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# Application of Transmission Electron Microscopy Techniques in the Veterinary Diagnosis of Viral Gastroenteritis in Livestock Animals

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## Abstract

Gastroenteritis caused by viruses is considered to be one of the most important diseases in livestock, being the main cause of morbidity and mortality in young animals, culminating in serious economic losses due to costs with prophylaxis and treatment, increased susceptibility of animals to secondary infections, developmental delay and death. Stressful factors may support the onset of illness. Several viral agents can cause gastroenteritis in various animal species. Rotaviruses are considered the main cause of enteric infections in various animals, including humans constituting important zoonosis. Due to genetic diversity and their ability to cross the species barrier, the coronaviruses infect many species. In cattle, they cause “Winter Dysentery” in adult animals and “Neonatal Diarrhea” in newborn calves. In swine, they are responsible for “Transmissible Gastroenteritis” and “Swine Epidemic Diarrhea.” Equines infected with coronavirus also develop severe gastroenteritis. Bovine viral diarrhea (BVD) caused by a flavivirus of the genus *Pestivirus* is related to digestive and reproductive disorders, affecting any productive sector, are it cut, milk or confinement. Transmission electron microscopy is an indispensable tool in the diagnosis of viral gastroenteric infectious diseases. Negative staining is a simple, fast and efficient technique, being ideal for the detection of gastroenteric viruses, being easily visualized. The immunoelectron microscopy (IEM) technique allows increasing the sensitivity of virus detection where low concentrations of virus are aggregated so that they may be more easily seen. The immunolabeling with colloidal gold technique utilizes specific antibodies tagged with particles of colloidal gold to label the antigen antibody reaction. Embedding resin technique allows obtaining information on the virus–cell interaction. The different transmission electron microscopy modalities promotes a fast and accurate diagnosis of the different gastroenteric viral agents, allowing prophylactic measures of control and prevention in the creations to be promptly instituted, avoiding animal losses and disastrous economic losses, and collaborating with the National Porcine and Bovine Agribusiness.

**Keywords:** viral gastroenteritis, livestock animals, veterinary diagnostic, transmission electron microscopy

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## 1. Introduction

Gastroenteritis caused by viruses is considered one of the most important diseases in livestock, being the main cause of morbidity and mortality in neonates. The food animal livestock industry estimated a multimillion dollar annual economic loss due to diarrheal diseases associated with a reduction in weight gain, costs with prophylaxis and treatment, increased susceptibility of animals to secondary infections, developmental delay and death of young animals. They represent an important sanitary problem compromising the herds, independently of the level of technification of the creation. Stressful factors such as long-distance travel, reproduction, nutritional deficiencies, environmental changes, etc., may support the onset of illness [1].

The main agents that can cause gastroenteritis in livestock animals are rotavirus, coronavirus and flavivirus.

### 1.1. Rotavirus

Rotaviruses are considered the main cause of enteric infections in cattle, swine, equines, canines, felines, birds and wild, including humans constituting important zoonosis [2, 3]. They are observed more frequently in neonates, with negative economic impact to the worldwide productive sector, causing high mortality, when it occurs in commercial creations. In livestock, rotaviruses are associated with severe enteric diseases in young calves [4, 5], weaning and postweaning piglets [6] and severe enteritis in foals [7].

Rotavirus is classified as a member of *Reoviridae* family, *Sedovirinae* subfamily and *Rotavirus* genus [8]. They have icosahedral symmetry and a nonenveloped capsid formed by three concentric layers of protein that is 70–90 in diameter. The genome of rotavirus comprises 11 segments of double-strand RNA of 16–21 kbp, encoding six structural proteins (VP1–VP4, VP6 and VP7) and five nonstructural proteins (NSp1–NSp5/6). They are classified into eight groups (A–H) based on the antigenic relationship of its VP6 protein. The most common groups that infect humans and animals are the A, B and C [9, 10].

#### 1.1.1. Bovine rotavirus

Bovine group A rotavirus (bovine RVA) is recognized as the most common cause of severe gastroenteritis in cattle, causing significant economic loss in the dairy and beef industry due to increased morbidity and mortality, treatment costs and reduced growth rates [11, 12].

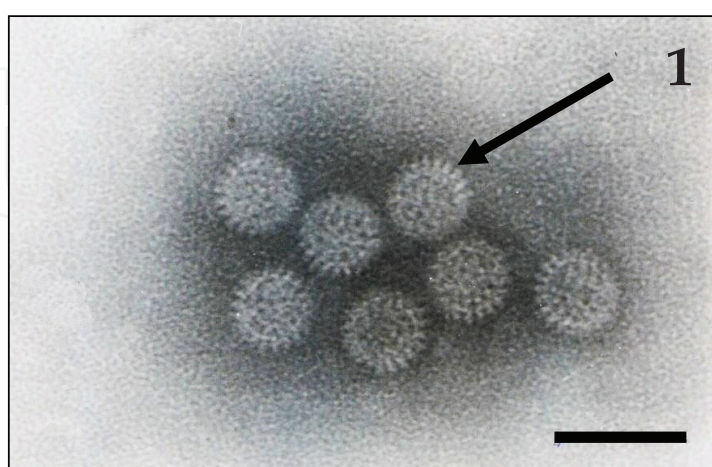
Infection with bovine rotavirus group A (RVA) has been reported in several countries, such as Brazil [12], the Netherlands [13], Australia [14], EUA [15], New Zealand [16] and Japan [17]. Rotavirus B strains also cause epidemic and sporadic cases of diarrhea in humans, pigs, cattle, limbs and rats [18]. Bovine RVB infections have been reported only in Japan [19], Indian [20], the United Kingdom [21] and the United States [22].

Rotaviral diarrhea usually affects calves between 4 days and 3 weeks old. The incubation period is 12–24 h, and the duration of diarrhea lasts from 2 to 5 days [19, 23–25].

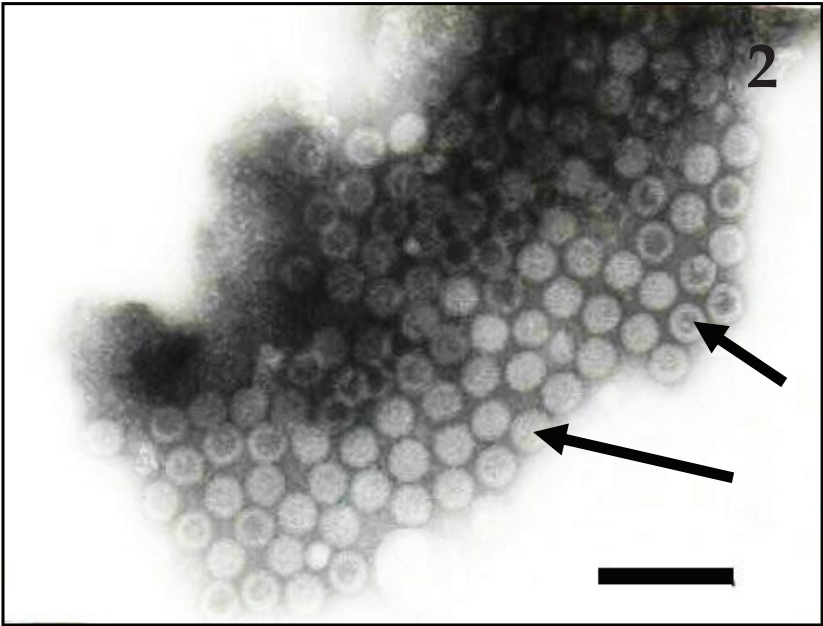
The animals presented depression, anorexia, excessive salivation, profuse diarrhea and severe dehydration [23, 24]. The abomasums typically contains milk curd and thick saliva. The virus replicates in the mature epithelial cells of the villi, and viral infection redirects the function of cells of the absorption for the partially digested milk and accumulates in the intestinal lumen [24, 26]. Other secondary agents often found in epizootics of rotavirus-associated diarrhea may contribute to the severity of the disease [26].

Transmission generally occurs when an unaffected animal has oral contact with infected feces and contaminated feed, or if they are exposed to living quarters with poor hygiene characteristics. Cows displaying signs and symptoms may shed the virus for as long as a week, while some cows can become reinfected and shed the virus throughout their life and remain asymptomatic [26].

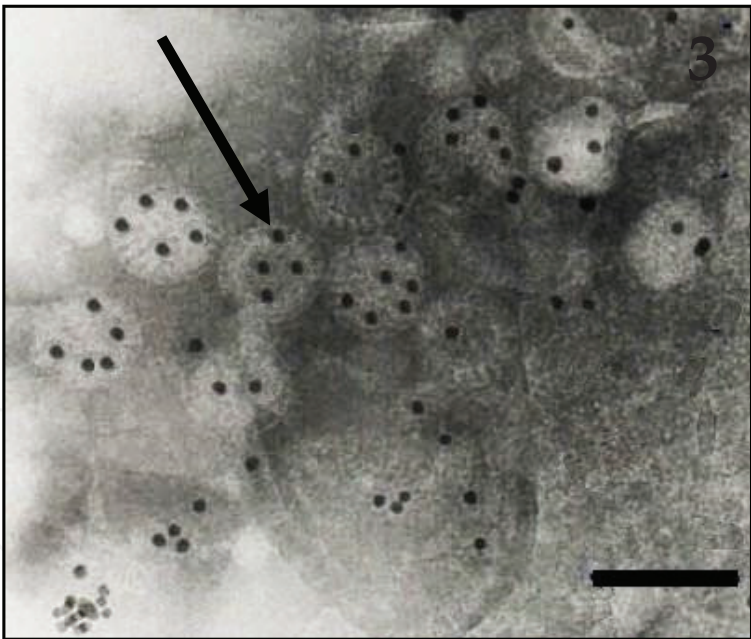
The methods of negative staining (rapid preparation) (**Figure 1**) and the immunoelectron microscopy (**Figure 2**) demonstrate the presence of rotavirus particles in fecal samples from calves with diarrhea [25], being named “the gold standard” in the diagnosis of viral enteritis in calves. Immune electron microscopy (**Figure 2**) and immunolabeling with colloidal gold particles (**Figure 3**) have a high sensitivity ranging from 87 to 100% of the different viral agents, which gives good diagnostic value [27, 28].



**Figure 1.** Negatively-stained rotavirus particles, showing individual capsomers (arrow) in feces of bovine. Bar: 80 nm.



**Figure 2.** Immunoelectron microscopy of rotavirus particles aggregated by antigen–antibody interaction in bovine feces. Observe “complete” (big arrow) and “empty” (minor arrow) particles. Bar: 240 nm.



**Figure 3.** Bovine rotavirus marked by the particles of colloidal gold (arrow). Bar: 100 nm.

General recommendations regarding decrease of RVA diarrhea include management practices, especially good hygiene and sanitation procedures, as well as pathogen-specific interventions, such as the use of vaccine prophylaxis [14, 29].



### 1.1.2. *Swine rotavirus*

In pig farms, rotaviruses are responsible for economic losses due to death of animals, poor growth performance and costs of diagnostic and treatment [30, 31].

The rotaviruses that affect pigs are differentiated as group A, B and C, based on the antigenicity and genetic characteristics of VP6 [9, 32]. Group H has already been described in Brazil [33]. Group A rotavirus is the most frequently isolated of piglets with 1- to 8-week-old diarrhea [34], but groups B and C are also described in piglets in both the maternity and nursery phases [35, 36].

Swine rotavirus has a worldwide distribution and has been reported in Africa [37], Vietnam [38], England [39], Italy [40] and Brazil [33, 41].

Since rotavirus can survive in the environment for an extended period and is transmitted via the fecal-oral route, outbreaks are difficult to control [42].

It affects piglets from the first to the sixth week of life, but occurs with a higher prevalence among animals from 2 to 4 weeks. Infection in neonates is associated with failure of passive immunity due to insufficient colostrum intake or the occurrence of a genotype different from that which occurs endemically in the herd [41, 43].

In pigs, the infection is characterized by vomiting, anorexia, slimming, prostration, diarrhea of liquid or pasty consistency and whitish coloration, which lasts for 2–5 days, and dehydration. In more severe cases, because of episodes of diarrhea, the animal may develop electrolyte imbalance, metabolic acidosis and death [43].

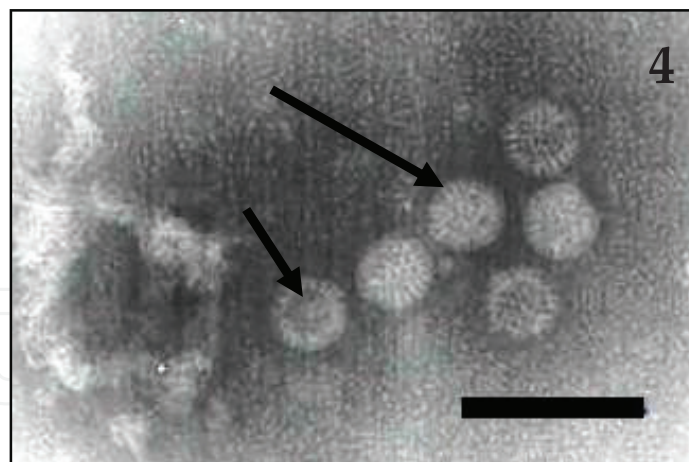
Rotavirus infects enterocytes from the apical and intermediate portions of the intestinal villi, causing lysis of enterocytes with decreased absorption capacity and digestive functions [44, 45].

Both asymptomatic animals and matrices, especially in the period of peri-parto, eliminate rotavirus in the environment and can be considered sources of infection [43].

Rotavirus particles are easily visualized by electron microscopy techniques (negative staining (**Figure 4**) and immune electron microscopy), considering that they are present in large quantities in feces and intestinal fragments of infected pigs. These techniques have been used in many studies of the swine rotavirus [46–48].

Inadequate management practices tend to increase the frequency of rotavirus diarrhea. These include weaning in younger animals, breeding pigs at multiple sites, and larger herds have a higher incidence of RV infection due to variation in the immunity level of females [49].

Delivery assistance and breastfeeding management are essential for newborn piglets to receive passive neutralizing antibodies through the ingestion of colostrum, which is the main form of protection of the newly born piglet against rotavirus.



**Figure 4.** Negatively-stained rotavirus, showing “complete” (big arrow) and “empty” (minor arrow) particles in feces of swine. Bar: 150 nm.

As with all viruses, there is no specific treatment for rotavirus. Treatment consists of controlling the progression of the clinical picture with the use of supportive therapy (electrolyte replacement) and of the secondary bacterial infections with the use of broad-spectrum antimicrobials [43].

#### 1.1.3. *Equine rotavirus*

Group A equine rotavirus (RVA)-associated diarrhea foals represent a main sanitary problem for the equine industry worldwide [50]. It is the main cause of diarrhea in foals up to 3 months of age, and the acute dehydration can incur severe economic burden due to morbidity in studs [51]. Diarrhea associated with rotavirus can also be a serious problem in areas of intensive breeding during the breeding season [52]. The G3P and P14P are the most prevalent equine rotavirus strain [53].

Clinical signs include diarrhea referred to as white diarrhea or milk diarrhea, lethargy, pyrexia, reluctance to suckle and abdominal tympani. Depression and colic are often observed in serious field cases [51, 54–56]. The malabsorptive watery diarrhea leads to severe dehydration and sometimes death, mainly in neonates with failure of passive antibody transference [50].

Transmission is by feco-oral route via contaminated feces or fomites, and the incubation period is of 1–2 days [57]. The virus invades the intestinal epithelium on the sides and the tips of the villi. The brush border epithelium of the small intestine synthesizes disaccharides to monosaccharides, which are absorbed in the gut. Destruction of the brush border villi results in a decrease in the formation of lactase, resulting in the absence of lactose digestion. This sugar remains in the lumen of the gut, osmotically attracting more fluid [58].

Treatment of foals with rotavirus diarrhea are directed to maintenance of hydration and electrolyte and acid-base balance, aiming to reduce abdominal discomfort or intestinal irritation, to prevent secondary bacterial infection and to avoid spread infection to other foals. The

administration of oral or intravenous electrolytic solutions, in addition to intestinal protectors, has been indicated as an auxiliary method in the treatment of rotavirus diarrhea. As prevention measures, isolation of foals with diarrhea, use of protective clothing for the handlers, hand hygiene, use of pedilavium, and appropriate vaccination of animals should be adopted [59, 60].

Direct electron microscopy readily detects rotavirus particles in feces and intestinal fragments of foals with diarrhea [61–64].

#### 1.1.4. *Sheep and goats rotavirus*

Rotaviruses A, B and C have been described in small ruminants [65–67]. Reports on ovine or caprine Rotavirus A are available from various countries worldwide, with detection rates reaching 60% and estimated 10–30% mortality [68–70].

During outbreaks of neonatal diarrhea by rotavirus A, prevalence in fecal samples and lamb, morbidity/mortality may be very high [71, 72]. During an outbreak of diarrhea occurred in a dairy herd of goats in Brazil, rotavirus A was detected in 80-day-old animals with watery diarrhea, anorexia, dehydration and death of one of the animals [73]. Rotavirus A has been associated with diarrhea in goats kids [66, 74]. Regarding the occurrence of rotavirus C, little information is obtained in these species [74].

Administration of colostrums is pivotal to protect lambs from rotavirus-induced diseases [75]. The colostrums and milk of ewes administered with an inactivated rotavirus A vaccine 2–3 weeks prior to mating contained high titers of antibody to the virus [76].

## 1.2. Coronavirus

### 1.2.1. *Bovine coronavirus*

Due to genetic diversity and their ability to cross the species barrier, the coronaviruses infect many animals species, including cattle, pigs, equines, rodents, dogs, cats, ferrets and domestic and wild birds [77].

#### 1.2.1.1. *Bovine enteropathogenic coronavirus*

Bovine coronavirus is widespread in the cattle population, resulting in economic losses to the beef and dairy industry in the world [78]. In both beef and dairy herds, BCoV can be associated with calf diarrhea, calf respiratory disease, winter dysentery, respiratory disease in adult cattle, and combined pneumonia and diarrhea in calves and adults [79, 80].

Bovine coronavirus belongs to the *Nidovirales* order, *Coronaviridae* family and *Betacoronavirus* genus [9]. They are simple-stranded positive sense RNA viruses, 32 kbp long, which associates with the nucleoprotein (N) forming a nucleocapsid with helical symmetry. The viral envelope of BCoV is formed by a lipidic double layer with five structural proteins (M, sM, HE, S and I) [81, 82].

Morphologically, they are pleomorphic, with radial projections with a form like-club giving an aspect of solar corona and they measure 75–160 nm of diameter [83].



Transmission of bovine enteropathogenic coronavirus occurs by the fecal-oral or respiratory routes, and most often transmission is horizontal and occurs from carrier dam to offspring postpartum [84].

BCoV causes severe hemorrhagic diarrhea, which is sometimes fatal in young animals, and the spiral colon is the host spot for viral replication in the gastrointestinal epithelium, leading to intestinal villi atrophy and osmotic diarrhea [85].

Once infected, a calf can secrete high levels of virus within 48 hours after experimental infection, and this may persist up to 14 days [86].

The clinical signs are represented by yellow to blood-stained mucus-containing diarrhea, which then progress to a profuse watery diarrhea. Subsequently the animals become dehydrated, depressed, weak and hypothermic, and their suckle reflex is loosened. Most of calves recover, but a few develop pyrexia, recumbency, coma and death [79].

#### *1.2.1.2. Winter dysentery (BCoV-WD) in adult cattle*

Winter dysentery (BCoV-WD) is a sporadic acute, contagious hemorrhagic enterocolitis of cattle that occurs in epizootic fashion in a herd [87].

BCoV-WD has been reported through the world including EUA [88], France [89], Spain [90], Canadian [91], Italy [92], Japan [93] and Brazil [94].

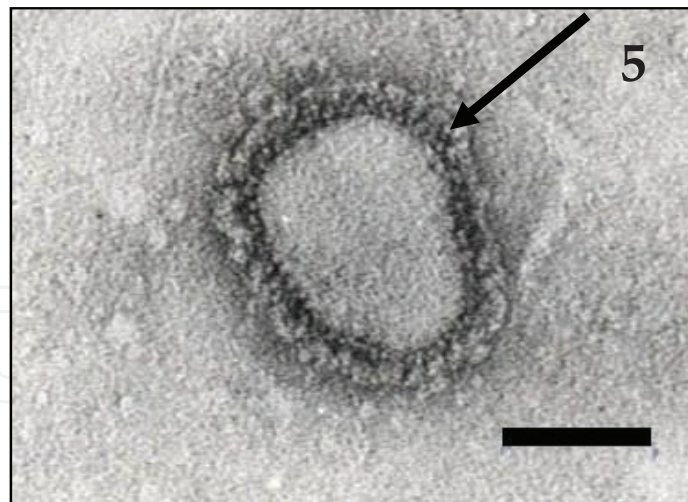
The incubation period for BCoV-WD ranges from 2 to 8 days [79].

The disease is characterized by a sudden onset of mucous dark, watery often-bloody diarrhea, which is accompanied by depression and anorexia in adult beef and dairy cattle. Mild to moderate signs of respiratory disease have been reported [92, 95]. The outbreaks occur during the winter season and result in high morbidity and low mortality rates. In an affected cattle herd, milk production may not return to normal for several weeks or even during that lactation period, resulting in significant economic losses for the milk industries. Cattle are more efficiently infected in winter, which increases the environmental contamination and justifies the high morbidity of winter dysentery during the cold months [96, 97].

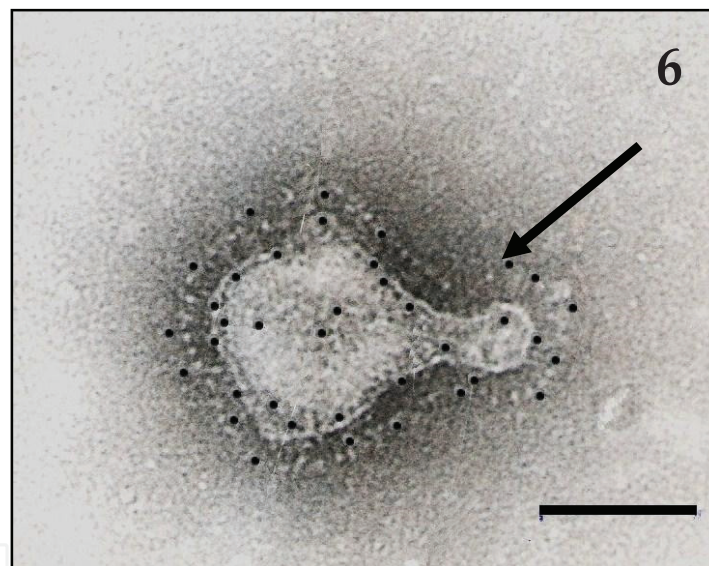
The intestinal lesions are comparable with those observed in calves with BCoV-induced diarrhea [79]. In calves with BCoV enteric infection, viral particles can be detected by electron microscopy in the feces 1–2 days before the onset of diarrhea and for several days after the diarrhea has resolved. BCoV can also be found in nasal secretions of calves with BCoV diarrhea. Recovered calves that are apparently immune to disease can still shed BCoV in their nasal secretions or feces [98].

Electron microscopy has been widely used to detect bovine coronavirus particles. Typically, coronavirus particles can be demonstrated in fecal samples by direct electron microscopy (**Figure 5**), immune electron microscopy or immunolabeling with colloidal gold particles (**Figure 6**) [27, 98–102].

In cases of coronavirus infection, the most indicated treatment is the symptomatic with electrolytes, antipyretics, antidiarrheals and probiotics, antimicrobial therapy to prevent secondary



**Figure 5.** Negatively-stained coronavirus particles containing distinctive club-shaped surface projections, in feces of bovine (arrow). Bar: 100 nm.



**Figure 6.** Buffalo coronavirus marked by the particles of colloidal gold (arrow). Bar: 100 nm.

infections, and the occurrence of outbreaks to vaccinate the animals. Colostrum intake has emerged as the natural and most useful method to control BCoV calf diarrhea [103]. The hygiene, management and sanity of the property are important factors for the prevention of neonatal diarrhea, thus avoiding serious damage to the producer [104].

### 1.2.2. Porcine coronavirus

#### 1.2.2.1. Porcine transmissible gastroenteritis (TGEV)

Porcine transmissible gastroenteritis is a highly severe contagious disease caused by virus of the *Coronaviridae* family and genus *Alphacoronavirus* [77].

As a notifiable disease, the TGEV causes significant economic losses in the pig industry and has been reported in several countries as Europe, American and Asia [105–107]. TGEV was detected for the first time in Brazil through histopathological techniques and the transmission electron microscopy in 19 (25.3%) small intestine samples of pigs from various municipalities in the State of São Paulo and the Minas Gerais, Brazil [108].

The disease affects pigs of all ages, and symptoms were represented by severe watery diarrhea accompanied by vomiting [109, 110], anorexia, prostration, dehydration, dyspnea and death [108]. In the TGEV epizooty, the high mortality rates, up to 100%, affecting piglets of less than 2 weeks of age is a result of severe dehydration [105].

The replication of the virus occurs in the digestory and respiratory tracts, and the target is the epithelial cells of the small intestinal villi that result in the atrophy of the infected epithelium focusing in severe intestinal disorders, which can be fatal in the neonatal period [111, 112].

The transmission route occurs by breast feeding, oral-fecal and fomites [113]. Exportation of fresh and frozen pork contaminated by TGEV allows that these types of food act as a potential source of viral transmission [105, 107, 114].

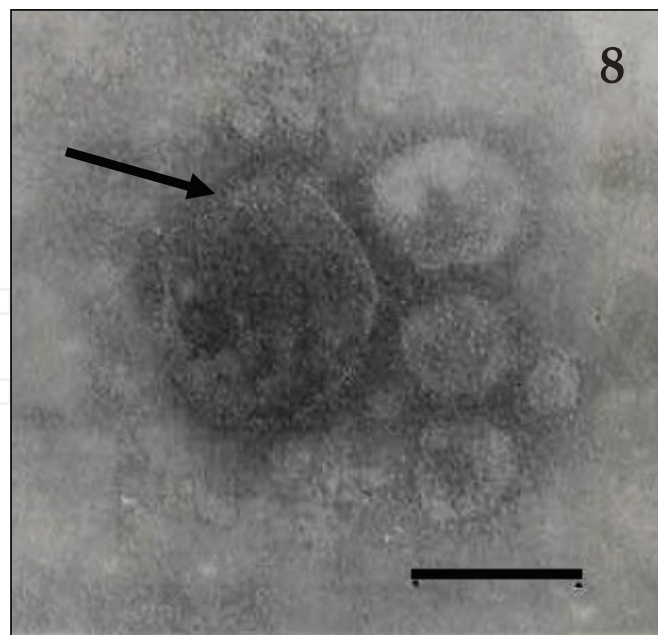
For the diagnosis of swine coronavirus, the negative-staining technique (**Figure 7**) has been widely used by many authors [115–119]. To confirm the viral strain (TGEV), immunoelectron microscopy (IEM) (**Figure 8**) and immunolabeling techniques with gold particles, performed with a monoclonal antibody specific for TGEV, can be used. Viral ultrastructural aspects can be studied through the resin embedding technique (**Figure 9**) [108].

According to the OIE [107], technique of transmission electron microscopy and *in situ* hybridization was chosen to identify TGEV. Using specific monoclonal antibodies it is possible to differentiate TGEV from the coronavirus that causes epidemic porcine diarrhea and the coronavirus that causes respiratory disease.

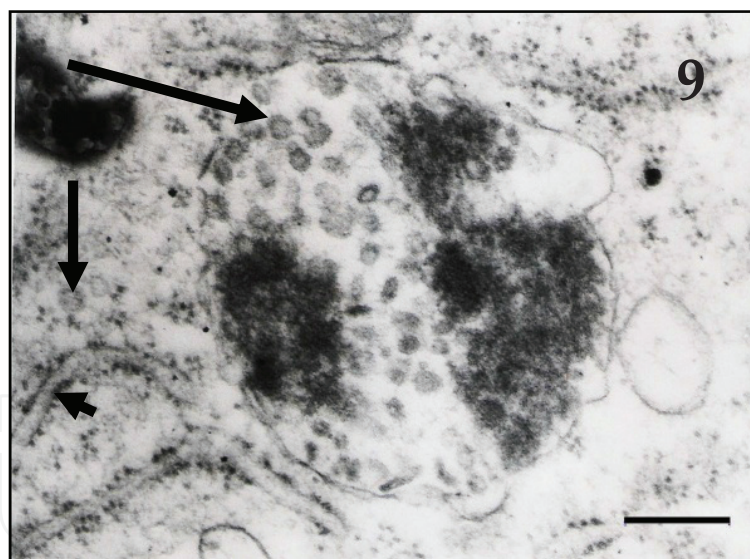


**Figure 7.** Negatively-stained coronavirus particles, showing characteristic envelope with radial projections forming a corona, in feces of swine (arrow). Bar: 100 nm.





**Figure 8.** Immunoelectron microscopy of TGEV (arrow) aggregated by antigen–antibody interaction in swine feces. Bar: 190 nm.



**Figure 9.** Resin embedding of swine small intestine. Observe coronavirus particles, incomplete, within intracytoplasmic vacuoles (big arrow), complete particle (minor arrow) and dilated cisternae of the rough endoplasmic reticulum (arrow head). Bar: 200 nm.

There is no specific treatment for TGEV. Treatment is symptomatic and seeks to avoid the spread and control of secondary infections, which may aggravate clinical signs. The most important prophylactic measure is to prevent the entry of TGEV in herds [120].

#### 1.2.2.2. *Epidemic porcine diarrhea (PEDV)*

The epidemic porcine diarrhea virus (PEDV) has been causing incalculable losses to the production of pigs in several countries, modifying the behavior of the swine market worldwide [121].

It was first observed in England, causing a devastating and sudden diarrhea during the winter, leading to losses in pig farms [122]. Subsequently, it was found in Belgium, Hungary, France, Italy, the Czech Republic, China and Asia, where more severe outbreaks were diagnosed. In the USA, it was first diagnosed in May 2013, and during the period from September 2013 to February 2014, losses of pigs in the United States by the swine epidemic diarrhea virus were estimated in 2.7 million or slightly more than 5% of the animals [123–126]. Recently, the presence of the virus has been reported in countries of South America, such as Peru [127] and Colombia [128].

The direct transmission occurs by fecal-oral route. Clinical signs of PEDV may occur within 4–5 days following introduction of infected swine to farms with susceptible animals. Following an outbreak, PEDV may subside but may become endemic if sufficient litters are produced overcome lactogenic immunity. Contaminated personnel equipment or other fomites may introduce the virus into a susceptible herd [121].

The incubation period of PEDV is 3–4 days [129].

Clinical signs of PEDV infection include anorexia, vomiting, diarrhea and dehydration. Morbidity and mortality in piglets less than 5 days is of almost 100% due to severe diarrhea and dehydration, but mortality in piglets older than 10 days is about 10% [130, 131].

PEDV replicates in the cytoplasm of villous epithelial cells throughout the small intestine, destroying target enterocytes because of massive necrosis or apoptosis. These processes lead to villous atrophy and vacuolation as well as a marked reduction in the enzymatic activity, causing malabsorptive watery diarrhea, followed by serious and fatal dehydration in piglets [132–134].

Ultrastructural colon lesions have been observed by transmission electron microscopy. At the cellular levels, PEDV protein E is localized in the endoplasmic reticulum with small amounts being found in the nucleus of infected cells [135].

Although an approved vaccine against the PEDV virus is not yet available, it is important to adopt biosecurity programs to prevent the spread of the disease in the country. These include the disinfection of environments susceptible to contamination, such as breeding, slaughtering and transport facilities, as well as water and food containers. It is also necessary to implement surveillance measures regarding the introduction of new animals in the herd, quarantine procedures and control of access to the farms [121].

#### 1.2.3. *Equine coronavirus (ECoV)*

Equine coronavirus (ECoV) which causes enteritis in foals is a disease of economic significance in equines for horse breeders [136].



They typically have a restricted host range, infecting only their natural host and closely related animal species but do have the capacity to cross the species barrier to infect new hosts. Equine coronavirus (ECoV) is the only coronavirus known to infect or cause disease in horses [137].

Equine coronavirus (ECoV) belongs to the *Coronaviridae* family and *Betacoronavirus* genus [138].

ECoV was first isolated in North Carolina (USA) from the feces of a diarrheic foal in 1999 [139] and was initially believed to only affect foals. Since 2010, there have been several reports of ECoV-associated respiratory and enteric infections in adult horses in Japan, Europe and the United States, but its global distribution is still poorly defined [10, 140–143]. In Brazil, the first outbreak of enteritis in horses was reported in 1988 by transmission electron microscopy in a horse livestock, in São Paulo, SP, affecting animals ranging from 1 week to 4 months that presented aqueous diarrhea [144]. Later, another outbreak was reported in a farm in Rio Grande do Sul, where 69 foals of 45–90 days were affected by severe enteritis [145].

The main clinical signs presented by the ECoV infection are represented by anorexia, apathy, lethargy, fever and neurologic abnormalities (ataxia, depression and recumbency). Respiratory problems, profuse aqueous diarrhea greenish to yellowish of putrid odor and discrete algia abdominal can also been observed [136, 141, 144–147]. It is transmitted by the fecal-oral route, and signs tend to resolve in 1–4 days, although animals can continue shedding for several weeks [147]. It is suggested that ECoV may spread among horses when they are stabled together or during transport [148, 149].

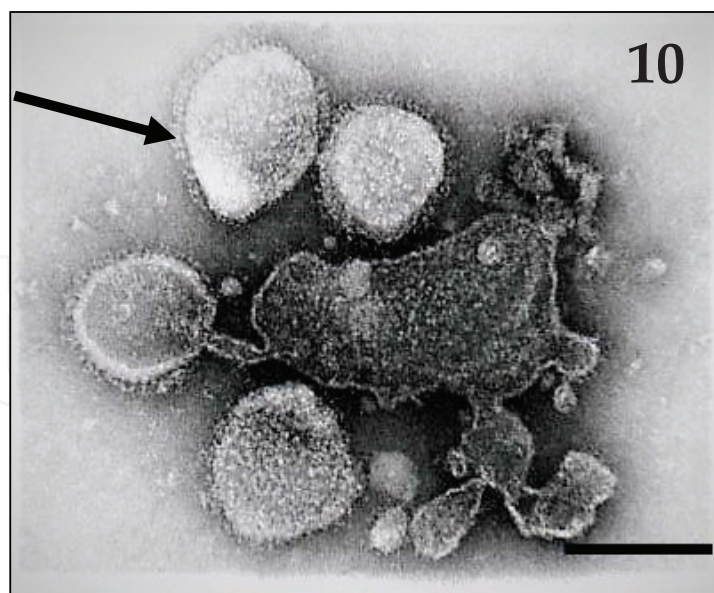
The virus has been diagnosed more frequently in adult animals over 2 years of age [146, 147, 150]. However, outbreaks have been reported in foals from 5 days to 4 months of age [139, 144, 145, 151] and in both adults and young animals [141].

Morbidity ranges from about 20–57%, and mortality is typically rare [147]; however, a high mortality rate has been described in foals [152].

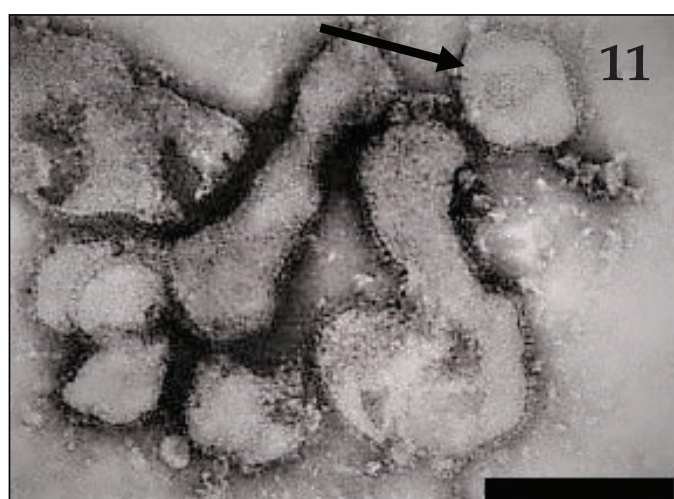
ECoV has been shown to produce cell death via apoptosis in Madin-Darby Bovine Kidney (MDBK) cell cultures [153].

With the aid of transmission electron microscopy techniques, the disease is more easily diagnosed. Negative-staining technique (rapid preparation) (**Figure 10**) and immunoelectron microscopy (**Figure 11**) has been widely used for direct visualization of viral particles [139, 144, 146, 151, 154–157].

Most adult horses with clinical ECoV infection recover spontaneously in a few days without specific treatment. Horses with persistent elevated rectal temperature, anorexia and depression are routinely treated with anti-inflammatory drugs intravenously. Horses with colic, persistent depression and anorexia and/or diarrhea have been treated more intensively with fluid and electrolyte until clinical signs have resolved. Additionally, antimicrobials and gastrointestinal protectants should be considered in horses with secondary bacterial infection. The use of BCoV vaccine in horses for the prevention of ECoV has not been investigated and cannot be recommended.



**Figure 10.** Negatively-stained coronavirus particles (arrow), in small intestine suspension of equine (arrow). Bar: 190 nm.



**Figure 11.** Immunoelectron microscopy of coronavirus particles (arrow), aggregated by antigen-antibody interaction in equine feces. Bar: 190 nm.

The prevention of ECoV infection should focus on the implementation of routine management practices aimed at reducing the likelihood of introducing and disseminating ECoV at any horse-based premise (boarding facility, show ground, and veterinary hospital). Once an ECoV infection is suspected, strict biosecurity measures including footbaths and the use of personal protective equipment should be provided and adequately maintained for sanitary purposes [158].

### 1.3. Bovine viral diarrhea (BVD)

Brazil currently occupies the position of the world's largest exporter of beef, with the production chain moving around 167.5 million/year, producing 9.5 million tons [159].

Bovine viral diarrhea virus (BVDV) is an important pathogen of ruminants causing severe economic losses to the cattle industry, affecting any productive sector, be it of cut, milk or confinement [160].

It has a worldwide distribution, having already been reported in several countries, such as Italy [161], Australia [162], EUA [163], China [164] and Japan [165].

In Brazil, the presence of BVDV has already been proven in several states, such as Pernambuco [166], Goiás [167], Maranhão [168], Minas Gerais [169] and Rio Grande do Sul [170].

Bovine viral diarrhea virus type 1 (BVDV-1) belongs to the *Flaviviridae* family and *Pestivirus* genus that comprises also the species bovine viral diarrhea virus type 2 (BVD-2), classical swine fever virus (CSFV) and border disease virus (BVD) [8].

The virions are 40–60 nm in diameter, spherical in shape, and contain a lipid envelope [171]. The *Pestivirus* genome composed of a positive-sense single-stranded RNA that is approximately 12.3 kb [172]. BVDV-1a and 1b are the most widely distributed BVDV-1 subtypes in the world and alternate as the most prevalent in different countries [173, 174]. BVDV-1 can be divided into at least 21 subgenotypes (1a–u). BVDV-1i is an uncommon subtype that has been reported in the United Kingdom and Uruguay, and recently, in Brazil [175, 176], and the subtype 1 h strain was isolated in Italy [175]. Bovine viral diarrhea virus is one of the most widespread cattle pathogens worldwide being considered emergent [177].

The clinical signs of BVDV infection are highly variable, ranging from unapparent or mild infection to fatal acute illness. These clinical signs or symptoms include acute or chronic gastrointestinal disorder, respiratory disease in calves, and a hemorrhagic syndrome with thrombocytopenia, skin diseases, immunosuppression and decreasing milk production. BVDV also had been related with infertility, return to estrus, embryonic or fetal mortality, abortion or mummification, fetal malformations or the birth of weak calves and infeasible [178–180].

The BVDV induces persistent fetal infection, generating a persistently infected animal with 50% calves lethality rate in the first year of life. The PI animals excrete the virus in large quantity in the secretions, being responsible for the maintenance of the virus in the herd [181].

Mucosal disease affects exclusively PI animals between 6 months and 2 years of age, with a fatal course. The acute form is characterized by a 10- to 14-day incubation period, followed by fever, anorexia, tachycardia, polypnea, erosions in the oral mucosa and nostrils, dehydration, watery diarrhea and rhinitis, nasal and ocular discharge and death within a few days. Animals that survive acute form develop chronic mucosal disease, whose signs are nonspecific [181].

Transmission may occur horizontally through direct contact between animals or indirectly through contaminated secretions, excretions and fomites or vertically leading to congenital infection of the fetus [180].

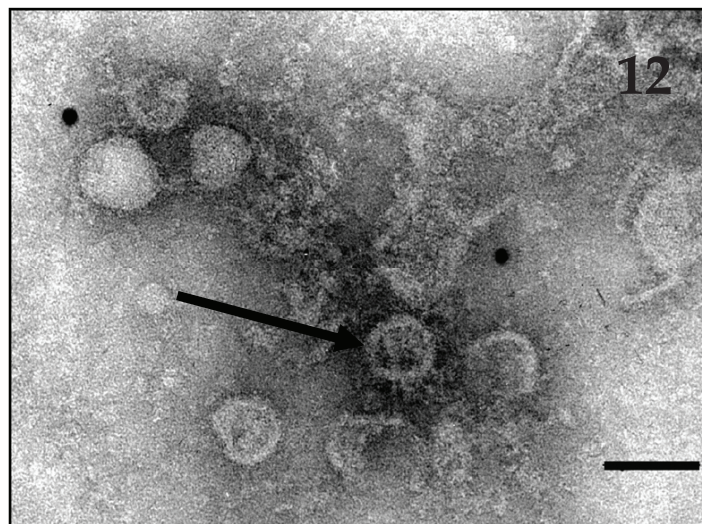
The introduction of BVDV in the herds occurs by the entry of PI animals on the farms, through the acquisition of cattle during the acute phase of the disease, persistently infected bulls or female breeding PI fetuses and contact between neighboring herds [182].



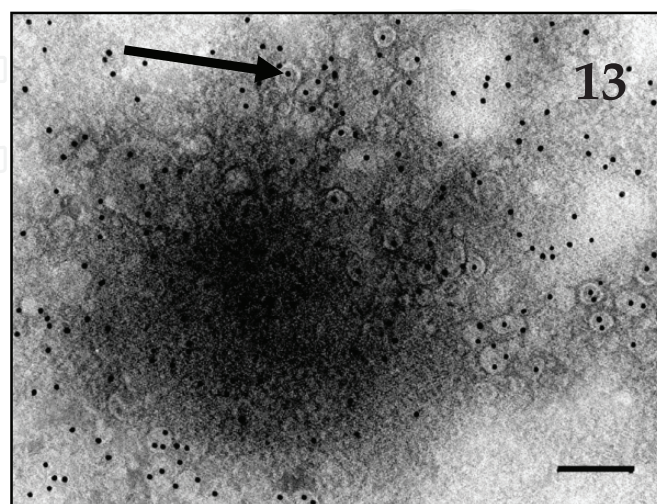
The laboratory diagnosis is performed through seroneutralization, cell culture isolation, PCR and immunohistochemistry [180].

Bovine virus diarrhea (BVD) particles have been identified by negative-staining electron microscopy (**Figure 12**) in feces, in purified virus preparations, in infected cell cultures and in tissues from infected animals [183–188], being this technique recommended by the OIE for the detection of BVDV [183].

Immunogold labeling technique was utilized for marking BVDV particles (**Figure 13**) and for locating both E (rns) and E2 proteins at the virus membrane [187]. The embedding resin technique was used to study the ultrastructural aspects of BVDV, showing that bovine viral diarrhea virus NS4B protein is an integral membrane protein associated with Golgi markers and rearranged host membranes [189].



**Figure 12.** Negatively-stained flavivirus isometric particles in bovine intestine suspension (arrow). Bar: 70 nm.



**Figure 13.** BVDV particles strongly enhanced by the dense colloidal gold particles (arrow). Bar: 140 nm.

The examination of suspected sample by immune electron microscopy procedures is feasible for BVDV virus detection, and it can be used as diagnostic tool, especially for screening of cell culture supernatants infected with suspected clinical specimens. The suitability of this assay for direct screening for identification of persistently infected animals, where the viral load is expected to be high and which are of great concern for control programs, needs to be explored [188].

Considering that BVDV infections cause significant economic losses to farms, biosafety, sanitary and hygiene measures should be implemented on farms to reduce the prevalence of the virus in herds. In addition, quarantine, vaccination and testing procedures should be instituted in the herds for identification and removal of persistently infected animals (IP), minimizing the spread of the virus [166, 178, 179, 190].

#### **1.4. Transmission electron microscopy**

Transmission electron microscopy is a perfectly adequate tool to investigate viral agents during outbreaks of gastroenteritis [191]. It is used when it is necessary to apply a fast and reliable diagnostic method to contain the infection and to quickly minimize the animal losses and consequently the economic damages that cause to the rational economic exploitation of the production animals, as much by the decrease of the productivity as in terms of treatment costs. Consideration should also be given to the zoonotic potential of some viruses, such as rotavirus, and the implications for public health [1].

Electron microscopy has led to the discovery of many new viruses, mainly those associated with gastroenteritis, for which it remained the principal diagnostic method [192].

Viruses are grouped into families based on their morphology. Viruses from various families look distinctly, and these morphological variances are the basis for identification of viruses by electron microscopy. The identification to the family level is already sufficient for the recognition of an unknown infectious viral agent and, to allow the immediate adoption of prophylactic measures, control and prevention of the disease [193].

##### *1.4.1. Negative-staining technique*

Negative staining has been a useful specimen preparation technique for biological and medical electron microscopists for almost 50 years since its introduction by Brenner and Horne in 1959 [194] as an established method [195].

The technique consists of an electron-dense stain that surrounds the biological specimen and penetrates in the structural crevices to give an image in which the biological specimen appears electron-lucent against the dark electron-dense background. The image is formed by the absorption or deflection of the electrons by the stain, giving opacity to those areas [195, 196].

Due to the simplicity of the preparation of the samples and the rapidity of the results (5–10 min), negative staining has been the most used technique, mainly in the detection and viral identification during outbreaks of gastroenteritis [197]. The large diversity of viruses potentially involved in gastroenteritis contributed to the use this technique in clinical virology [198].



In addition to the rapidity, it has several other advantages, such as to enable the detection of different viral particles in a single sample without the need for specific reagents, to allow the discovery of new viruses, and to require a small amount of sample, besides when detecting the agent to exclude the possibility of obtaining false-positive results [193].

Negative staining can be applied to various types of biological samples. In cases of gastroenteritis, viruses can be easily visualized in feces, small intestine fragments, fecal swab and peritoneal fluid where they are found in large quantity.

Several types of contrasting (heavy metal salts) are used; however, 2% ammonium molybdate and pH 5.0 provide the best contrast to viral agents.

The diagnosis is made by comparing the dimensions and specific morphology of the visualized particles and other taxonomically combined viral families.

#### *1.4.2. Immunolectron microscopy technique*

Immunolectron microscopy (IEM) technique that consists in the direct visualization of antigen and antibody complexes by negative stain which promotes increased sensitivity in 100-fold, cujo resultado positivo, is indicated by the presence of virus-antibody aggregates [192].

The technique was initially developed to quantify plant by Derrick [199] and was subsequently used in several types of clinical samples [200–202].

Immunolectron microscopy (IEM) is utilized when the number of viral particles in a sample is very low, when virions are pleomorphic and difficult to identify because they do not have a typical viral morphology or when the samples are “dirty” because the aggregated complexes are more easily observed [203]. It allows identification of the virus for specific antigen-antibody reaction and such identification is achieved by its morphology. It is also used to serotype morphologically similar (but antigenically distinct) particles [195, 204, 205].

Several variations of the method such as immune clumping or direct immunolectron microscopy (DIEM) [206, 207] or immune aggregation electron microscopy (IAEM) [203], solid phase immune electron microscopy (SPIEM) [199] and decoration [208] have been used. Hyperimmune sera, monoclonal antibodies or convalescent sera can be used in performing the technique [193, 203]. The SPIEM has been utilized to detect most of the viruses that cause gastroenteritis such as bovine rotavirus, swine, equine, canine, bovine coronavirus, swine, canine parvovirus and BVDV.

IAEM was used to detect porcine rotavirus (PoRV), porcine torovirus (PoToV) and porcine epidemic diarrhea coronavirus (PEDV) in pigs with enteritis utilizing convalescent sera [203].

#### *1.4.3. Immunolabeling with colloidal gold particles by negative-staining technique*

In this technique, the antigen-antibody reaction is enhanced by antigen labeling by colloidal gold particles associated with protein A, using type- and genus-specific antibody. The method also allows detection and identification of antigen structures induced by the virus and its localization in infected cells, serotype viral strains [209], and determines antigenic variants in isolated strains [210].

This technique was used to label TGEV particles in feces and small intestine fragments of infected pigs [108], type A rotavirus and coronavirus in samples from diarrheic calves and winter dysenteric cattle [143], the simultaneous presence of coronavirus and rotavirus in feces of calves with diarrhea [211] and BVDV in feces of cattle with diarrhea [184].

#### *1.4.4. Immunolabeling in ultrathin section technique*

Immunolabeling in ultrathin sections are powerful tools for detecting and localizing proteins in cell and tissues and to detect virus or viral antigen on the surface of or within ultrathin sections of the cells [195, 212, 213]. The two most widely used techniques are pre-embedding and post-embedding techniques. 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 [214]. Antibodies coupled to electron-dense markers such as colloidal gold can reveal the localization and distribution of specific antigens in various tissues. The colloidal gold has been the most widely used marker [215].

Immunolabeling in ultrathin sections has been widely applied to elucidate ultrastructural pathological aspects of gastroenteric viruses. Payne et al. [216] studied bovine coronavirus antigen in the host cell plasmalemma in cells traced with colloidal gold particles. Risco et al. [115] investigated the presence of two types of virus-related particles that are found during transmissible gastroenteritis virus (TGEV) morphogenesis, whereas Salanueva et al. [217] reported aspects of the structural maturation of the transmissible gastroenteritis coronavirus (TGEV). This technique has also been applied to check the exploitation of microtubule cytoskeleton and dynein during canine parvoviral traffic toward the nucleus [215].

#### *1.4.5. Resin embedding technique*

The resin embedding technique followed by ultrafine sections is especially important to reveal fine details of the ultrastructure of all types of cells and tissues [218], and in an infectious process, it allows observing pathogenesis of infection and the identification of the agent [205]. The thin sectioning 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 [219].

The ultrastructural set of details not only determines the infection, but also the course of the disease in the creations [220].

Resin embedding technique allowed to study several ultrastructural aspects of the intracellular behavior of the TGEV in intestinal fragments of infected pigs [108] and of the parvovirus in intestinal fragments of newborn dogs with diarrhea [220]. This technique also allows studying the efficiency of the vaccines based on in situ produced, noninfectious rotavirus-like particles (RVLPs) [221].

## 2. Conclusion

The different transmission electron microscopy modalities promote a fast and accurate diagnosis of the different gastroenteric viral agents, allowing prophylactic measures of control and prevention in the creations to be promptly instituted, avoiding animal losses and disastrous economic losses, and collaborating with the National Porcine and Bovine Agribusiness.

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