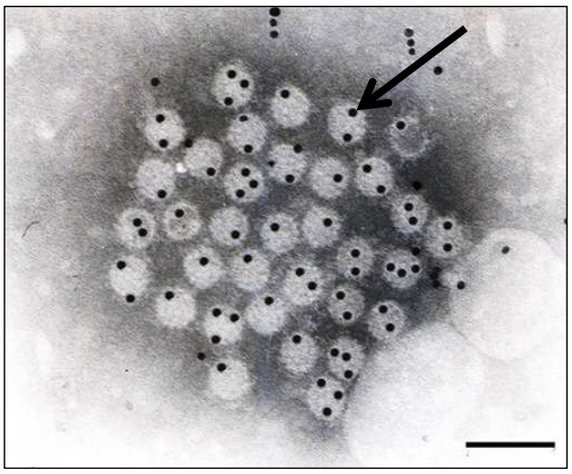


Figure 16. Bovine papillomavirus marked by the particles of colloidal gold (arrow). Bar 100 nm.

2.4. Immunolabeling in ultrathin sections technique

The immunoelectron microscopy is one of the best methods for detecting and localizing proteins in cells and tissues and to detect virus or viral antigen on the surface of or within ultrathin sections of the cells [9,60,61].

The type of marker used depends on the type, location, and stability of the antigen under study [9].

Various types of markers have been used in ultrathin sections. The best known are the red ruthenium, lanthanum nitrate, horseradish peroxidase, ferritin, colloidal thorium dioxide (thorotrast) and colloidal gold [24].

The ruthenium red has been applied to study ultrastructural aspects of retroviruses, such as details of the structure of peplomers, fusion or entry, assembly, release, and budding in infected cells [62,63,64].

Ruthenium red staining also promotes the ultrastructural visualization of glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis* [65].

Applications of immunolabeling with ferritin allowed investigation of the complex antigenic interactions induced in cells by infection with herpesvirus [66] and by influenza and vaccinia viruses [67], as well as aspects of maturation and budding of African swine fever virus [68].

This marker facilitated electron microscopic study on the penetration of Newcastle disease virus into cells leading to the formation of polykaryocytes [69]. However, the immunolabeling antibody against the surface hemagglutinin spike on the viral surface of Newcastle disease virus showed that the size of the ferritin particles resulted in confusion with natural ferritin in the cell cytoplasm [57].

Electron microscopic observations of bacteria and Mycoplasma ultrastructure has been made by immunolabeling with ferritin [70,71].

Horseradish peroxidase-labeled antibody was used for localization of viral precursor antigens of reovirus [72], herpes simplex and vaccinia viruses [73], and rotavirus [74] and to study the organization of the endosome compartment of Semliki Forest virus [75].

There has been a recent report on the development of "Apex" (monomeric 28-kDa peroxidase), a genetically encodable EM tag that is active in all cellular compartments that withstands strong EM fixation to give excellent ultrastructural preservation [76].

The application of lanthanum in culture of *Scherichia coli*, allowed the study of the components of the cell envelope, the periplasm, and the energized inner membrane [77].

Ultrastructural aspects of the attachment and penetration of herpesvirus in BHK21 cells [78] and loci of viral ribonucleic acid synthesis of arbovirus [79] were described by immunolabeling with colloidal thorium dioxide.

Combined methods of ferritin tracing, lanthanum staining, and acid phosphatase localization was employed to demonstrate active process of retrovirus phagocytosis [80].

The association of tracers, ruthenium red with lanthanum nitrate was utilized as a marker for scrapie particles [81].

The gold particle labeling technique was first described by Faulk & Taylor in 1971 [82], when they were able to tag gold particles to anti-salmonella rabbit gamma globulins in one step in order to identify the location of the antigens of salmonella.

The use of primary antibodies conjugated with gold particles allows high resolution detection and localization of a multiplicity of antigens, both on and within the cells, revealing the distribution of molecular components at various structural levels [60,83].

This technique allows immunolocalization of viral proteins and their association with cell membranes of infected cells [84].

Two methods of immunolabeling were developed, the pre-embedding method and the post-embedding method. The pre-embedding method primarily detects determinants exposed at the surface of infected cells such as virus receptors or envelope glycoproteins of budding viruses that are freely accessible to antibodies and reagents. The post-embedding labeling of thin sections allows access to determinants present in the different compartments of the cell and to internal viral structures since they become exposed at the surface of the section [85].

The major advantage offered by the post-embedding method is that each antigen molecule at the surface of the section should stand the same chance of being immunodetected, regardless of its cellular or sub-cellular localization of bacterial cells proteins [86].

In post-embedding methods of immunogold staining, the cells or tissues are fixed chemically or cryo-immobilized, dehyidrated, and embedded in epoxy or acrylic resins. The sections are then immunochemically stained with primary antibodies raised against antigens exposed on the surface of the sections. The primary antibodies are visualized by staining immunochemically with secondary antibodies raised against the species and isotype of the primary antibodies, conjugated to colloidal gold particles. The ultrathin sections are stained with uranyl acetate and lead citrate [9,60,87].

This technique was used to label glycoproteins GP1 and GP4 of the bovine herpesvirus type 1 epitopes exposed at the surface of the cytoplasmic membrane or the envelope of the budding viral particles [85].

Immunolabeling using VP8-specific antiserum and colloidal gold labeled protein A as the electron-dense marker was applied to identify tegument protein VP8 of bovine herpesvirus-1 [88].

Salanueva et al. [89] showed that the two types of particles of the porcine transmissible gastroenteritis virus, large annular virus and small dense viruses are closely related, since both large and small particles reacted equally with polyclonal and monoclonal antibodies specific for TGEV proteins.

Another application of this technique showed that the collapse of the endoplasmic reticulum cisternae observed during African swine fever virus infection is dependent on viral envelope protein, J13Lp [90].

2.5. Resin embedding technique

The introduction of the epoxy resins for electron microscopy in the 1950s was a major step in the development of thin section electron microscopy for ultrastructural analysis [91].

The resin embedding technique consists of glutaraldehyde or paraformaldehyde fixation (2,5%), osmic acid post-fixation (1%), uranyl acetate en bloc staining, dehiydration with acetone, embedding in epoxy resin, thin sectioning and staining [92,93,94].

Ultrathin sections is an important tool to reveal fine details of the ultrastructure of all types of cells and tissues [95]. In an infectious process, it allows observing pathogenesis of infection and the identification of the agent [43].

The thin sectioning technique has the advantage of allowing the observation of virus cell interaction, which reveals the site of virus replication and maturation in the host cells, a pertinent information in the identification of unknown viruses [96].

Many ultrastructural aspects of this interaction can be observed in infections by several genera of poxviruses and papillomavirus. Amorphous, fibrillar, homogeneous, crystalline and A-B types inclusion bodies (fig. 17), nuclei with aspect dentate, membrane-bound vacuoles and mature and immature particles budding from cellular membranes, can be distinctly visualized in ultrathin sections after the resin inclusion of lesion or crust fragments in cases of ecthyma contagious, myxomatosis, swinepox, and avianpox, among others [32,33,34,97,98].

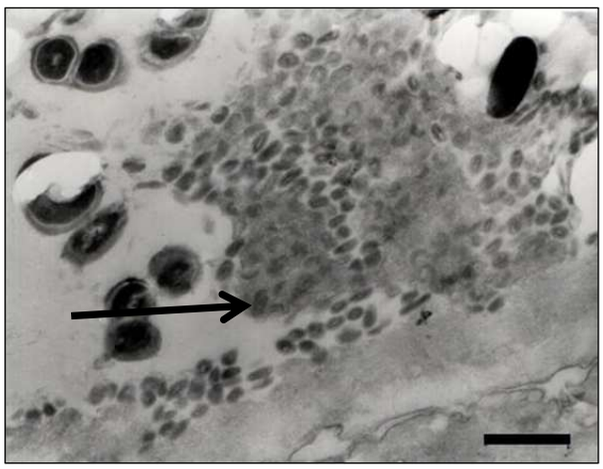


Figure 17. Ultrathin section of the scabs fragments infected by Avian pox. Type A or Bollinger intracytoplasmic inclusion bodies, containing in its Interior mature particles (arrow). Bar: 800 nm.

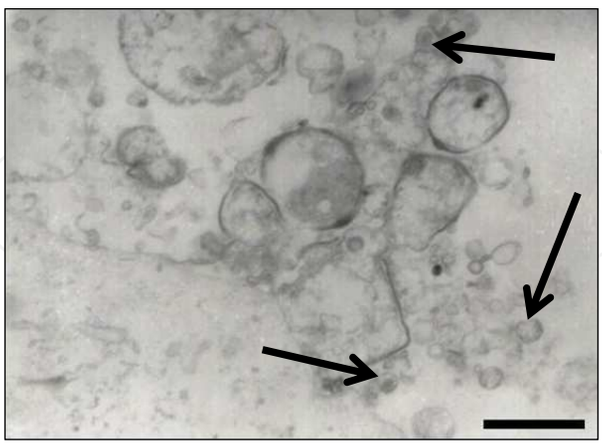


Figure 18. herpesvirus particles in various stages of development (arrow) in cells inoculated with brain suspension of monkeys infected with herpesvirus type 1. Bar: 660 nm.

Several aspects of morphogenesis herpesvirus were seen in Vero cell monolayers inoculated with brain suspension of monkeys infected with herpesvirus type 1 (fig. 18).

These ultrastructural details not only determine the infection, but also the course of the disease in the creations.

The embedding technique was used to describe a new *Rickettsia* species isolated from the tick *Amblyomma incisum* from the Southeast of Brazil, the *R. monteiroi*, showing characteristic Gram-negative morphology, with a cell wall and a cytoplasmic membrane separated from the cell wall by the periplasmic space [99].

Different methods for thin section electron microscopy have been developed for detection of mycobacteria [100].

3. Discussion

Electron microscopy is undoubtedly an indispensable tool in the diagnosis of animal infectious diseases and to investigate the structural analysis of cells and tissues at various levels of resolution [1, 101].

The negative staining is a traditional technique that allows a quick, efficient, simple and conclusive diagnosis. The "open view" of the direct electron microscopy can visualize all agents on the specimen grid, also unknown microbes as well as unsuspected ones [6].

No simple method for an unequivocal and rapid diagnosis of infectious disease is available [19].

The success of the diagnosis depends on the quality of the sample taken, preparation and experience of the ultramicroscopist [102].

A positive detection requires of 10^5 particles per ml in the diagnostic suspension [4,6].

In the feces, however, the viral particles are present in high concentrations and are easily visualized [35].

One of the main applications of negative staining is the investigation of outbreaks of viral gastroenteritis [11] that cause high mortality in the creations.

This technique has also been used to detect the presence of enteric viruses such as poult enteritis complex (PEC), a disease economically important in poultry, characterized by enteritis, diarrhea, poor weight, and high mortality [103].

The technique also allows the identification of multiple agents in a same sample [19].

Herpesvirus, poxvirus, and papillomavirus particles are also found in large numbers in crusting, blistering, vesicular fluid or epithelium [4,6].

Some viruses may have low viral titer; however, this problem can be circumvented by applying the techniques of immunoelectron microscopy and immunolabeling with colloidal gold.

The electron microscopy has been utilized as a front-line method in emergency infectious diseases and/or in suspect cases of bioterrorism [19, 104].

The sudden appearance of vesicular lesions or respiratory illness in farm animals may be evidence of an emerging disease, a possible zoonosis or an agriterrorist act [19].

Exotic infections in several animal species have also been identified by electron microscopy [20]. Coronavirus particles were detected in ferrets with clinical status of diarrhea [105].

Several outbreaks of viral diseases were detected in wild animals using electron microscopy techniques. An enteric coronavirus was detected in capybaras [106] and the presence of poxvirus and paramyxovirus was confirmed in wild birds [32, 98].

Electron microscopy plays a fundamental role in assisting veterinary clinics and hospitals, Ecological Parks, Zoos, and breeding farms.

The immediate results of examinations of electron microscopy allows the rapid introduction of therapeutic, preventive and control measures in breeding sites and plan strategies for fighting infection, avoiding unnecessary loss of animals and economic damages.

The gain in time is an important factor in the control of infections [1,107].

In the event of new outbreaks caused by infectious agents, electron microscopy allows to assess the possibility of developing specific vaccine for the protection of the creations.

The introduction of electron microscopy techniques in diagnostic routine during outbreaks in farm animals allows to helping determine the risk areas at the site to be studied, collaborating in this way with the National Agribusiness, giving a base for health programs.

Another important use of electron microscopy is the identification of an unknown virus that has been isolated in tissue culture [20] or those viral agents particularly difficult to cultivate [3] and when alternative standard diagnostic methods fail to produce reasonable results [26].

The obtained electron micrographs are widely used to illustrate practically any text scientific papers, monographs, atlases and books in cell biology, anatomy, and pathology [95].

Future applications in this area include negative staining and cryo-negative staining, cryo-preparation methods of vitreous sections (CEMOVIS) and digital images for transmission electron microscopy that can be processed by software programs may contribute to the improvement of veterinary diagnostic by electron microscopy [57,101,108,109].

The electron microscope is an expensive (costs about 600,000 euros), sophisticated, and extremely efficient equipment for rapid veterinary diagnosis, the annual cost spent on maintenance, should not be considered a disadvantage.

The main requirement of an Electron Microscopy Laboratory, however, is the need for highly trained operators with knowledge and skills to handle the equipment, prepare the sample, and realize the diagnosis accurately [6,12,26,43,110].

The preparation of a negative staining sample amounts to less than 0.5 euros, which is much less than the costs for alternative molecular tests [6].

The cost to the user is around $ 16.50 per sample processed by this technique in our laboratory.

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Considering the importance of electron microscopy in the diagnosis and research preparation is key to the new generation of ultramicroscopists with the appropriate technical skills [11].

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