Universidade Estadual de Londrina

THALITA EVANI SILVA DE OLIVEIRA

PATHOGENESIS OF *SENECAVIRUS A*: ROLE OF THE BLOOD BRAIN BARRIER IN NEWBORN PIGLETS WITH THE EPIDEMIC TRANSIENT NEONATAL LOSSES SYNDROME

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Dissertation submitted to the Animal Health Science Graduate Programme (area of concentration Animal Health Science) of Universidade Estadual de Londrina as partial requirement for the acquisition of the Masters of Science Degree.

Supervisor: Prof. Dr Selwyn Arlington Headley

Co-supervisor: Prof. Dr Amauri Alcindo Alfieri

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Não importa o que fizeram com você. O que importa é o que você faz com aquilo que fizeram com você.
Jean Paul Sartre

Não é sobre chegar no topo do mundo e saber que venceu. É sobre escalar e sentir que o caminho te fortaleceu.
Ana Vilela

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ABSTRACT

Senecavirus A (SVA), Picornaviridae family, is the aetiological agent of a vesicular disease that is clinically indistinguishable from the other known vesicular diseases of the pigs, highlighting the foot-and-mouth disease, an animal disease of foremost epidemiological importance worldwide. SVA is the aetiological agent of two syndromes affecting different age groups of swine: Porcine Idiopathic Vesicular Disease (PIVD) and Epidemic Transient Neonatal Losses (ETNL). The objective of this study were to describe the gross, histopathological, immunohistochemical (IHC) and ultrastructural findings associated with natural infection by SVA, and to determine the possible tropism of the virus for different tissues of newborn piglets from Brazil. Autopsies of 54 piglets with clinical signs associated with ETNL were done. Of these, 80% (43/54) piglets contained the antigens of SVA IHC and SVA RNA was identified by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for SVA. Most (81%; 35/43) of the piglets that died of ETNL were between 2-6 days of age. The most frequent macroscopic lesions during autopsy were liquid faeces (91%; 39/43), renal petechial haemorrhages (79%; 34/43), interstitial pneumonia (77%; 33/43), ulcerative lesions at the coronary band (35%; 15/43), oedema of the mesocolon (32%; 14/43), vesicles at the snout (30%; 13/43) and lymphadenopathy (28%; 12/43). Histopathology revealed ballooning degeneration of the urothelium of the bladder (100%; 43/43), urinary pelvis (95%, 41/43), atrophic enteritis (93%, 40/43), and nonsuppurative meningoencephalitis and plexus choroiditis (7%; 3/43). Positive immunostaining for SVA was observed at the endothelium and epithelial of the choroid plexus (CP) of the brain and in epithelial tissues of all organs evaluated, except in the lungs and lymphoid tissues. Transmission electron microscopy demonstrated hydropic degeneration of superficial enterocytes of the small intestine, hydropic degeneration, apoptosis, and endothelial necrosis of fenestrated capillaries and ependymocytes of the CP associated with intralesional viral particles consistent with SVA. This study has demonstrated that SVA is an epitheliotropic virus, has positive immunoreactivity for the transitional epithelium of the renal pelvis, ureter, urinary bladder, and with endothelial identification of viral antigens in the capillaries of the cerebral CP, tongue epithelium, and the superficial enterocytes of the small intestine. These results demonstrated that the ballooning degeneration observed in different tissue/organs were caused by SVA and suggest that the neurological signs observed in some piglets were due to SVA-induced nonsuppurative meningoencephalitis. In addition, initial results suggest that SVA may act as an enterovirus, producing an initial enteric disease with subsequent neurological dissemination.

Key words: Central nervous system. *Picornavirus*. Seneca Valley virus. Swine. Vesicular diseases.

OLIVEIRA, Thalita Evani Silva. **Patogênese do *Senecavirus A***: papel da barreira hematocefálica em leitões com a síndrome das perdas neonatais epidêmicas transientes. 2018. 81 f. Dissertação (Mestrado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

Senecavirus A (SVA), família Picornaviridae, é o agente etiológico de uma doença vesicular clinicamente indistinguível das outras doenças vesiculares dos suínos, destacando-se a febre aftosa, doença animal de maior importância epidemiológica mundial. O SVA é o agente etiológico de duas síndromes, acometendo suínos de diferentes idades: Doença Vesicular Idiopática Suína (PIVD) e Perdas Neonatais Epidêmicas Transientes (ETNL). O objetivo desse estudo foi descrever os achados macroscópicos, histopatológicos, imuno-histoquímicos (IHQ) e ultra-estruturais associados a infecção natural por SVA e determinar o possível tropismo do vírus em diferentes tecidos de leitões neonatos do Sul e Suldeste do Brasil. Foram realizadas autopsias de 54 leitões com sinais clínicos associados a ETNL. Destes, (80%; 43/54) leitões foram positivos na IHQ e confirmados na transcrição reversa seguida de reação em cadeia da polimerase (RT-PCR) para SVA. A maioria (81%; 35/43) dos leitões que morreram por ETNL tinham entre 2-6 dias de idade. As lesões macroscópicas mais frequentes durante as autopsias foram fezes líquidas (91%; 39/43), hemorragias petequiais renais (79%; 34/43), pneumonia intersticial (77%; 33/43), lesão ulcerativa na banda coronária (35%; 15/43), edema de mesocólon (32%; 14/43), vesículas no focinho (30%; 13/43) e linfadenopatia (28%; 12/43). Na histopatologia foi observado degeneração balonosa do urotélio da bexiga (100%; 43/43), pelve urinária (95%; 41/43), enterite atrófica (93%; 40/43), meningoencefalite não supurativa e plexo coroidite (7%; 3/43). A imunomarcação positiva ao SVA foi observada no endotélio e células epiteliais do plexo coroide (CP) e nos tecidos epiteliais de todos os órgãos avaliados, exceto, nos tecidos pulmonar e linfoide. A microscopia eletrônica de transmissão revelou degeneração hidrópica dos enterócitos apicais do intestino delgado, degeneração hidrópica, apoptose e necrose do endotélio dos capilares fenestrados e ependimócitos do CP associados a partículas virais intralesionais compatível com SVA. Este estudo demonstrou que o SVA é um vírus epiteliotrópico, apresentando imunomarcação positiva para o epitélio de transição da pelve renal, ureter e vesícula urinária, endotélio dos capilares do CP cerebral, no epitélio da língua e nos enterócitos apicais do intestino delgado. Esses resultados demonstram que a degeneração balonosa observada em diferentes tecidos/órgãos foram causadas pelo SVA e sugerem que os sinais neurológicos observados em alguns leitões foram decorrentes da meningoencefalite não supurativa induzida pelo SVA. Além disso, os resultados iniciais sugerem que o SVA pode atuar como um enterovírus, produzindo uma doença entérica inicial com posterior disseminação neurológica.

Palavras-chave: Sistema nervoso central. *Picornavirus*. Seneca Valley virus. Suínos. Doenças vesiculares.

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LIST OF ABBREVIATIONS

BBB	Blood-brain barriers
BCSFB	Blood-cerebrospinal fluid barrier
CNS	Central nervous system
CP	Choroid plexus
CSF	Cerebrospinal fluid
DAB	3,3'-diaminobenzidine-tetrahydrochloride-dihydrate
Dpi	Days post inoculation
ETNL	Epidemic transient neonatal losses
FMD	Foot-and-mouth disease
FMDV	*Foot-and-mouth disease virus*
IHC	Immunohistochemistry
PCR	Polymerase chain reaction
PIVD	Porcine idiopathic vesicular disease
qRT-PCR	Real time quantitative reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SV	Vesicular stomatitis
SVA	*Senecavirus A*
SVDV	*Swine vesicular disease virus*
SVV-001	Seneca Valley virus
TEM	Transmission electronic microscopy
VESV	*Vesicular exanthema of swine virus*
VES	Vesicular exanthema of swine
VSV	*Vesicular stomatitis virus*

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51 ## 1 INTRODUCTION

52 *Senecavirus A* (SVA), is the only representative of the genus
53 *Senecavirus*, family Picornaviridae [1], and is the aetiological agent of an emerging
54 infectious vesicular disease that is clinically indistinguishable from other known vesicular
55 diseases of swine [2-9], such as foot-and-mouth disease, an animal disease of major
56 epidemiological importance worldwide [10].

57 This virus is the aetiological agent of two distinct but overlapping
58 syndromes that affects swine of different age groups: Porcine Idiopathic Vesicular Disease,
59 PIVD [3-5, 9, 11-16], and Epidemic Transient Neonatal Losses, ETNL [11, 12, 15, 17-19].

60 In 2014, outbreaks of PIVD were reported in piglets, as well as weaned
61 and adult pigs from the largest pig producing States of Brazil [4, 9]. In 2015, SVA
62 outbreaks occurred in the USA [13, 20, 21], and China [16, 22], with subsequent outbreaks
63 in Colombia [8], and Thailand [6] in 2016.

64 In contrast, ETNL, is a multisystemic disease of piglets that has multiple
65 clinical signs including diarrhoea, sudden death [9, 11, 12, 15, 17-19], lethargy, and
66 neurological manifestations [11, 12, 17-19]. It has an elevated mortality rate that varies
67 between 30 to 70% [4, 9]. Usually, it ETNL has a sudden onset is transient, with vesicular
68 lesions that may develop within two weeks of spontaneous outbreaks [23].

69 This study describes the clinical, gross, pathological,
70 immunohistochemical, and ultrastructural aspects related to SVA-induced diseases in pigs.

71 **2 LITERATURE REVIEW**

72 **A systematic review of the clinical and pathological aspects**

73 **of *Senecavirus A*-induced diseases[1]**

74 2.1 ABSTRACT

75 *Senecavirus A* (SVA), a member of the Picornaviridae family, is associated with two
76 syndromes: Porcine Idiopathic Vesicular Disease (PIVD) and Epidemic Transient Neonatal
77 Losses (ETNL). This virus has tropism for epithelia, resulting in ballooning degeneration
78 with subsequent vesicular formation. While PIVD is associated with vesicles and
79 ulcerative lesions at the feet and snouts of pig regardless of age, ETNL is observed
80 exclusively in piglets that are up to 10-day-old. The predominant histopathological
81 findings in piglets with ETNL include ballooning degeneration of the urothelium of the
82 renal pelvis, ureter, and urinary bladder, interstitial pneumonia, atrophic enteritis with
83 vacuolization of the superficial enterocytes of the villi of the small intestine, lymphoid
84 depletion of the lymph nodes and tonsil. Transmission electron microscopy demonstrated
85 viral particles at the choroid plexus of one piglet that had nonsuppurative
86 meningoencephalitis and choroid plexitis. Initial studies suggest that SVA may use the
87 Trojan horse mechanism to enter the brain. The tonsils were considered as one of the
88 primary sites of replication for SVA. In newborn piglets the period of viraemia was
89 transient (3-7 days), and the peak of SVA genomic copies coincided with elevated piglets
90 mortality due to ETNL. It is proposed that immunological piglet immaturity may be a key
91 feature of viral replication, and that SVA probably acts as an enterovirus producing an
92 initial enteric disease with subsequent nonsuppurative meningoencephalitis in piglets with
93 neurological manifestations associated with ETNL, via the Trojan horse mechanism.

[1] This paper was written according to the guidelines of the European Journal of Clinical Microbiology & Infectious Diseases. http://www.springer.com/biomed/medical+microbiology/journal/10096 Impact factor 2.727.

2.2 KEYWORDS

Central nervous system, Epidemic Transient Neonatal Losses, Picornaviridae, Seneca Valley virus, Swine.

2.3 INTRODUCTION

Epitheliotropic viruses induce the formation of vesicles that may progress to ulcers in epithelia and mucosae [10, 24]. Among the viral pathogens that cause vesicular diseases in swine, *Foot-and-mouth disease virus* (FMDV, Picornaviridae family), *Vesicular stomatitis virus* (VSV, Rhabdoviridae family), *Swine vesicular disease virus* (SVDV, Picornaviridae family), *Vesicular exanthema of swine virus* (VESV, Caliciviridae family) [25], and *Senecavirus A* (SVA, Picornaviridae family) must be highlighted [3-6, 8, 9]. Vesicular diseases caused by these viral pathogens have great economic impacts on production animals, and are of extreme epidemiological importance due to the associated clinical signs [3, 5], that are not easily distinguished from those frequently observed in Foot-and-Mouth Disease, FMD [25, 26], which is an important global animal health problem with regular occurrence of disease epidemics [27].

The family Picornaviridae contains several pathogens that infects humans and animals [24, 27, 28]. Viruses within this family can cause a wide range of disease manifestations that are of variable severity in mammals, resulting in cardiac, gastrointestinal, hepatic, mucocutaneous, neurological, respiratory, and systemic diseases [28]. A comparative analysis of the forms of dissemination, incubations periods, and target tissues of viral agents of importance to pig farms worldwide is given in Table 1.

In 2002, Seneca Valley virus (now renamed, *Senecavirus A*) was serendipitously isolated in the laboratories of Neotropix Inc. [2] that are located in the proximity of the Seneca Creek State Park, Gaithersburg, MD, USA [29], while cultivating adenovirus-5-based vectors in the PER.C6 cell line (transformed foetal retinoblastoma), then known as

120 SVV-001. This virus accidentally contaminated porcine trypsin or foetal bovine serum [2],
121 and was initially considered as non-pathogenic, and thereafter observed SVV-001 inducing
122 selective cytotoxicity and high selectivity for in tumour cells of neuroendocrine features
123 [30].

124 Cases of vesicular disease of unknown origin, denominated Porcine Idiopathic
125 Vesicular Disease (PIVD), were reported in swine herds, between 1979 to 2008, from
126 different countries, including the USA [31, 32], Australia [33], Canada [34], Italy [35],
127 New Zealand [36], and the UK [37]. In 2012, SVA was initially associated with PIVD in a
128 six-month-old boar from Indiana, USA, that had vesicles at the oral cavity, coronary band,
129 and ulcers on all hooves [7]. In 2014, SVA was associated for the first time with cases of
130 PIVD in pigs from the feeding and finishing phases in several herds from different
131 geographical regions of Brazil [4, 9, 17, 18].

132 The second syndrome, associated with SVA infection is known as Epidemic
133 Transient Neonatal Losses (ETNL), which is characterized by diarrhoea, neurological
134 signs, and sudden death in piglets up to 10 days of age [11, 12, 17-19], as the main clinical
135 features. The objectives of this review are to discuss the clinical, gross, and pathological
136 aspects related with SVA-induced disease syndromes in pigs.

137

138 2.4 PICORNAVIRUS

139 1.4.1 Physical Properties and the Virus Structure

140 The name Picornaviridae is due to the small size of the virus (*pico*, a small unit of
141 measurement [10^{-12}]), and the type of nucleic acid that constitutes the viral genome
142 (ribonucleic acid - RNA) [28]. Picornaviruses are approximately 25-30 nm in diameter,
143 have an icosahedral non-enveloped capsid. The viral genome is composed of single-
144 stranded linear RNA with positive polarity, whose length may vary between 7 and 9 kb
145 [38].

146 Picornaviruses virions are spherical, and the particles consists of a non-enveloped
147 RNA genome surrounded by protein, with a diameter of about 30 nm [28]. Replication of
148 picornaviruses occurs exclusively within the cell cytoplasm [24, 28, 39]. The viral particles
149 are deficient of a lipid envelope, are resistant to organic solvents [28], and can remain
150 viable for several days and often weeks in the environment [27].

151 Each *Picornavirus* has specific requirements to successfully replicate and
152 complete its cycle, including: (i) entry pathway, (ii) viral tropism, (iii) target cells, and (iv)
153 pH. Simple and stratified epithelial cells and mucosa-associated lymphoid tissue
154 (lymphocytes, macrophages, and dendritic cells) are generally used as targets cells by these
155 viruses at the site of entry [40], resulting in cell-associated viremia or leukocytic
156 trafficking [40]. Among the picornaviruses that affect pigs, FMDV is highly labile and
157 rapidly loose its infectivity at a pH that is lower than 6.0 [28]. While cardioviruses,
158 enteroviruses (except rhinoviruses) [28, 41], and teschoviruses are acid stable and retain
159 their infectivity at pH values that are below 3 [28, 42].

160 The viral genome contains a single, long open reading frame, encoding a
161 polyprotein that is cleaved to form 11 distinct proteins [24, 27, 28, 43, 44]. In addition to a
162 leader peptide which is not always present, the polyprotein consists of three regions,
163 designated P1, P2, and P3. The P1 region encodes the structural proteins composed of four
164 proteins referred to as VP4, VP2, VP3, and VP1, that form the viral capsid [43, 45]. Three
165 non-structural proteins form the polyprotein P2 (2A, 2B, 2C), and four non-structural
166 proteins are the components of P3 (3A, 3B, 3C, 3D) [24, 27, 28, 43, 44]. The functions of
167 the non-structural proteins encoded in P2 and P3 are not fully elucidated [43]. In all
168 *Picornavirus*, the capsid proteins 1B, 1C, and 1D, non-structural proteins 2C, 3C, and 3D
169 are completely conserved [45].

170 The first complete sequencing of the SVA genome occurred in 2008 [2], and these
171 results have shown that SVA has close genomic similarity with *Encephalomyocarditis*
172 *virus* (EMCV) and *Theilovirus* (TMEV), both of the *Cardiovirus* genus, with a sequence of
173 similar polypeptides (P1, 2C, 3C, and 3D) [2]. The RNA genome of SVA consist of 7,280
174 nucleotides, and the genomic regions 2A, 2B, 3A, and 3B of SVA differ significantly from
175 those of all other known picornaviruses [2].
176
177 2.5 CHARACTERISTICS OF PICORNAVIRUSES OF IMPORTANCE TO SWINE
178 The Picornaviridae family is composed of 40 genera [1, 45], and 94 species [46]
179 that infect vertebrates [28]. Among these, the *Aphthovirus*, *Cardiovirus*, *Enterovirus*,
180 *Kobuvirus*, *Pasivirus*, *Sapelovirus*, *Senecavirus,* and *Teschovirus* are viral genera of
181 importance to pigs [24]. The methods of dissemination, target tissues, and disease
182 processes of the main Picornaviridae of importance to swine are resumed in Table 1.
183 Interestingly, most of these viral pathogens, except *Aphthovirus,* gain entry to the organism
184 via the oral (or oral-nasal) pathway, and all produce lesions in epithelial structures.
185 *Cardiovirus, Enterovirus, Sapelovirus*, SVA, and *Teschovirus* are associated with
186 nonsuppurative encephalitis and/or meningoencephalitis in newborn and weaned piglets
187 [17-19, 47-58]. In newborn piglets naturally infected with SVA that demonstrated
188 neurological manifestation associated with ETNL, immunoreactivity to SVA by
189 immunohistochemistry (IHC) was observed in the epithelial cells of the choroid plexus
190 (CP) and ependymal cells of the lateral ventricle, with gliosis, neuronal necrosis, malacia
191 of the brainstem, and perivascular cuffings. Furthermore, in 2017, our group observed by
192 transmission electronic microscopy (TEM), that the endothelial cells of the CP and
193 ependymocytes infected with SVA had ballooning degeneration, apoptosis or necrosis
194 surrounded by virus particles of 17–30 nm in diameter morphologically compatible with
195 SVA [19].

196 2.6 SPECIFIC ASPECTS OF THE CENTRAL NERVOUS SYSTEM RELATIVE TO
197 INFECTION BY SVA

198 2.6.1 Blood-Brain Barriers Systems and Defence Mechanisms

199 The central nervous system (CNS) is composed of the brain, spinal cord, optic
200 nerves, and retina [59], and consists of blood vessels with structural peculiarities. The
201 capillaries are unique, since they are surrounded by perivascular astrocytic fibers within
202 the microcirculation, and form a barrier between the brain parenchyma and the vascular
203 system [60, 61]. This result in the blood-brain barrier (BBB), an important anatomical
204 structure, which constitutes the largest interface between the blood and the brain, and
205 selectively limits the entry of molecules and the leucocytes into the meninges and to the
206 brain [61-65].

207 The BBB is associated with: (i) selective transport of essential nutrients to the
208 brain; (ii) defend the CNS from neurotoxic substances circulating in the blood; (iii)
209 prevents entry of infectious disease agents (iv) and large proteins from the lumen of blood
210 vessel; (v) ion regulation; and (vi) transport of gases in and out the CNS [62-66].

211 In addition to the BBB, there are other defence mechanisms of the brain,
212 including: (i) the blood-cerebrospinal fluid barrier (BCSFB), that exist between the blood
213 and the cerebrospinal fluid (CSF), which is formed by the CP and arachnoid, and (ii) the
214 ependymal barrier along the brain and CSF, formed by ependymal cells and astrocytic feet
215 processes [62, 64, 65].

216 The glia limitans located in the arterioles, capillaries and venules of the CNS, are
217 separated from the endothelial layer by pericytes and smooth muscle cells forming the
218 Virchow-Robin space, except in the capillaries, where this space does not exist [61, 65].
219 The close contact between astrocytes, resident cells of the CNS (neurons, microglia,
220 oligodendrocytes, and other astrocytes) and with microcirculation vessels, results in the

221 activation of astrocytes, and the microglia during inflammatory processes, since these cells
222 have an important role in innate and adaptive immune[67].

223 The CP is composed of capillaries with fenestrated endothelia, a monolayer of
224 epithelial cells, interconnected by dense tight junctions, which are supported by a thin
225 stroma in the ventricular system [59, 68]. The main function of the CP is the production of
226 CSF [59, 62, 64, 68]. The CP is located in the lateral, third and fourth ventricles of the
227 brain [68], and protects these delicate structures as well as regulate metabolism and
228 homeostasis in the CNS [59, 62, 64, 68].

229 Moved by pulsations of the CP and action of cilia on ependymal cells, the CSF
230 circulates from the ventricles to the brainstem to cover the external surfaces of the brain
231 and spinal cord, and serve as protection against mechanical shocks [59]. Reabsorption of
232 CSF in to the blood occurs in the major veins of the superior sagittal sinus, through
233 arachnoid granulations or villi [69].

234 It was previously thought that the immune privilege of the CNS was based on
235 multiple factors, including: i) presence of the BBB; ii) lack of lymphatic drainage; iii) low
236 expression levels of major histocompatibility complex molecules class I and II; iv)
237 absence of antigen-presenting cells [70]; and, v) many anti-inflammatory soluble
238 modulators [62, 64].

239

240 2.6.2 Lymphatic Vessels are Present in the Brain

241 It was proposed that the immune privileged of the CNS is complex and not to
242 absolute [66, 69, 71]. Neuroimaging done in the brain of the mouse has identified
243 meningeal lymphatic vessels that drains the CSF and immune cells directly to the deep
244 cervical lymph node [66]. This then suggest that the CNS is probably connected to the
245 peripheral immune system through a network of lymphatic vessels present in the meninges.

246 The meningeal lymphatic system probably begins at the eyes and connects to the olfactory
247 bulb before aligning to adjacent sinuses. These lymphatic vessels are in the transverse
248 sinuses and the superior sagittal sinus [66].

249 The production of antibodies in the deep cervical lymph nodes four days after
250 administration of a detectable antigen in the CSF, in which there was a peak in antibody
251 production on the sixth day and that lasted up to 10 days after administration [71]. This
252 same study, revealed that there was a small production of antibodies in the superficial
253 cervical lymph nodes and spleen, at least sixth days post-administration. The first region
254 affected in the deep cervical lymph nodes by the ink was the medullar sinus, suggesting
255 that the antigen was phagocytosed and transported by macrophages via leukocytic
256 trafficking [71].Immune privilege of the CNS is not the consequence of limited antigen
257 [72]. Neuroinflammation and CNS autoimmune disease induce T cell accumulation in the
258 CNS, demonstrating that there is continuous surveillance of CNS antigens in peripheral
259 lymphoid tissues. T cells have high affinity for CNS antigens under various conditions,
260 which indicates that immune privilege is not the result of limited antigen sampling [72].
261 Immune cells (B, T, and dendritic cells) were identified in the meningeal lymphatic vessels
262 of healthy mice, suggesting that the meningeal lymphatics may participate actively in
263 leucocytic trafficking [66]. Moreover, cell-mediated immunosurveillance of the CNS [66,
264 69, 73], may have high affinity for antigens of the brain [72], and be fundamental in
265 protecting these tissues against cancer [74], infections [75], and inflammatory processes
266 [72].

267

268 2.7 INFECTIONS OF THE CENTRAL NERVOUS SYSTEM ASSOCIATED WITH 269 PICORNAVIRIRUS

270 Encephalitis and/or meningoencephalitis are histopathological findings frequently
271 associated with several viruses of the Picornaviridae family, such as *Cardiovirus* [52, 54,

272 76], *Enterovirus* [55], *Sapelovirus* [47, 53, 57], *Teschovirus* [48-51, 58, 77], and
273 *Senecavirus* [17-19]. These viruses are significant causes of morbidity and mortality,
274 particularly in newborn piglets [17-19, 47-58].

275 Viruses can cross the BBB, and invade the CNS by different mechanisms [68].
276 The first is associated with transcellular traversal passage without evidence of disruption of
277 the tight junctions between the cells as the virus crosses the cytoplasm. Secondly, there is
278 paracellular traversal passage, which involves virus penetration between adjacent barrier
279 cells with and/or without evidence of disruption of tight junctions [68, 78, 79]. Both
280 mechanisms result in elevated levels of viremia and inflammation [80]. Furthermore, there
281 is the Trojan horse mechanism, in which the virus penetrates the barrier by transmigration
282 within infected phagocytes [68, 78, 79]. The Trojan horse mechanism has been proposed as
283 the most likely method used by SVA to enter the brain and produces nonsuppurative
284 meningoencephalitis in ETNL [19].

285

286 2.8 PATHOLOGICAL SYNDROMES ASSOCIATED WITH *SENECAVIRUS A* IN
287 SWINE

288 2.8.1 Porcine Idiopathic Vesicular Disease

289 2.8.1.1 Introduction and historical overview

290 Outbreaks of PIVD were reported sporadically since 1979 [31, 33, 36]. In a herd
291 from Florida, one adult swine had vesicular lesions on the muzzle and feet, but viruses
292 were not identified in samples derived from vesicular epithelium, and clinical signs were
293 indistinguishable from those observed in FMD [31].

294 In 2004, Amass and colleagues [32] reported outbreaks of vesicular diseases in
295 sows, newborn piglets, and finishing pigs from several swine herds in Indiana, USA. These
296 authors observed that diseased swine had one or more clinical signs including lameness,
297 fever, lethargy, intact and/or ruptured vesicular lesions the oral and gingival mucosae,

298 tongue, snout, hoof, and coronary bands [32]. Viruses of classical vesicular diseases
299 (FMDV, VSV, SVDV, and VESV) were not identified in this cases. The aetiological agent
300 of this outbreak was not characterized, and because of its unknown aetiology, the disease
301 was referred to as porcine idiopathic vesicular disease [32].

302 In 2007, pigs from a farm in Manitoba, Canada, transported to Minnesota, USA,
303 developed vesicular and/or ulcerative lesions on the snout and the coronary bands, tissues
304 separating from the edge of the coronary band and dewclaws. Approximately 80% of the
305 187 affected pigs had lameness, and four pigs (2%) were febrile [34]. These authors
306 indicated that the vesicular lesions were indistinguishable from those of foreign vesicular
307 animal diseases, but all testing were negative for FMDV, VSV, SVDV, and VESV [34].
308 This was the first identification of SVA RNA in an outbreak affecting pigs, but the
309 pathogen was not associated with the disease observed in these pigs [34]. In fact, the first
310 description of SVA isolated from swine vesicular disease occurred in 2012, in a 6-month-
311 old boar at an agricultural exhibition in Indiana, USA [7]. The infected boar had a history
312 of anorexia, lethargy, claudication with intact and ruptured vesicles and erosive lesions in
313 the oral cavity, around the snout and coronary bands, with ulcerations of both limbs. The
314 SVA was detected by reverse transcription-polymerase chain reaction (RT-PCR) from
315 these vesicular lesions. This was the second study that identified SVA in vesicular disease
316 of swine in which other classical infectious vesicular disease agents were not detected,
317 confirming that these lesions were associated with SVA-induced infection [7].

318 Until early 2014, PIVD was a disease exclusively observed in swine, sporadically
319 in young pigs, and not associated with pig mortality in any production phase [7, 31-36].
320 However, since late 2014, SVA-induced PIVD with associated vesicular lesions were
321 identified in two states in China [16, 22], seven states in Brazil [4, 9], and nine states in the

322 USA [3, 5, 13, 14], and the disease occurred simultaneously in newborn piglets and adult
323 pigs [5, 6, 8, 11, 12, 17-19, 23, 29].

324 Tousignant and colleagues (2017) [23], conducted an epidemiological
325 longitudinal study with 34 parity sows and 30 suckling piglets from 15 litters, that had
326 clinical signs associated with PIVD outbreaks. These pigs were analysed by real time
327 quantitative RT-PCR (qRT-PCR), and had a high load of SVA RNA in the tonsil and rectal
328 swabs from suckling piglets and sows, with the highest percentage of SVA positive
329 samples up to six weeks post-outbreak [23].

330

331 2.8.1.2 Epidemiological findings

332 Between 1988-2005, 12 different viral strains, serologically similar to the SVV-
333 001 prototype were detected by the National Laboratory of Veterinary Services in Ames,
334 Iowa, USA, in pigs, cows, and mice from different states (California, Illinois, Iowa,
335 Louisiana, Minnesota, New Jersey, and North Carolina), of the USA suggesting that SVV-
336 001 and other strains are common and are widely distributed both in time and
337 geographically in the USA [81]. In these seven states, more than 60 serum samples from
338 farmers and non-farmers were evaluated, and one farmer had a serum neutralization title of
339 1:8, demonstrating that humans are not probable hosts for SVA, confirming that this virus
340 was not prevalent in human populations [30, 81].

341 The first outbreaks of SVA infection in Brazil were reported in 2014, but it was
342 not known whether the virus was circulating in swine populations within this country. A
343 retrospective serological investigation was performed by Saporiti and colleagues (2017)
344 [82], using 594 serum samples collected before and after outbreaks of SVA. This study
345 utilized 347 samples from 19 herds, between 2007-2013, and 247 samples from four herds,
346 between 2014-2016, after the SVA outbreaks in Brazil. These investigators demonstrated

347 that all serum samples obtained before 2013 were negative for anti-SVA antibodies, with
348 neutralizing antibodies to SVA being detected only in serum samples obtained after 2014.
349 Moreover, after the SVA outbreaks in Brazil these herds had 36.4% (90/247) of positive
350 serum samples (neutralizing antibody titter ≥ 64) from pigs with clinical signs of PIVD
351 [82].

352 A study done with flies (*Musca domestica*) and mice (*Mus musculus*), from
353 positive swine herds located in Minnesota, Midwest region of the US, and Santa Catarina,
354 Southern region of Brazil [83]. The SVA was detected by qRT-PCR in faeces (19%; 5/27)
355 and small intestine (4%; 1/27) of the mice, suggests that these pests may play a role on the
356 epidemiology of SVA [83]. Interestingly, mice can carry and, perhaps, viable SVA in
357 faeces, and small intestine, suggest that these species may play a role on SVA
358 epidemiology [83]. These results demonstrate that faeces is a route of elimination of the
359 agent into the environment, and mice are natural hosts of the SVA, as previously noted by
360 Knowles et al. who detected neutralizing antibodies against SVA in this species [81].

361 The morbidity associated with PIVD seems to be different in distinct geographical
362 regions, being 1.5-15% in Minnesota [83], 38-92% in Iowa [11], and 20 to 90% in
363 Southern Brazil [4]. In fact, in 2015 there were a total of 200 cases in the entire USA,
364 while in 2017, there were 300 cases in Wisconsin alone, where there is more trafficking of
365 pigs [84]. It must be highlighted that Iowa, North Dakota, and Minnesota in the USA [85],
366 and Southern the Brazil [86] are the largest pork producers in these countries.

367

368 2.8.1.3 Clinical manifestations

369 Affected breeding herds, feeding and finishing pigs infected with PIVD can
370 demonstrate several clinical manifestations (Table 2). Clinic signs include lameness,
371 lethargy, intact or ruptured vesicles, fever, coalescing erosions and/or ulcerative lesions on

372 the coronary bands, hooves, snout, footpad, interdigital area, and heel of the feet, dewclaws
373 [3-5, 9, 23, 84], and reduced feed intake [23].
374 Several outbreaks of vesicular disease associated with SVA-induced infections
375 were described in pig herds from diverse geographical regions of the USA with effect from
376 2015 [11-13, 20, 21]. Since most of these infections were diagnosed in boars, it may be a
377 possible form of transmitting this disease to other countries through the commercial usage
378 of semen [16], or other fluid contaminated with the agent, because SVA is a contaminating
379 virus.
380 During 2016, SVA-associated outbreaks occurred in countries that had no prior
381 identification of PIVD; with infections being diagnosed in Colombia [8] and Thailand [6],
382 with the spread of the disease to another state of China [22].
383
384 2.8.1.4 Pathological findings
385 Ulcerative lesions of the coronary bands (Fig. 1A) and snout (Fig. 1B) are the
386 most common pathological findings in PIVD [3, 4, 6-9, 11-13, 15, 29, 83, 84]. The most
387 important histopathological features associated with PIVD are shown in Table 3, with
388 atrophic enteritis and degenerative changes to multiple epithelia being some of the most
389 frequently observed lesions in affected pigs.
390 There are few studies that evaluated PIVD in adult pigs. Lymphoid hyperplasia in
391 the tonsils, spleen (Fig. 1C), and lymph nodes were histopathologically described in 15-
392 week-old finishing pigs inoculated with SVA [3]. Other histopathological alterations are
393 not commonly observed in pigs with PIVD [3, 5]. Lymphoid hyperplasia (Fig. 1D),
394 probably correlated with immunogenicity of the SVA infection caused by high activation
395 of B lymphocytes in plasma cells producing (64 to 4096 anti-SVA antibody titters) in
396 spontaneous [82] and experimental studies with SVA [5, 87, 88].

397 2.8.2 Epidemic Transient Neonatal Losses

398 2.8.2.1 Introduction and historical overview

399 The first reports of ETNL occurred in November 2014 [9, 17, 18], in piglets from
400 farms in Brazil, during which piglets that were less than 10-day-old were more frequently
401 affected, with elevated piglet morbidity (up to 70%) and mortality (up to 30%) [4, 9]. One
402 year later, new cases were diagnosed in pig farms from Brazil [17-19], China [16, 22], and
403 the USA [9, 11, 12, 15].

404 Due to severe diarrhoea, piglets with ETNL can be easily confused or
405 misdiagnosed with diarrheal-associated diseases caused by pathogens such as *Porcine*
406 *epidemic diarrhea virus*, *Transmissible gastroenteritis virus*, *Porcine deltacoronavirus*,
407 *Porcine reproductive and respiratory syndrome virus*, *Escherichia coli*, *Porcine rotavirus*,
408 and/or *Clostridium* spp. [89]. Cachexia is not a clinical sign frequently observed in ETNL,
409 and stomachs of affected piglets are frequently filled with milk when autopsied [18, 19],
410 suggesting that feed intake is not a major problem in ETNL.

411

412 2.8.2.2 Epidemiological findings

413 Morbidity and mortality rates are significantly more elevated when compared to
414 PIVD, especially in one to ten-day-old piglets [9, 11, 17]. Morbidity levels can attain 70%
415 [9], but mortality rates are comparatively reduced, and may vary from 15 to 30% [4, 9, 15,
416 19].

417 In contrast to PIVD, elevated mortality rates due to ETNL were observed in
418 piglets worldwide [4, 9, 11, 12]. In the USA, 34% piglet mortality were identified in in
419 farms from Minnesota [83], and with 29% mortality in Iowa [11]. In the USA, lesions were
420 initially described in sows, and a week thereafter in piglets, with elevated neonatal
421 mortality [12].

422 Piglet mortality due to SVA in Brazil has been estimated as 20-30% in the
423 Southern [17], and 30-70% in the Southeastern and Midwestern [9] regions of the country.
424 In Brazil, ETNL seems to occur simultaneously in piglets and sows [4, 9], and not
425 separated by one week as described in the USA. The reason for this one-week delay in the
426 onset of disease between sows and piglets is unknown, but might be related to management
427 practices that are characteristic to each geographical location.
428 A phylogenetic study revealed that there are genetic differences in the genome in
429 the three clades of SVA [90]. However, it is currently unknown, if the changes in the
430 contemporary SVA strains may contribute to the emergence of SVA-associated diseases in
431 various countries.
432
433 2.8.2.3 Clinical manifestations
434 The ETNL syndrome affects newborn piglets (0 to 10-day old), which have
435 similar clinical signs as those observed in PIVD. The clinical signs in newborn piglets,
436 especially those between three to seven days of age, are more severe [9, 11, 16-19], and
437 coincide with the viral peak of SVA replication [3]. These clinical signs may include
438 dehydration [23], lameness, weakness, lethargy, cutaneous hyperaemia, reluctance to
439 suckle, sialorrhea, diarrhoea, neurological manifestations, and/or sudden death [9, 11, 12,
440 16-19]. This syndrome has since been described in pig farms from Brazil [9, 15, 17-19],
441 China [16, 22], and North America [11, 12, 15]. Table 2 summarizes the main clinical
442 signs associated with ETNL.
443
444 2.8.2.4 Pathological findings
445 In our studies with newborn piglets naturally infected with SVA, we demonstrated
446 that the main gross pathological findings associated with SVA are liquid faeces, lymphoid

447 hyperplasia (Fig. 2A), vesicular or ulcerative lesions on the lips (Fig. 2B), snout (Fig. 2B,
448 2C), oral mucosa and tongue, coronary band (Fig. 2D), palmar/plantar regions, and sudden
449 death [17-19]. In addition, we have demonstrated that SVA has tropism for epithelia,
450 particularly the urothelium of the renal pelvis, ureter and urinary bladder, with severe
451 ballooning degeneration (Fig. 3A), and hyperplasia of the epithelium (Fig. 3B) [17-19].
452 Additionally, the epidermis adjacent to vesicular lesions may have parakeratotic
453 hyperkeratosis (Fig. 3C) with rete peg formation and ballooning degeneration [3, 7].

454 In some piglets with neurological manifestations associated with infection
455 induced by SVA, observed nonsuppurative meningoencephalitis with cerebrocortical
456 necrosis of the brain, rare malacia of the brainstem and perivascular cuffing formed by
457 lymphocytes. In the CP, nonsuppurative choroid plexitis, with necrosis and ballooning
458 degeneration at the fenestrated capillary, hyperplasia, necrosis and ballooning degeneration
459 in ependymocytes can be observed [19].

460

461 2.9 POSTULATED PATHOGENESIS FOR *SENECAVIRUS A*

462 Significant genetic diversity has been observed in SVA isolated from different
463 countries and continents [4, 9, 16, 20, 34, 35, 91]. In comparison with other picornaviruses,
464 the life cycle of SVA is not fully elucidated [3, 5]. Several studies have suggested that
465 SVA has tropism for epithelia, resulting in ballooning degeneration which coalesce to form
466 vesicles [3, 5, 17-19, 92]. The predominant histopathological features identified with SVA-
467 induced spontaneous infections were ballooning degeneration of the urothelium of the
468 renal pelvis, ureter and urinary bladder, interstitial pneumonia, atrophic enteritis with
469 vacuolization of superficial enterocytes of the villi of the small intestine, as well as
470 lymphoid depletion of lymph nodes (unpublished data) and the palatine tonsil [17-19].

471 Using a combination of RT-PCR and IHC with newborn piglets [17-19, 92], we
472 have demonstrated that there is hyperplasia of the epithelium of the CP cells in piglets with

473 (Fig. 4) or without nonsuppurative meningoencephalitis (Fig. 3D). Several studies have
474 shown that there is a high genomic load of SVA at the tonsil of infected piglets [15, 92];
475 the palatine tonsils are drained by the regional lymph node (deep cervical lymph node)
476 [71]. Moreover, SVA antigens were identified in the brain of piglets with nonsuppurative
477 meningoencephalitis and neuronal necrosis and with clinical manifestations of neurological
478 impairment, and TEM analysis confirmed the participation of SVA in the development of
479 nonsuppurative choroid plexitis [19]. We have proposed that SVA enters the brain via the
480 Trojan horse mechanism, probably by infecting phagocytes that dislocate from the deep
481 cervical lymph node directly to the brain, via lymphatic vessels in the transverse sinuses
482 and the superior sagittal sinus [66]. Experimental studies should be performed to validate
483 this hypothesis.

484 Interstitial pneumonia in domestic animals is a characteristic lesion that is
485 frequently associated with viral agents and results in proliferation of type II pneumocytes
486 and interstitial accumulation of lymphoplasmacytic infiltrate [93]. Our group has
487 demonstrated that the lungs of naturally infected pigs have elevated loads of SVA (6.78-
488 9.96 log^{10} genomic copies per g of tissue) [92]. However, *in situ* hybridization has
489 identified SVA in the alveolar septum of newborn piglets without evidence of
490 histopathological alterations [15]. Moreover, we have detected high loads of the SVA by
491 qRT-PCR, and positive immunolabelling of SVA by IHC (Fig. 4A-C) in piglets with
492 interstitial pneumonia [92]. These findings suggest that SVA contributes actively towards
493 the development of interstitial pneumonia in affected swine.

494 Immunohistochemical studies have demonstrated that SVA has tropism for
495 epithelia [19], and frequently there is positive immunostaining on the epithelium of the
496 renal pelvis, ureter, urinary bladder, with endothelial identification of viral antigens in the
497 capillaries of the cerebral CP, tongue, and the superficial enterocytes of the small intestine

498 by IHC [18, 19]. Collectively, these findings demonstrate the multisystemic nature of
499 SVA. Moreover, vascular injury seems not to be the major histopathological findings
500 associated with SVA, since there are no reports of vascular-associated diseases in ETNL or
501 PIVD [3, 15, 17-19, 92]. Therefore, dissemination via the lymphatic system seems to be
502 the most probably method of entry to affected tissues/organs and may explain the elevated
503 viral load of SVA observed in lymphoid organs by ISH [3].

504 There are few descriptions of neurological signs with concomitant nonsuppurative
505 meningoencephalitis in piglets infected with SVA [16, 18, 19]. How SVA enters the brain
506 is not fully elucidated, but initial studies have suggested that SVA is probably transmitted
507 via the faecal-oral route [19, 23], due to the identification of the viral particles within the
508 superficial enterocytes of the small intestine by TEM [19] and antigens by IHC [18, 19],
509 and simultaneously in the epithelia of the CP [19]. In addition, qRT-PCR has identified the
510 highest percentage of SVA in the tonsil and rectal swabs of weaned pigs up to six weeks
511 post outbreak [23]. This may suggest that SVA can be eliminated via faeces, and
512 demonstrates the potential of this virus to disseminate within a farm during the movement
513 of infected pigs with subclinical signs [23].

514 In addition, the elevated high viral load of SVA was identified in the tonsils [3,
515 92]. Experimental study has shown that the incubation period of SVA varied between 4-5
516 days [3], which corresponded to the period of clinical signs and high mortality in
517 spontaneously infected newborn piglets [19]. We have shown that lymphoid tissues, such as
518 the tonsil and spleen, were associated with the highest SVA viral load, varying from 6.74
519 to 10.38 $\log^{10}$ genomic copies per g of tissue [92]. In addition, experimental studies done in
520 15-week-old finishing pigs, revealed positive immunolabelling for SVA in these lymphoid
521 organs (mediastinal and mesenteric lymph nodes, spleen, and tonsil), and small intestine by
522 ISH [3], suggesting that SVA uses these organs as likely entry portals to the brain, and may

523 result in nonsuppurative meningoencephalitis via CPs in piglets with neurological
524 manifestations of ETNL [19]. These findings are suggestive of the oral-neurological
525 dissemination of SVA as is characteristic of several neurotropic enteroviruses such as
526 *Porcine teschovirus* [77], *Poliovirus* [94], and *Enterovirus* 71 [95].

527 The tonsils were considered as one of the primary sites of replication for SVA [3,
528 92], and we have shown that superficial enterocytes [18, 19], and other lymphoid tissues
529 (regional lymph nodes and spleen). The SVA may infect macrophages, B cells, T cells and
530 dendritic cells, disseminate SVA via leucocytic trafficking. Dissemination of SVA to other
531 tissues may also use this route, considering that viral RNA is constantly observed in the
532 brainstem, cerebellum, cerebrum [17, 18, 92], heart, kidney, liver, lungs, small intestine,
533 urinary bladder, spleen, tonsils [3, 17, 18, 92], large intestine, and the mediastinal and
534 mesenteric lymph nodes [3], demonstrating that SVA is a multisystemic viral pathogen.

535 The period of viremia associated with SVA is short (3-7 days) and transient
536 followed by rapid reduction [3, 23], while the peak of SVA genomic copies ($\approx 1\times10^7$/mL)
537 detected in serum on three days post experimental inoculation (dpi) with a subsequent
538 progressive reduction up to 10 dpi [3]. In a study with 43 pigs naturally infected with SVA,
539 most of the piglets (81%; 35/43) that died due to ETNL had between 2-6 days of age [19].
540 This may suggest that the clinical manifestations of SVA are more intense in newborn
541 piglets, resulting in sudden death [9, 11, 16-19], partially due to the immature immune
542 system of these animals at birth [19], and the absence of passive antibodies in the
543 colostrum [96], or it may suggest that there is some interference with the active transfer of
544 antibodies from infected sows to their immature offsprings. Further, immunological
545 maturity of the pigs is attained between 5 and 7 weeks after birth [96], and may be the
546 reason to explain the absence of reports describing spontaneous death of pigs in the
547 weaned, fattening or finishing phase, as well as in sows and boars.

548 With the use of transmission electron microscopy, we have demonstrated viral
549 particles of SVA (Fig. 4) in the degenerated endothelial cells of fenestrated capillaries of
550 the CP, with intraluminal accumulation of macrophages and lymphocytes in a newborn
551 piglet with nonsuppurative meningoencephalitis, and have suggested that this lesions could
552 have been induced by the leukocytic trafficking by means of fenestrated capillary of the
553 CP, after an initial nonsuppurative choroid plexitis with subsequent damage to the BBB
554 [19].

555 Apoptosis is a defence mechanism of intrinsic and innate immunity to prevent
556 viral replication [39, 40, 63]. The follicular hyperplasia observed in lymphoid tissues
557 occurs due to intense immunological reaction due to antigenic stimulation, since viral
558 replication stimulates the humoral response [88]. These findings may suggest that the SVA
559 infection is highly immunogenic, since serum samples of naturally infected pigs high (≥
560 4096) anti-SVA antibody titters [82].

561

562 2.10 CONCLUSION

563 The main lesions associated with SVA are vesicles and/or ulcerative dermatitis on
564 the snout and feet. It must be emphasized that lesions on the coronary bands and
565 interdigital spaces seem to occur before those on the snout, independent of the age of the
566 affected pig. In newborn piglets, the peak of viremia appears to be associated with elevated
567 mortality rates, suggesting that immunological immaturity is a key feature associated with
568 the replication of SVA. Experimental studies must be conducted to elucidate the intricate
569 mechanisms of death observed in ETNL. The nonsuppurative meningoencephalitis
570 observed in piglets with ETNL was related to the presence of SVA in the brain, probably
571 after an initial intestinal lesion, suggesting that this virus may produce encephalitis after
572 enteric colonization, thereby acting as an enterovirus. Nevertheless, how the SVA enters
573 the brain is not well understood, but the Trojan horse mechanism is been proposed.

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872

873 **Table 1.** Comparative summary of the forms of dissemination, incubations periods, and target tissues of the principal Picornavirus of importance
874 to pigs.
875

Genus	Virus name[1]	Dissemination	Incubation period (days)	Repication site	Principal disease manifestations
Aphthovirus	Foot-and-mouth disease O (FMDVO)	Respiratory tract [26, 27, 97, 98]	2-14 [26]	Virus replication occurs in the pharynx [27]	Claudication, vesicles on the coronary bands of the feet and on the snout [27, 98]
Cardiovirus	Encephalo-myocarditis 1 (EMCV1)	Oro-nasal, viruses are thought to spread from tonsils [52] and epithelial cells of the intestinal tract [27]	2-29 [56]	The virus can be isolated from the heart, brain, small intestine, kidneys, liver, lungs, lymph nodes, pancreas, and spleen [52, 54, 76]	Encephalitis, hind limb paralysis, myocarditis, and / or type 1 diabetes [52, 56, 76, 99-102]
Enterovirus	Enterovirus E (EVE1)	Oral [103]	6-12 [28]	Initial viral replication is presumed to occur in the tonsils and Peyer's patches, with posterior multiplication in regional lymph nodes [103]	Enteric [28], neurological [55, 95, 104], and type 1 diabetes [105]
Sapelovirus	Porcine sapelovirus (PSV1)	Oral [53]	1-5 [53]	The virus penetrates the intestinal barrier from the luminal side through destruction of enterocytes at the villi, initiating viral replication [53]	Enteritis, pneumonia, reproductive disorders [53], and polioencephalomyelitis [47, 53, 57]
Senecavirus	Seneca Valley virus (SVV1)	Probably, oro-nasal, viruses are thought to spread from tonsils [3, 92] and epithelial cells of the intestinal tract [19]	4-5 [5]	Tonsils were indicated as one of the primary sites of replication [15, 92]	Porcine Idiopathic Vesicular Disease [4, 5, 9, 13] and Epidemic Transient Neonatal Losses [5, 11, 15, 17-19]
Teschovirus	Porcine teschovirus 1 (PTV1)	Oro-nasal [27]	4-28 [27]	Primary replication in the tonsils and Peyer's patches [27]	Atrophic enteritis [49, 106], interstitial pneumonia [58, 106], and polioencephalomyelitis [48-51, 58, 77, 106]

876 Legend: [1]Official name as defined by the International Committee on Taxonomy of Viruses [1].

877 **Table 2** The relative frequency of the gross lesions described in swine infected by
878 *Senecavirus A* in Epidemic Transient Neonatal Losses (ETNL) and Porcine Idiopathic
879 Vesicular Disease (PIVD).
880

Principal gross manifestations described	ETNL	PIVD	References
Concomitant vesicles at the muzzle with ulcerative lesions at the coronary band	Frequent	Frequent	[5, 19, 34, 35]
Faint rib impressions at the pleural surface of the lungs	Frequent	*	[18, 19]
Hypertrophy of Peyer's patch	Not frequent	*	[19]
Liquid faeces	Frequent	*	[3, 11, 15, 17-19, 29, 83, 107]
Lymphadenopathy	Not frequent	Rare	[16, 19, 88]
Mesocolonic oedema	Not frequent	*	[11, 19, 29]
Renal petechial haemorrhage	Frequent	*	[16-19]
Skin abrasion at the carpus	Not frequent	Not frequent	[5, 16, 18, 19]
Sudden death	Frequent	*	[3, 11, 15, 17-19, 29, 83, 107]
Ulcerative cheilitis	Not frequent	Not frequent	[5, 19, 32]
Ulcerative gingivitis	Not frequent	Not frequent	[7, 18, 19, 32, 35]
Ulcerative glossitis	Not Frequent	Frequent	[15, 17-19, 29, 32, 35]
Ulcerative lesion at the coronary bands	Frequent	Frequent	[3-7, 11, 12, 15-19, 29, 32, 34, 35, 83, 88, 91]
Ulcerative lesions at the interdigital area	*	Not frequent	[5, 12, 29]
Ulcerative lesion of the hoof	Rare	Not frequent	[3, 4, 11, 16-19, 29]
Vesicles at the snout	Frequent	Frequent	[3-5, 7, 11, 15-19, 29, 32, 34, 83, 88, 91]

881 Legend: *, not previously described is this category of pigs.

882

883 **Table 3.** The relative frequency of the histopathological lesions described in swine
884 infected by *Senecavirus A* in Epidemic Transient Neonatal Losses (ETNL) and Porcine
885 Idiopathic Vesicular Disease (PIVD).
886

Principal histopathological features	ETNL	PIVD	References
Atrophic enteritis	Frequent	*	[15, 17-19]
Ballooning degeneration of the transitional epithelium of the bladder and of the epithelium of the renal pelvis	Frequent	*	[18, 19]
Cortical laminar necrosis, malacia and nonsuppurative perivascular cuffing of the central nervous system	Rare	*	[16, 19]
Follicular lymphoid hyperplasia of mesenteric lymph nodes	Frequent	Frequent	[3, 19]
Interstitial pneumonia	Frequent	Not frequent	[16-19]
Ballooning degeneration of keratinocytes with formation of intraepidermal vesicles	Frequent	Frequent	[3, 7, 15, 19]
Lymphocytic myocarditis	Not frequent	Rare	[16-18]
Lymphoid depletion in tonsil	Frequent	Not frequent	[15, 17-19]
Necrotizing cheilitis	Not frequent	*	[17-19]
Necrotizing dermatitis at the hoof and snout	Frequent	Frequent	[3, 7, 11, 12, 15, 17-19, 29]
Necrotizing gingivitis	Not frequent	*	[17-19]
Necrotizing glossitis	Not frequent	*	[17-19]
Neuronophagy of the central nervous system	Rare	Rare	[16, 19]
Nonsuppurative choroid plexitis	Rare	*	[19]
Nonsuppurative hepatitis	Rare	Rare	[16-18]
Nonsuppurative meningoencephalitis	Not frequent	Rare	[16, 19]
Nonsuppurative nephritis	*	Rare	[16]
Severe hyperplasia of the urothelium of the renal pelvis, ureters, and urinary bladder	Frequent	*	[17-19]
Vacuolization of superficial enterocytes	Not frequent	*	[17-19]

887 Legend: *, not previously described for this category of pigs.

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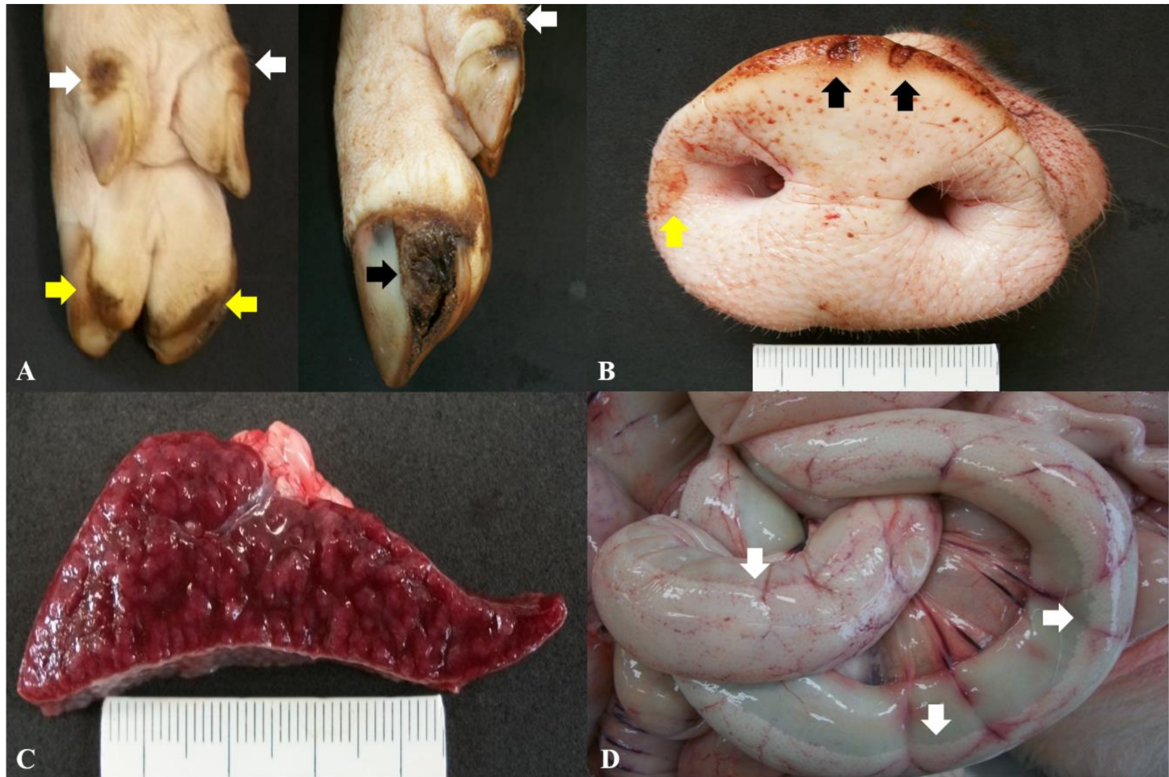
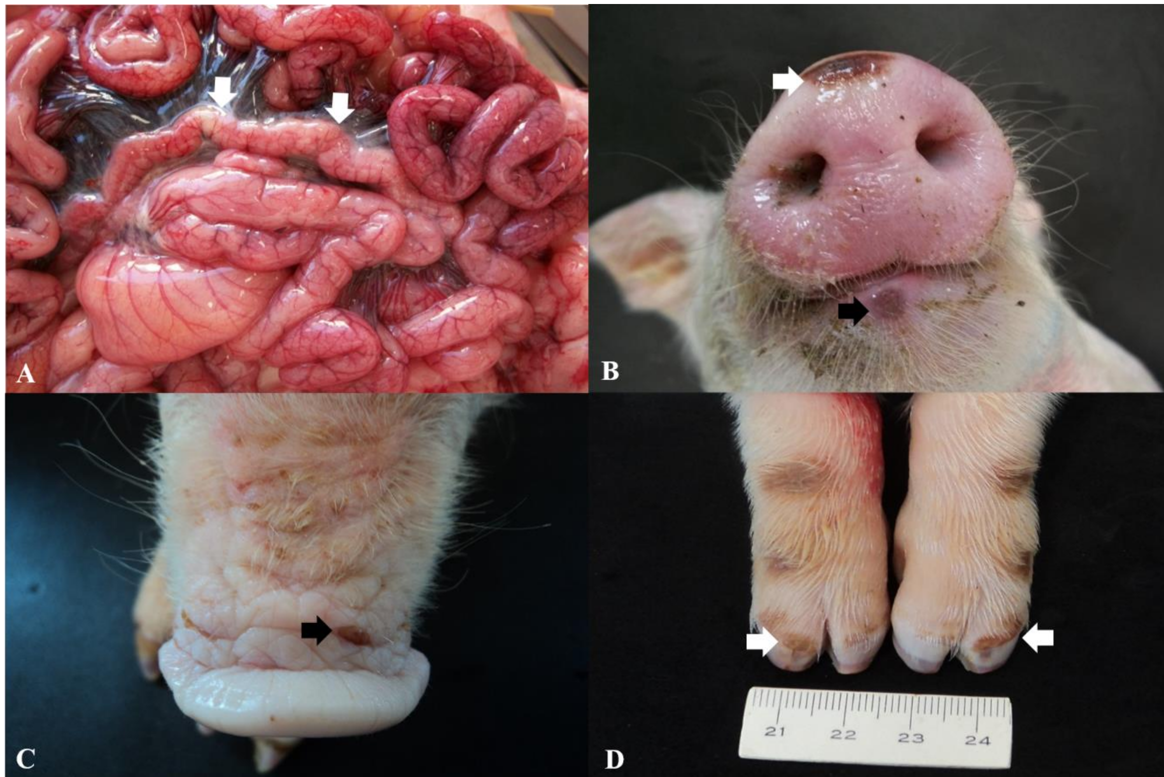


890
891 **Fig. 1** Gross findings observed in finishing and feeding pigs with PIVD. 170-day-old pig;
892 ulcerative lesions at the plantar foot (yellow arrows), dewclaws (white arrows), and
893 coronary band (black arrow) of the hindlimbs (A). Multifocal ulceration at the snout after
894 rupture of older vesicles (black arrows); rupture a recently formed vesicle (yellow arrow)
895 (B). Spleen with white pulp hyperplasia (C). 60-day-old pig; diffusely on the
896 antimesenteric border of intestinal loops, there is marked hypertrophy of the lymphoid
897 tissue (white arrows) (D).
898



899
900 **Fig. 2** Gross findings observed in newborn piglets with ETNL. Five-day-old piglet;
901 intestinal loops are diffusely congested and dilated by liquid feces and there is marked
902 hyperplasia of the mesenteric lymph nodes (white arrows) (A). Six-day-old piglet,
903 ulcerative lesion at the lower under lip (black arrrow) and necrotizing dermatitis at the
904 muzzle after rupture of a vesicle (white arrow) (B). Four-day-old piglet; there is intact
905 vesicle the snout (black arrows) (C), ulcerations and crusting lesions at the metacarpus and
906 coronary band (white arrows) of the forelimbs (D).
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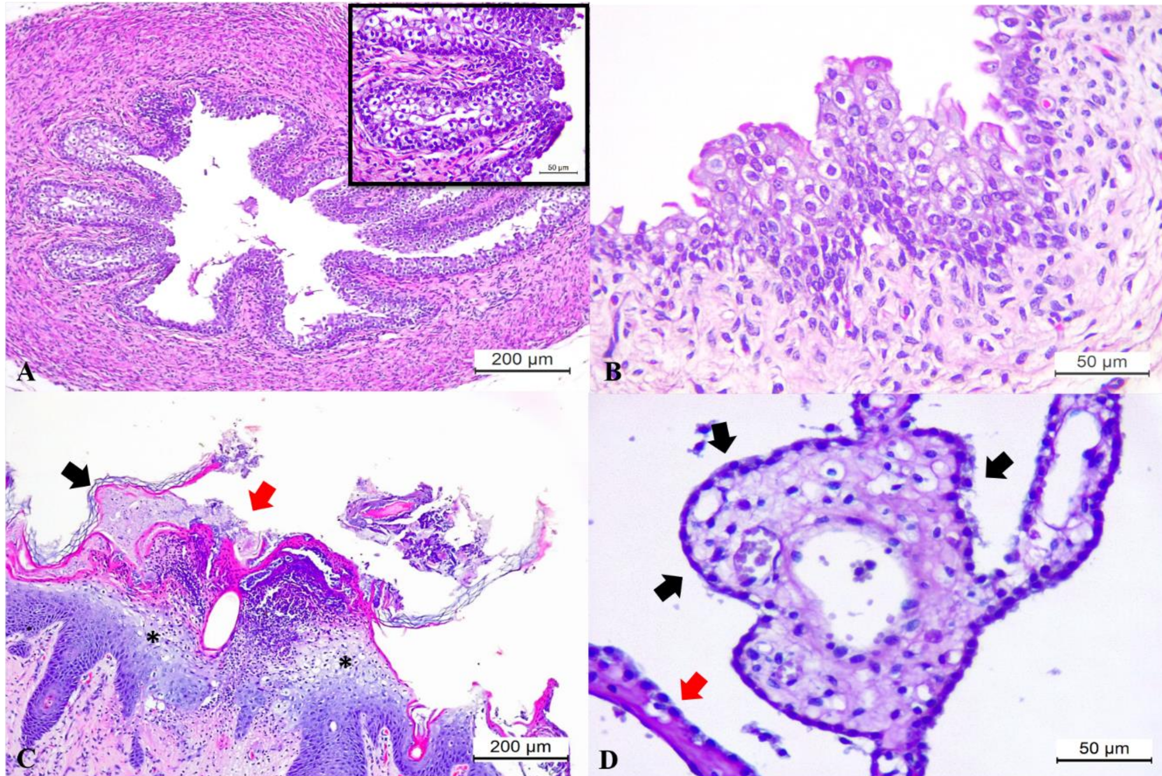

910
911 **Fig. 3** Histopathological findings observed in newborn piglets naturally infected by SVA.
912 Ureter, there is diffuse, severe hyperplasia and ballooning degeneration of the urothelium.
913 Insert: higher magnification of hyperplasia and ballooning degeneration urothelium (A).
914 Urinary bladder, observe hyperplasia and ballooning degeneration of the transitional
915 epithelium (B). Epidermis of the nasal planum; there is ballooning degeneration (*) of
916 keratinocytes at the stratum spinosum with the formation of intraepidermal intact (black
917 arrow) and rupture (red arrow) of vesicles (C). Haematoxylin & Eosin stain. Choroid
918 plexus, there is mild hyperplasia and ballooning degeneration of ependymocytes (black
919 arrows), as compared with normal (red arrow) ependymocytes (D). Periodic Acid Schiff
920 stain. Bar, A and C 200 µm; B and D 50 µm.

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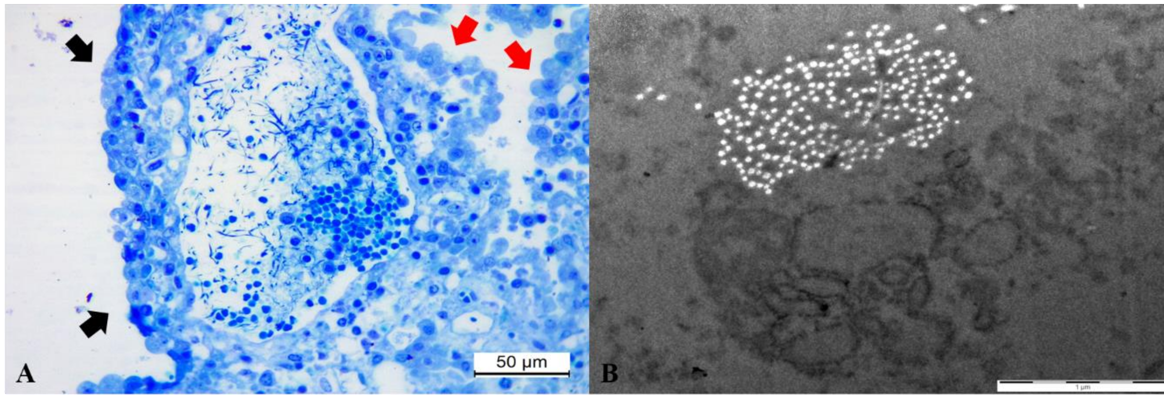
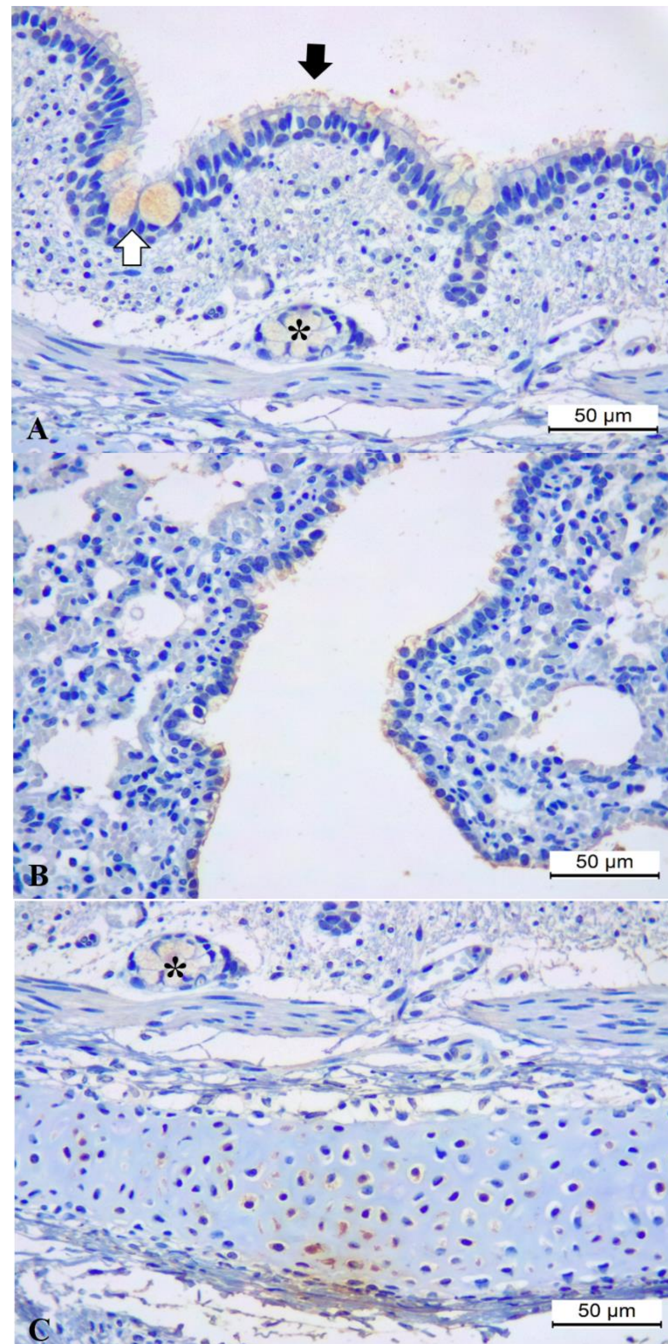


922 **Fig. 4** Semithin and transmission electron micrograph of the choroid plexus of newborn
923 piglet naturally infected with SVA. There is moderate hyperplasia of ependymocytes
924 (black arrows), as compared with normal (red arrow) ependymocytes in a nonsuppurative
925 choroid plexitis. Observe severe accumulations of macrophages and lymphocytes admixed
926 with fibrin in the lumen of a venule, and ballooning degeneration of ependymocytes next
927 to venule (**A**). Negatively stained cytoplasm of ependymocytes observe Golgi complex
928 degeneration surrounded by aggregates of viral particles (**B**). New methylene blue stain.
929 Bar, A - 50 µm; B - 1 µm.
930



931
932 **Fig. 5** Immunohistochemical detection of antigens of SVA in epithelial tissues of newborn
933 piglets. Lung; there is positive immunostaining (black arrow) at the respiratory epithelium,
934 at the goblet cells (white arrow) and bronchial glands (*) of the bronchus (A). Observe
935 immunoreactivity to SVA and vacuolization of respiratory epithelial cells of the bronchiole
936 (B) and is positive immunolabelling of SVA at chondrocytes the hyaline cartilage (C).
937 Immunoperoxidase. Bar, A-C 50 µm.
938

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

- The purpose of this study was to describe the gross, histopathological, immunohistochemical (IHC), and ultrastructural features, as well as to determine the tropism of *Senecavirus A* (SVA) in different tissues of neonatal piglets with epidemic transient neonatal losses (ETNL).

3.2 SPECIFICS OBJECTIVES

- Describe gross findings that are more frequently identified in piglets spontaneously infected with ETNL;
- Characterize the lesions caused by SVA in new born piglets affected by ETNL using histopathological, IHC, and transmission electron microscopy techniques.

4 ARTICLE - HISTOPATHOLOGICAL, IMMUNOHISTOCHEMICAL, AND ULTRASTRUCTURAL EVIDENCE OF SPONTANEOUS *SENECAVIRUS A*-INDUCED LESIONS AT THE CHOROID PLEXUS OF NEWBORN PIGLETS

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Histopathological, immunohistochemical, and ultrastructural evidence of spontaneous *Senecavirus A*-induced lesions at the choroid plexus of newborn piglets

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Epidemic Transient Neonatal Losses (ETNL) is a disease of piglets caused by Senecavirus A (SVA) in which the method of dissemination and associated lesions are not well-defined. This study investigated the possible SVA-induced lesions by examining spontaneous infections in newborn piglets. Histopathology revealed ballooning degeneration of transitional epithelium, nonsuppurative meningoencephalitis, plexus choroiditis, and atrophic enteritis. RT-PCR identified SVA in all tissues evaluated and sequencing confirmed these results. Positive immunoreactivity to SVA was observed in endothelial and epithelial tissues of all organs evaluated. Semithin analysis revealed vacuolization of apical enterocytes of the small intestine, balloon degeneration and necrosis of endothelial cells of the choroid plexus (CP) and nonsuppurative choroid plexitis. Ultrathin evaluation demonstrated hydropic degeneration of apical enterocytes, degeneration and necrosis of endothelium of CP fenestrated capillaries, degeneration of ependymocytes associated with intralesional viral particles. It is proposed that SVA initially infects apical enterocytes of newborn piglets and probably enters the circulatory system with entry to the brain via the CP, by first producing an initial inflammatory reaction, with subsequent encephalitic dissemination. Consequently, SVA probably uses an enteric-neurological method of dissemination.

Senecavirus A (SVA), formerly known as Seneca Valley virus, is the only representative of the genus *Senecavirus*, family *Picornaviridae*[1]. SVA is currently associated with the syndrome known as Epidemic Transient Neonatal Losses (ETNL), which is clinically manifested by lethargy, cutaneous hyperaemia, diarrhoea, neurological signs and/or sudden death in newborn piglets in pig farms from Brazil[2–5], North American[4,6,7], China[8,9], Thailand[10], and Colombia[11]. Furthermore, ETNL was reported as affecting piglets under 10 days of age[3]. Piglet mortality due to SVA in Brazil has been estimated as 20–30% in the states of Paraná and Santa Catarina[2], and 30–70% in the states of Minas Gerais and Goiás[5]. Additionally, in Brazil the disease in piglets and sows occur simultaneously[2,5], while

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in the USA, sows are affected initially causing vesicular lesions with subsequent newborn mortality occurring weeks thereafter[6].

The detection of a pathogen in tissue by Polymerase Chain Reaction (PCR) and/or Reverse Transcription-Polymerase Chain Reaction (RT-PCR) in the absence of related pathological alterations does not necessarily imply that the identified agent is associated with a specific lesion or disease. Consequently, it is of extreme importance to identify the presence of the pathogen associated with histopathological lesions in affected tissues[4]. Immunohistochemistry (IHC) is an important diagnostic technique frequently used for the detection of a specific protein associated with the antigen of infectious disease agents in the microscopic lesions of formalin-fixed paraffin embedded (FFPE) tissues[12]. Another important technique that assists in the diagnosis of infectious diseases is *in situ* hybridization (ISH), due to the detection of specific nucleic acid (DNA, RNA, or messenger RNA) segments of an infectious agent in the sample under evaluation[13,14].

Currently, investigations related to ETNL have been restricted to few studies[3,4,6], that involved small populations of animals, varying from nine[6] to ten[3,4] necropsies of piglets affected by this syndrome. It must be highlighted that in one study 66.7% (6/9) of the brain samples evaluated were negative for SVA by ISH[4], while 80% (8/10) of the piglets investigated by our group contained antigens of SVA in the choroid plexus (CP) by IHC, and in three of these SVA was amplified from the brain by RT-PCR[3]. We hypothesized that the elevated detection of SVA in the brain is probably associated with obtaining brain fragments containing the CP, and that this viral pathogen may have the ability to alter the integrity of vessels of the CP, since we have consistently identified antigens of SVA at the vascular endothelia of capillaries at this anatomic location[3]. Furthermore, since we had identified atrophic enteritis and histopathological evidence of lesions to the CP in piglets from a previous study[3], we hypothesized that viral particles might have been present in the ballooning degeneration identified in cases of atrophic enteritis and at the CP of these diseased piglets. The CP is the location of the blood-cerebrospinal fluid barrier (BCSFB), one of the barriers within the brain that prevents the entry of infectious agents to the central nervous system[15–18].

This study describes the gross, histopathological, IHC, and transmission electron microscopy (TEM) findings associated with spontaneous SVA-induced ETNL infections in 54 newborn piglets from Southern Brazil, validates the findings described in an earlier study by our group with a smaller population of piglets[3], and provides initial evidence for the possible method of dissemination of SVA in newborn piglets.

Results

Clinical manifestations and gross findings.

Of the 54 piglets with clinical manifestations suggestive of ETNL, in 80% (43/54) of these, at least one of the tissues/organs evaluated had positive immunolabelling for SVA by IHC and SVA RNA was identified by RT-PCR. Consequently, only the results of piglets with at least one tissue that was IHC positive for SVA were considered during this study. Diarrhoea was the most common clinical manifestation (91%; 39/43) observed in these piglets. The stomach of all piglets, including those with cachexia (9%; n = 4/43), were filled with milk; only 24% (13/54) of these piglets had neurological signs. The distribution of the age of the piglets is given in Supplemental Fig. 1; age was considered as the time of death. Consequently, most (81%; 35/43) piglets that died due to ETNL were between 2–5 days of age.

The most frequent gross manifestations observed during autopsy were liquid faeces (91%; 39/43), renal petechial haemorrhage (79%; 34/43), faint rib impressions on the pleural surface of the lungs (77%; 33/43), pulmonary oedema and congestion (60%; 26/43). In addition, there were cases of ulcerative lesion at the coronary band (35%; 15/43; Fig. 1A), mesocolonic oedema (32%; 14/43; Fig. 1B), vesicles at the snout (30%; 13/43; Fig. 1C), and lymphadenopathy (28%; 12/43). Less frequently occurring lesions included hyperplasia of Peyer's patch (16%; 7/43), ulcerative glossitis (16%; 7/43; Fig. 1D), skin abrasion at the carpus (14%; 6/43; Fig. 1E), ulcerative gingivitis (14%; 6/43), ulcerative lesion of the hoof (12%; 5/43; Fig. 1F), and ulcerative cheilitis (9%; 4/43). Furthermore, there were concomitant vesicles at the muzzle with ulcerative lesions at the coronary band in 21% (9/43) of the piglets investigated.

Histopathological findings associated with Senecavirus A observed in newborn piglets.

The principal histopathological findings are graphically summarized in Fig. 2. Ballooning degeneration of the transitional epithelium of the urinary bladder (100%; 43/43) and of the epithelium of the renal pelvis (95%; 41/43), villous atrophy (atrophic enteritis) of the small intestine (93%; 40/43), and interstitial pneumonia (84%; 36/43) were the predominant histopathological alterations observed. In addition, there was severe hyperplasia (involving 3 to 13 layers of epithelial cells) of the urothelium of the renal pelvis, ureters and urinary bladder (Fig. 3A). Furthermore, rare intracytoplasmic eosinophilic structures, measuring 6–7 μm, and suggestive of viral inclusion bodies of SVA were observed in areas of ballooning degeneration of the urinary bladder (Fig. 3B) and in neurons within areas of nonsuppurative meningoencephalitis.

Neurological disease: evidence of brain disease was observed in 46% (6/13) of the piglets with clinical manifestations suggestive of ETNL. These histopathological alterations varied from nonsuppurative meningoencephalitis ($n = 3$; Fig. 3C), cortical laminar necrosis ($n = 3$; Fig. 3D), nonsuppurative choroid plexitis ($n = 3$; Fig. 3E,F), gliosis and discrete cortical oedema ($n = 3$), malacia ($n = 1$; Fig. 3G), and areas of perivascular cuffing ($n = 1$, Fig. 3H).

Cutaneous lesions: the histopathological lesions observed in the skin of these piglets consisted of vesicular lesions that varied from dermal ulcers and/or crust formations. These alterations were observed at the coronary bands (35%; 15/43), snout (35%; 15/43), metacarpal (19%; 8/43), and hoof (9%; 4/43), and consisted predominantly of moderate, multifocal to coalescing necrotizing dermatitis. Necrotizing dermatitis with crusting formations was characterized by severe necrosis of keratocytes at the stratum spinosum of the epidermis with inflammatory influx composed of intact and degenerated neutrophils, rare lymphocytes, and some histiocytes. Moreover, at the skin adjacent to this region of necrotic dermatitis, there was orthokeratotic hyperkeratosis, intracellular oedema (severe, multifocal, hydropic degeneration, and multifocal, moderate ballooning degeneration),

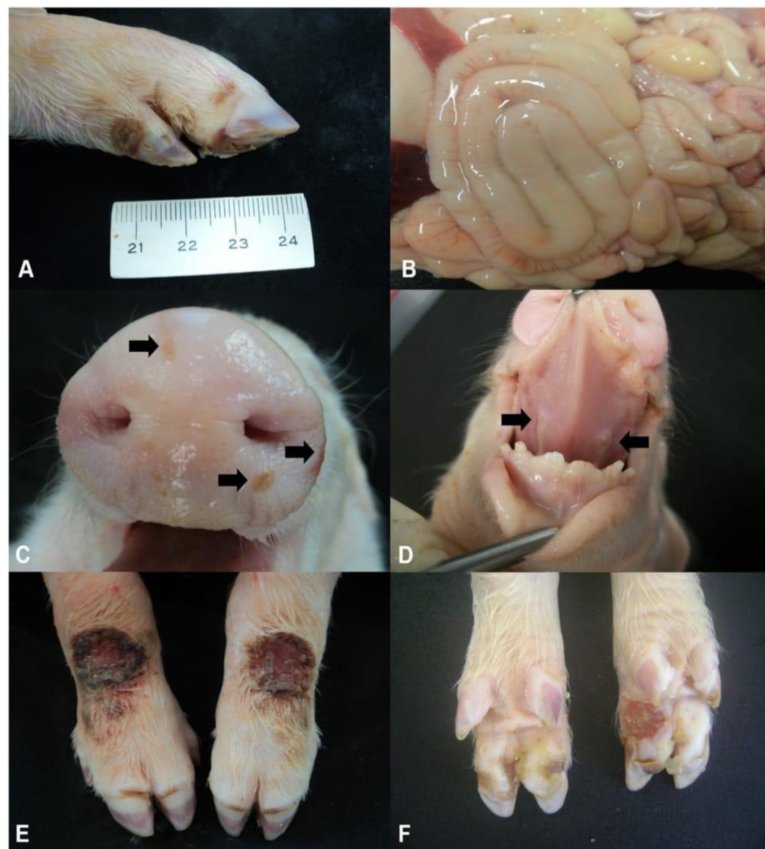

Figure 1. Gross findings observed in newborn piglets naturally infected by SVA. Coronary band, 4-day-old piglet; observe the erosive lesion (0.7 cm in diameter) at the coronary band of the right forelimb (**A**). Abdominal cavity, 3-day-old piglet; there is mesocolonic oedema; the intestinal segment is dilated by diarrhoea and the mesocolon is swollen (**B**). Snout, 4-day-old piglet; there is multifocal ulceration of the skin (arrow) of the snout after rupture of a vesicle (**C**). Tongue, 6- day-old piglet; there is symmetric ulcerative glossitis (arrow) at the ventral face of the tongue (**D**). Fore limbs and hooves, 7-day-old piglet; observe ulcerations and crusting lesions (2 cm diameter) at the coronary bands and metacarpus with an erosive lesion at the margin of the coronary band of the forelimbs (**E**). Palmar footpad, 3-day-old piglet; there is a large ulceration (1.2 cm diameter) at the right footpad (**F**). Scale in cm.

irregular epidermal hyperplasia with rete pegs formation, anastomoses, and mild acantholysis. In rare cases, it was possible to observe intact intradermal pustules, formed by mild parakeratotic hyperkeratosis with an underlying accumulation of degenerate neutrophils.

Oral cavity: consisted of lesions classified as necrotizing glossitis (16%; 7/43), gingivitis (14%; 6/43), and cheilitis (12%; 5/43). These lesions were characterized by focally extensive erosions and/or ulcerations with severe accumulations of intact and degenerate neutrophils. In addition, at the mucosa adjacent to areas of necrotizing glossitis, gingivitis or cheilitis, there was intracellular oedema (multifocal, moderate hydropic, and ballooning degeneration) of the superficial epithelium.

Lymphoid tissue: lesions were observed in the mesenteric lymph nodes and mucosa-associated lymphoid tissue. These alterations consisted of follicular lymphoid hyperplasia (53%; 23/43) and depletion (35%; 15/43) of mesenteric lymph nodes; follicular lymphoid hyperplasia (37%; 16/43) and depletion (44%; 19/43) of the tonsils; and follicular lymphoid hyperplasia of Peyer's patch (28%; 12/43). The lymphoid tissue of the spleen was not investigated in this study, since it was not possible to differentiate between the red and white pulp due to the poorly developed organs in young piglets[19].

Intestinal and hepatic disease: lesions observed in the small intestine consisted primarily of atrophic enteritis (93%; 40/43), followed by fusion of villi (35%; 15/43). In addition, there were necrosis (30%; 13/43) and vacuolization (12%; 5/43) of apical enterocytes in piglets with clinical manifestations of diarrhoea. Furthermore, there was random lymphoplasmacytic hepatitis (42%; 18/43).

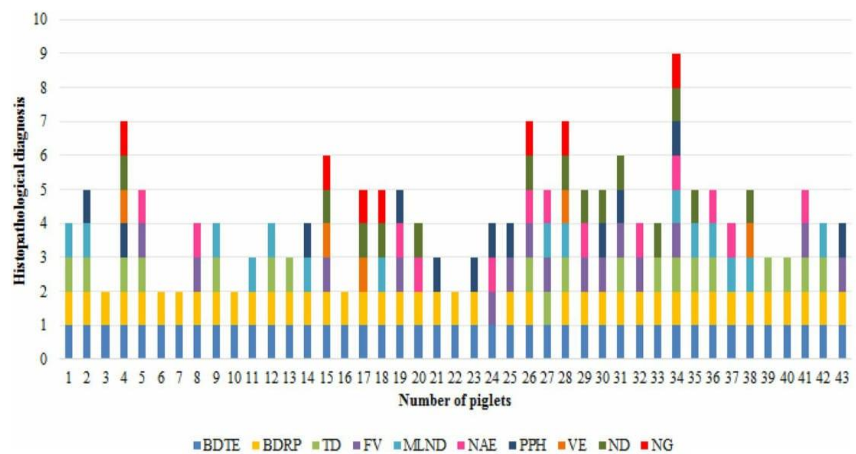

Figure 2. Graphical distribution of the principal histopathological findings in 43 piglets with SVA. Legend: BDTE, ballooning degeneration of transitional epithelium; ballooning degeneration of renal pelvis (BDRP), tonsilar depletion (TD), fusion of villi (FV), mesenteric lymph node depletion (MLND), necrosis of apical enterocytes (NAE), Peyer's patch hyperplasia (PPH), vacuolization of enterocytes (VE), necrotizing dermatitis (ND), necrotizing glossitis (NG).

Immunohistochemistry findings associated with *Senecavirus A* in newborn piglets. The principal tissues that demonstrated immunoreactivity to SVA were the urothelium of the urinary bladder (100%; 43/43), the renal pelvis (95%; 41/43), and the capillaries of the CP (Fig. 4A) of the cerebrum (81%; 35/43), with positive immunolabelling in areas of ballooning degeneration and epithelial hyperplasia of transitional epithelium (Fig. 4B). There was positive immunostaining of SVA at the epithelial cells of all piglets evaluated.

Furthermore, all piglets (# 1, 2, 3, 13, 26, 27) with neurological signs associated with ETNL and with histopathological evidence of brain disease demonstrated positive immunoreactivity to SVA at the CP and ependymal cells. In addition, there was severe vacuolization of epithelial cells of the CP (Fig. 4C) in all piglets with clinical manifestations of neurological disease.

There was positive immunoreactivity at the oral mucosa to SVA in 70% (30/43) of the piglets with severe hydropic degeneration (Fig. 4D,E), and positive immunolabelling for SVA in the apical enterocytes of the small intestinal in 63% (27/43) of all piglets with atrophic enteritis. Five piglets (# 4, 15, 17, 28, 38) presented intracytoplasmic vesicles at the apical enterocytes (Fig. 4F).

Furthermore, there was immunoreactivity to antigens of SVA in lesions at the coronary band (35%; 15/43), snout (35%; 15/43), metacarpal (19%; 8/43), tongue (16%; 7/43), gingiva (14%; 6/43), lips (12%; 5/43), and hoof (9%; 4/43). All pulmonary fragments (alveolar septum and respiratory epithelium) and lymphoid tissue of the tonsils evaluated were negative for SVA.

Semithin and transmission electron microscopic findings. Semithin (ST) evaluation through the apical region of the small intestine demonstrated severe vacuolization of apical enterocytes, with intracellular degeneration resulting in peripheral nuclear dislocation (Fig. 5). This virus-induced lesion must be differentiated from the physiological vacuolization of enterocytes associated with the ingestion of colostrum by piglets.

Semithin sections of the CP revealed nonsuppurative choroid plexitis characterized by the accumulation of macrophages and lymphocytes admixed with fibrin within the ventricular lumen and fenestrated capillaries (Fig. 6A,B). In addition, there was marked ballooning degeneration and necrosis of endothelial cells of fenestrated capillaries with foci of swelling and degeneration of ependymal cells of the CP (Fig. 6B,C). Moreover, in some areas, the swollen ependymocytes resulted in discrete thickening of the ependymal layer (Fig. 6C).

Transmission electron microscopy (TEM) of the choroid plexus and the apical region of the small intestine revealed information that confirmed the participation of SVA in the development of ETNL in newborn piglets despite the quality of the images which was affected by the method of collection (see below). Consequently, essential details of cell morphology were compromised and thus not observed. Nevertheless, TEM evaluation of positively stained sections of the small intestine demonstrated that normal apical enterocytes of newborn piglets have different-sized, enlarged macromolecules associated with the intestinal absorption of colostrum (Fig. 7A), which when negatively stained did not contain viral particles (Fig. 7B). However, infected apical enterocytes demonstrated hydropic degeneration and were surrounded by viral particle aggregates (Fig. 7B,C); viral particles were observed only in apical enterocytes (Fig. 7D). Furthermore, there was swelling and rupture of the cisternae of the rough endoplasmic reticulum of infected apical enterocytes (data not shown).

Transmission electron microscopy of positively stained CP revealed an influx of lymphocytes and macrophages within the fenestrated capillaries of the CP, and that these leucocytes contained intracytoplasmic phagolysosomes (Fig. 8A); infected macrophages were not seen translocating via the tight junctions. Furthermore, negatively stained endothelial and ependymocytes infected by SVA demonstrated balloon degeneration, characterized as cytoplasmic vesicles (Fig. 8B,C) surrounded by viral particles of 17–30 nm in diameter (Fig. 8D). In

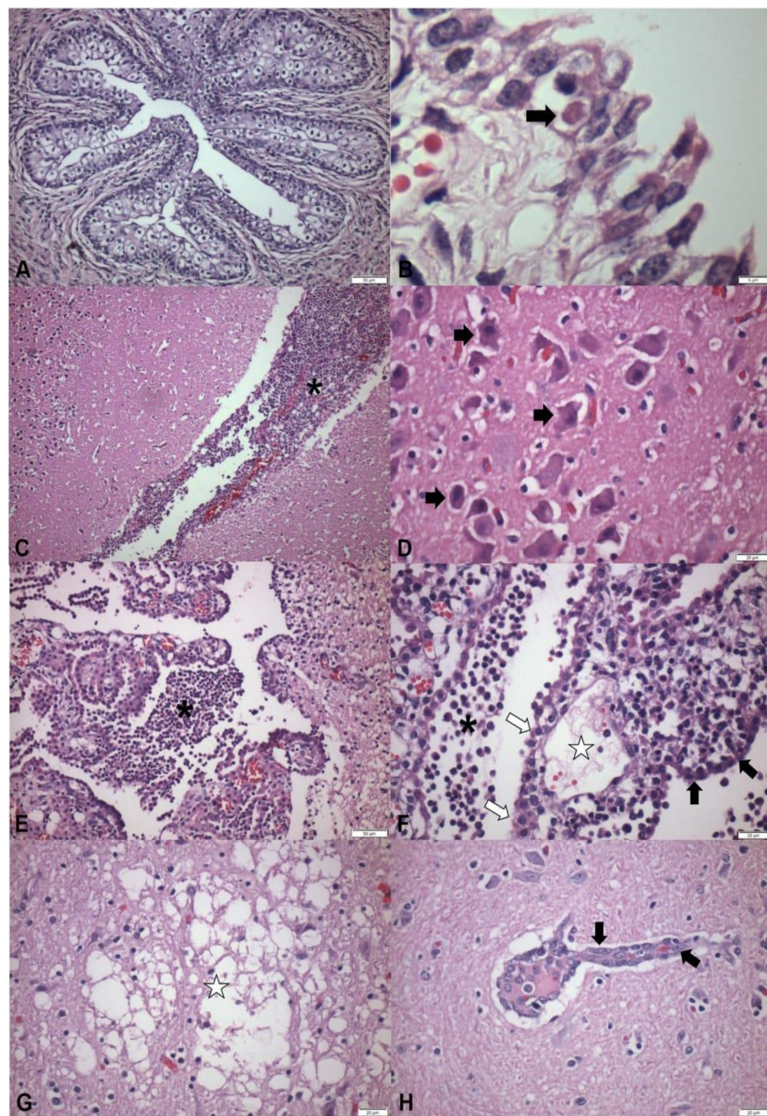

Figure 3. Histopathological findings observed in newborn piglets naturally infected by SVA. Ureter, there is severe hyperplasia and balloon degeneration of the urothelium (**A**). Urinary bladder, observe ballooning degeneration of the transitional epithelium associated with an intracytoplasmic, eosinophilic inclusion body (arrow) (**B**). There is severe focally extensive nonsuppurative meningoencephalitis (*, **C**) and cerebrocortical necrosis of the brain (**D**); observe several ischemic neurons (arrows). There is severe multifocal nonsuppurative choroid plexitis (*) of the lateral ventricle (**E**); observe the intraluminal accumulation of fibrin (★) in a fenestrated capillary, degeneration of ependymocytes and (open arrows), ballooning degeneration (arrow head) as compared with normal (closed arrow) ependymocytes (**F**). There is malacia (★) of the brainstem with gliosis (**G**), and discrete perivascular cuffing formed by lymphocytes and macrophages at the cortex (**H**). Haematoxylin & Eosin stain. Bar, A, E 50 μm; B 5 μm; C 100 μm; D,F–H 20 μm.

addition, in an endothelial cell of the CP, the nucleus was displaced to the margin of the cell, with detachment of the nuclear membranes, enlargement of the perinuclear space, deformation of the nuclei, and condensation and fragmentation into an apoptotic body (Fig. 8C). These findings are hallmarks of apoptosis suggesting that the balloon degeneration caused by SVA progressed to individual cell death.

The viral particles observed in the small intestine and CP were similar in size and morphology, demonstrating the pleomorphic non-enveloped viral particles of approximately 17–30 nm in diameter which is consistent with that of a picornavirus. The overall findings are compatible with SVA infection in these cells, and probably suggests an enteric-neurological dissemination pathway for SVA.

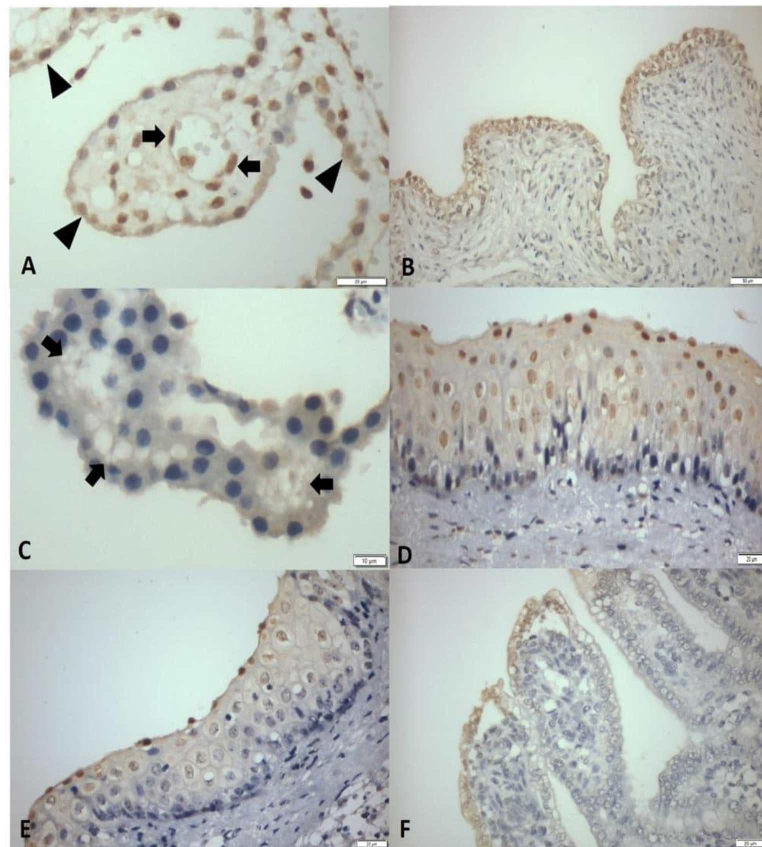

Figure 4. Immunohistochemical detection of antigens of SVA in epithelial tissues of newborn piglets. Cerebrum; there is positive immunostaining (arrow) at the capillary endothelium (**A**) and at the ependymal cells (arrow heads) of the choroid plexus (**C**). Urinary bladder; observe immunoreactivity to SVA at transitional epithelial cells (**B**). Observe the vacuolization of epithelial cells of the choroid plexus (**C**). Oral mucosa; observe positive immunolabelling of mucosal epithelium within areas of hydropic degeneration (**D,E**). Small intestine; there is positive immunolabelling of SVA at apical enterocytes within areas of intracytoplasmic vacuolization (**F**). Immunoperoxidase. Bar, A 50 μm; B,D-F 20 μm, and C 10 μm.

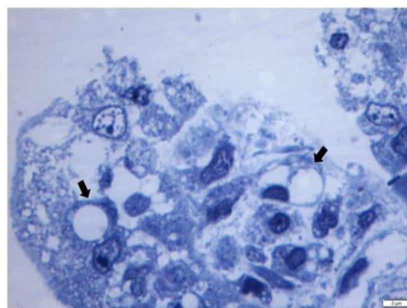

Figure 5. Semithin sections of the small intestine of piglets naturally infected with SVA. Observe SVA-induced balloon degeneration (open arrow) of the apical enterocyte of the small intestine resulting in displacement of the nucleus to the periphery. Bar, 5 μm.

Discussion

The results of the TEM and ST investigation have demonstrated that the vacuolization observed at the apical enterocytes of the small intestine during this and in a previous report of our group by histopathology[3], were in fact viral induced, since there were degeneration and vacuolization of enterocytes, representing early manifestations

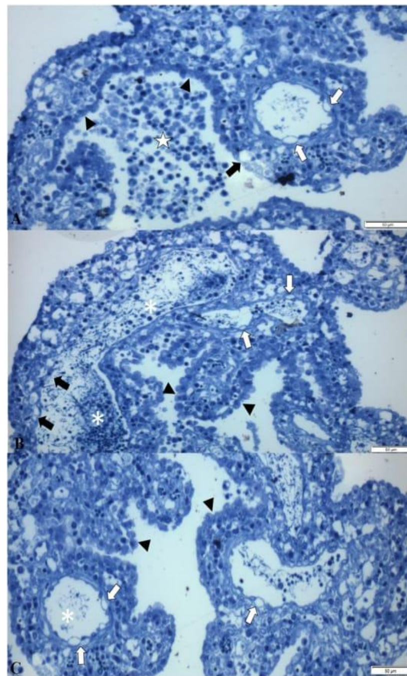

Figure 6. Semithin evaluation of the choroid plexus of piglets naturally infected with SVA. There is non-suppurative choroid plexitis (★), ballooning degeneration of endothelial cells (open arrows) of fenestrated capillary, degeneration (closed arrow) and swelling of (arrow heads) ependymocytes (**A**). Observe severe accumulations of macrophages and lymphocytes admixed with fibrin in the lumen (*) of a fenestrated capillary, necrosis (closed arrow) and degeneration (open arrows) of endothelial cells, with swelling and thickening (arrow heads) of ependymocytes (**B,C**). New methylene blue stain Bar, A-C, 50 μm.

of cellular injury. Similarly, the TEM and ST findings of degeneration and swelling of epithelial and endothelial cells of the CP (that were immunoreactive to SVA by IHC and contained viral RNA by RT-PCR) associated with intralesional viral particles confirmed the participation of SVA in the development of ETNL in these piglets.

An interesting finding during this investigation was the positive immunoreactivity of SVA to epithelial cells of the CP and ependymal cells of the lateral ventricle in six piglets with neurological manifestation associated with ETNL; similar the Haematoxylin & Eosin and IHC findings with combined RT-PCR amplification of SVA were previously described in newborn piglets[3]. SVA is a member of the family *Picornaviridae*, which includes several human and animal neurotropic enteroviruses (EV) such as porcine teschovirus (PTV)[20], poliovirus, and enterovirus 71[21], that produce diseases that begin in the gastrointestinal tract with subsequent neurological disorders, including nonsuppurative encephalomyelitis with perivascular cuffing[20], aseptic meningitis[21], and neuronal necrosis[20,21].

In human pathology, it was postulated that viral dissemination of EV may occur via several mechanisms including disruption of the blood-brain-barrier, BBB[22], and the blood-cerebrospinal fluid barrier, BCSFB, located between the cerebrospinal fluid and blood vessels of the CP[15,17]. Moreover, experimental studies have shown that PTV enters the brain hematogenously[20]. Furthermore, we have previously demonstrated antigens of SVA at the endothelial cells of capillaries within the CP[3], which may indicate that SVA has the capacity to alter the permeability of the BCSFB in newborn piglets and probably result in nonsuppurative meningoencephalitis and cerebral cortical oedema with neuronal necrosis as herein described; cerebral oedema is a manifestation of dysfunction to the BBB[16,18]. Consequently, these theories are in accordance with the ST and TEM findings during this study which demonstrated degeneration and necrosis of endothelial cells of fenestrated capillaries of the CP and nonsuppurative choroid plexitis associated with intralesional viral particles. This then suggests that SVA produces an initial inflammatory response at the CP, enters the brain probably due to disruption of the BCSFB, and produces nonsuppurative meningoencephalitis and neuronal necrosis in newborn piglets. However, it is currently unknown what mechanism is used by SVA to cross the BCSFB. Infectious agents may cross the BCSFB via tight junctions between CP epithelial cells[16–18], probably by infecting macrophages[17,18], resulting in leucocytic trafficking. Alternatively, organisms may use the "Trojan horse" effect by infecting circulating leukocytes and then the brain parenchyma[23], or colonize and infect epithelial cells of the CP before gaining entry to the brain[17]. Nevertheless, members of the *Picornaviridae* family are known to enter the brain via the BBB or neuromuscular junctions[23]. Although we were unable to observe infected leucocytes translocating through tight junctions of CP epithelial cells by TEM, this might be the most likely form of entry of SVA to the brain, and probably only observed in controlled experimental studies. Therefore, additional investigations are needed to confirm this hypothesis.

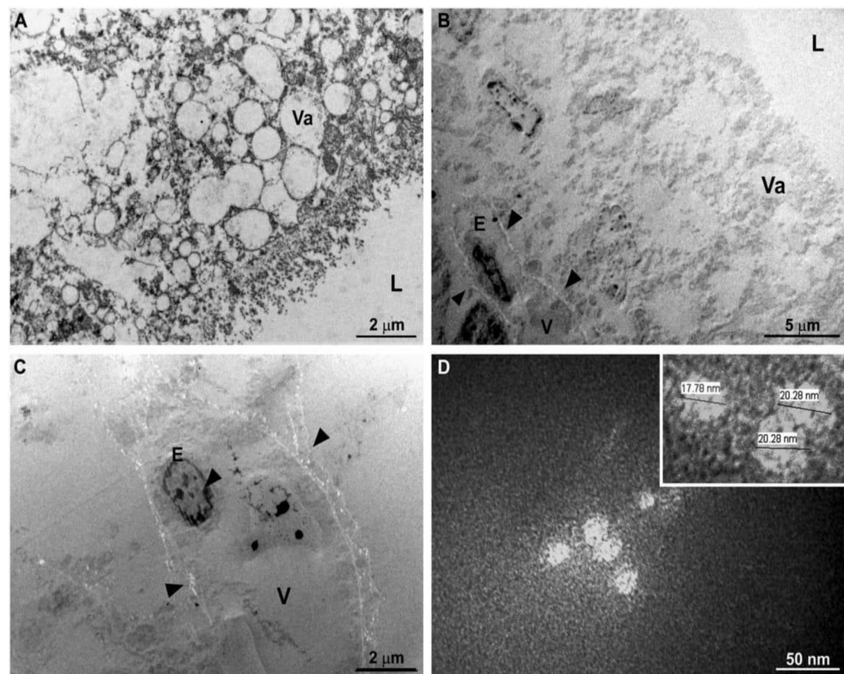

Figure 7. Transmission electron micrograph of the apical enterocytes of the small intestine. Electron microscopy of positive stained demonstrating that normal non-infected apical enterocytes of the small intestine contained numerous enlarged vacuoles (Va), intestinal lumen, L (**A**). Negative staining revealed that normal enterocytes had vacuoles (Va) of different sizes without viral particles, while infected cells had marked hydropic degeneration (vesicle, V) and viral particle aggregates surround (arrow heads) (**B,C**). Non-enveloped virus particles (**D**). Inset: high magnification of virus particles shown in (d) measuring 17 to 20 nm.

In newborn piglets[3] and adult pigs[24] SVA has been associated with gastrointestinal disease. In addition, the apical cells of the small intestine of newborn piglets with SVA-associated enteric disease have demonstrated histopathological evidence of injury, were immunoreactive to SVA by IHC and SVA RNA was identified by RT-PCR[3]. In the current study, atrophic enteritis was identified in 93% (40/43) of the piglets with gastrointestinal disease, and positive immunolabelling for SVA in the intestine of 63% (27/43) of these piglets. Consequently, we postulate that SVA may produce disease in newborn piglets by first affecting the intestine, after colonizing and replication in the tonsils[24], with the possibility of subsequent dissemination to the brain by disruption of the BCSFB.

During this study, vacuolization of the villi of apical enterocytes of the small intestine was observed in five piglets (# 4, 15, 17, 28, 38) by histopathology; all lesions contained antigens of SVA by IHC and SVA was identified by RT-PCR. The results of the current study can now confirm that these lesions are in fact SVA-induced ballooning degeneration of enterocytes as demonstrated by ST and TEM, and represents an early phase of viral infection. We have described similar findings in the small intestine of piglets in which there was positive immunolabelling for SVA and viral detection by RT-PCR[3]. This is additional evidence in support of the enteric-neurological dissemination theory proposed for SVA. Nevertheless, more studies are needed to understand the mechanism involved with this degenerative lesion and the dissemination of SVA to the brain. However, caution must be taken to differentiate the SVA-associated apical degeneration of enterocytes with that observed in newborn piglets. In normal piglets, macromolecules of colostrum passively obtained from the sow is absorbed via enterocytes[25], resulting in enlarged vacuoles that are enhanced by swelling of apical enterocytes[26]. However, TEM evaluation from this study has demonstrated that enterocytes infected by SVA are swollen due to intracytoplasmic vesicles surrounded by viral particles, while macromolecules of entreocytes derived from passively obtained colostrum did not demonstrate evidence of viral infection.

This is the first study that evaluated the combined pathological, immunohistochemical, molecular, and TEM findings associated with SVA-induced ETNL in a large population of piglets. The results from this investigation confirmed the findings of a previous study by our group that used relatively fewer piglets[3], ratifies that the tissues recommended for the IHC detection of SVA antigens are the urothelium, brain with the CP, in addition to ulcerative lesions at the hooves, coronary band, lips or snout, and erosive lesions of the tongue. Furthermore, these findings demonstrate that piglets under 10 days of age can be infected by SVA and develop a multisystemic disease[3–6]. The ballooning degeneration observed in this study by histopathology and confirmed by ST and TEM evaluations, is one of the most characteristic histopathological features described in SVA-induced infections[2–4], and represents initial invasion of the virus. The widespread immunolabelling of epithelial tissues observed in this study, and as was previously demonstrated[3], suggests that SVA has tropism for epithelia.

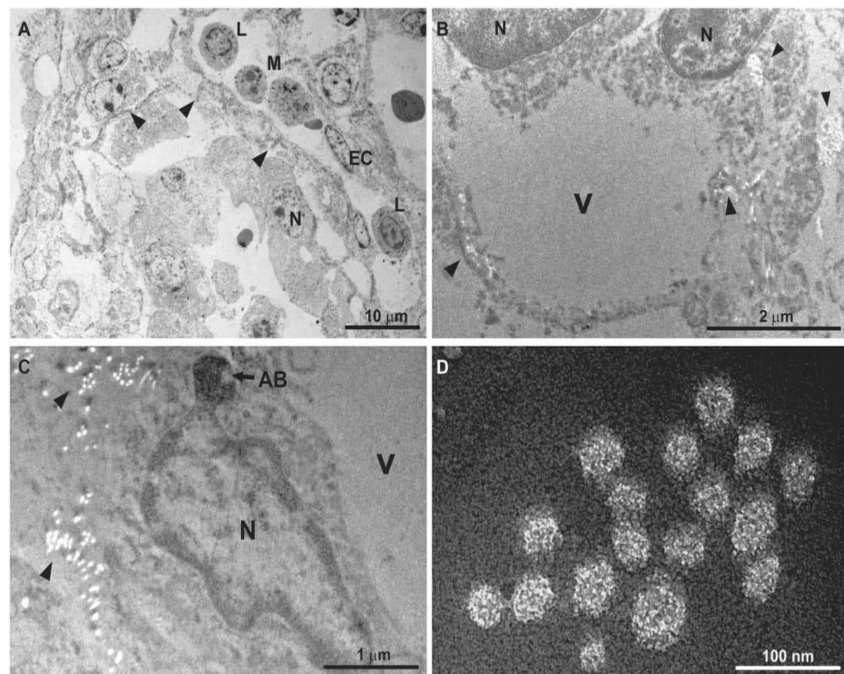

Figure 8. Transmission electron micrograph of the choroid plexus of newborn piglets naturally infected with SVA. Positive staining of the CP revealed the fenestrated capillary (arrow heads) containing an influx of lymphocytes (L) and monocytes (M); the tight junction (arrow heads) between adjacent endothelial cells (EC) is shown. N, nucleus (**A**). Observe the negatively stained balloon degeneration of ependymocytes characterized by intracytoplasmic vesicle (V) that is surrounded by aggregates (arrow heads) of SVA (**B**). There is an infected endothelial cell of the CP with an apoptotic body (AB), a vesicle (V) and aggregates of SVA (head arrow) (**C**). Closer demonstration of the non-enveloped virus observed in the cytoplasm of the infected endothelial cell of the CP (**D**).

There are few experimental studies inoculating SVA in finishing pigs[24,27], weaned piglets[28], but there is no experimental study with newborn piglets thus far. Nevertheless, experimental observations have shown that SVA induces a transient viremia in pigs, that is observed 3–10 days post inoculation, and coincides with the acute phase of this disease[24]. Interestingly, most of the piglets (81%; 35/43) from this study that died due to ETNL were between 2–5 days of age. This may suggest that the clinical manifestations of SVA are more intense in newborn piglets, resulting in sudden death[2,3,5,6,9] due to the immature immune system of these animals at birth. The immune system of pigs is developed during the perinatal period and becomes mature when piglets are between 5 and 7 weeks of age[29]; consequently, neonatal piglets would then be more susceptible to infection which may explain the elevated incidence of sudden death in ETNL.

In summary, it is proposed that SVA infects the apical enterocytes produces atrophic enteritis with consequent alteration of cellular permeability which may result in diarrhoea, that is frequently observed in piglets with ETNL[3,5]. Alteration of cellular stability of apical enterocytes was demonstrated by semithin and ultrathin evaluations during this study. The virus probably then disseminates through lymphatic vessels, replicates in lymphoid organs (primarily the tonsil), and produces a transient viremia[24]. Evidence from this study demonstrated that SVA produces lesions at the BCSFB of the choroid plexus of newborn piglets, as demonstrated by IHC, ST and TEM, and may suggest that this virus enters the brain via the BCSFB, considering that this via is used by members of the *Picornaviridae* family[23]. The virus produces an initial inflammatory response at the CP which results in disruption of the BCSFB due to SVA-induced degeneration of endothelial and epithelial cells of the choroid plexus with subsequent encephalitic dissemination resulting in cerebrocortical necrosis and nonsuppurative encephalitis. However, addition studies are being carried out to confirm this hypothesis.

Conclusions

The results from this investigation have demonstrated that ETNL is a multisystem disease of newborn piglets. It is proposed that SVA infects the apical enterocytes and then produces nonsuppurative meningoencephalitis and neuronal necrosis after possible disruption of the BCSFB at the choroid plexus. This would then suggest that SVA probably infects the brain in a manner that is similar to that of enteroviruses.

The gross, histopathological, molecular, and IHC findings of the 54 pigs analyzed corroborate with the findings of the study previously realized by our group with reduced number of piglets. We suggest that the gross alterations

more frequently associated with SVA are ulcerative lesions at several anatomical locations (including the tongue, muzzle, coronary band, and hooves), which must be differentiated from those observed in other infectious diseases of pigs. There is evidence to indicate that the ballooning degeneration of the transitional epithelium is the most frequent histopathological lesion associated with SVA in newborn piglets and that IHC can be used to identify antigens of SVA in affected tissues. We recommend the collection of fragments of the urinary bladder, CP, renal pelvis, oral mucosa, and ulcerative lesions for histopathological and IHC diagnoses of SVA in newborn piglets.

Methods

Animals and gross pathology. During June 2015 to November 2016, 54 piglets, between 1 to 10 days of age, from 23 farms located in the South and Southeast regions of Brazil with presumptive diagnosis of SVA were submitted to the Laboratory of Animal Pathology, Universidade Estadual de Londrina, for diagnostic investigation. These piglets had reported clinical signs suggestive of ETNL, including diarrhoea, neurological manifestations, reduced weight gain, and sudden death. All autopsies were done soon after death; tissue fragments (brain, heart, lung, kidney with ureters, liver, mesenteric lymph node, spleen, small intestine, skin and/or tongue when injured, tonsil, and urinary bladder) were collected for histopathology. Caution was taken to include the brain with the CP. All fragments were immersed in 10% buffered formalin solution for 48 h and then routinely processed for histopathological evaluation with the Haematoxylin and Eosin (H&E) stain.

Immunohistochemical identification of *Senecavirus A*. Selected FFPE tissue fragments of the brain with CP, lung, renal pelvis, small intestine, tonsil with oral mucosa, urinary bladder, skin, and tongue with erosive or ulcerative lesions were processed for IHC. These tissues were selected since they were constantly positive for SVA by RT-PCR in a previous study by our group[3], where it was demonstrated that this virus has tropism for epithelial organs. Therefore, this study only presents the IHC findings since, other related viral agents known to produce similar lesions in piglets[2,3] were not identified by RT-PCR assays in these piglets. In addition, multiple tissues from all 43 piglets were positive for SVA RNA by RT-PCR as previously described[3].

Commercial silanized slides (StatLab, McKinney, TX, USA) containing FFPE tissue fragments were deparaffinized, hydrated in alcohol baths and subjected to antigen retrieval, using citrate buffer (pH 6.0) with the pressure cooker system (Electrolux Pressure Cooker PCC10, São Paulo, SP, Brazil) for 2 min. Subsequently, there was blocking of endogenous peroxidase with methanol and hydrogen peroxide (3%) for 25 min. The primary incubation was done with a monoclonal antibody (1:50 dilution), kindly provided by Dr. M. Yang, National Centre for Foreign Animal Disease, Manitoba, Canada[30], with overnight incubation at 4 °C. Incubation with the secondary antibody (SuperPicture™ Polymer Detection kit; Invitrogen Corporation, Camarillo, CA, USA) was done in a humid chamber for 25 min at 25 °C, after which the chromogen, 3,3′-diaminobenzidine (DAB, Invitrogen® Life Technologies, Frederick, MD, USA), was added for 3 min. Finally, all slides were counter-stained with Harris haematoxylin and assembled with a commercial resin. Positive (uroepithelium of the urinary bladder the piglets) and negative controls were used in all IHC assays, since the urothelium is the tissue of choice for the diagnosis of SVA by RT-PCR and IHC[3].

Ultrathin and transmission electron microscopic. Tissue fragments of the CP and small intestine of one piglet with nonsuppurative meningoencephalitis and vacuolization of apical enterocytes that were collected during autopsy for routine histopathological analysis and maintained in 70% alcohol were selected for TEM processing. This was done primarily to identify viral particles in these tissues, based on our previous findings in newborn piglets with ETNL[3]. These sections (measuring 1 mm^3) were rehydrated in ethanol gradient (50–30° GL) and washed three times in 0,1 M PBS. The samples were fixed in 3% glutaraldehyde in 0.1% cacodylate buffer, post-fixed in 1% osmium tetroxide in 0,1 M cacodylate buffer, dehydrated in ethanol gradient and, after two baths in propylene oxide, embedded in Araldite resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections (60 nm) were obtained by using a ultramicrotome (Leica Ultracut UCT, Solms, Hesse, Germany), transferred to 200 mesh grids, stained with 2% uranyl acetate and lead citrate (Reynold's solution) or 3% phosphotungstic acid, then analyzed and photographed using a transmission electron microscope (FEI Tecnai G2, FEI Company, Hillsboro, OR, USA).

Animal welfare issues

All methods used during this investigation were approved by and carried out in accordance with the guidelines and regulations of the Universidade Estadual de Londrina relative to the usage of animals submitted for autopsy. The owners of all animals used during this study gave consent for their usage in diagnostic and scientific activities.

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Author Contributions

Oliveira, T.E.S. and Headley, S.A. substantially contributed to the conception and design of the study; drafted the manuscript, and contributed to the analysis, and interpretation of all pathological, immunohistochemical, transmission electron microscopy (TEM) data. Oliveira, T.E.S., and Michelazzo, M.M.Z. performed all autopsies and histopathological evaluations. Fernandes, T., and de Oliveira, A.G. contributed to the preparation, evaluation, and analyses of TEM data. Leme, R.A., Alfieri, A.F., and Alfieri, A.A. contributed to the acquisition of all piglets and evaluation of virological and molecular data. All authors have read, critically analysed, approved the final draft of this manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Additional Information

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Competing Interests: The authors declare that they have no competing interests.

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5 CONCLUSION

- Vesicular and/or ulcerative lesions at feet and snout, often concomitantly, were the most frequently observed gross features.
- Kidney with renal pelvis, urinary bladder, brain containing CP, vesicular and/or ulcerative lesions at feet and snout, oral mucosa, small intestine are the tissues of choice for diagnosis of SVA.
- Immunohistochemistry using the SVV-60 monoclonal antibody for the detection of SVA antigens in different organs of the pig was successfully standardized, demonstrating that it is a specific technique for the diagnosis of this viral infection.
- SVA has been shown to be an epitheliotropic virus, presenting high occurrence in choroid plexus epithelium, oral mucosa epithelia, urothelium of the renal pelvis, ureter and urinary bladder.
- The ballooning degeneration observed in different tissues/organs and the vacuolization of superficial enterocytes were caused by SVA, as confirmed by transmission electron microscopy.
- The neurological signs observed in some piglets with ETNL were due to non-suppurative meningoencephalitis induced by SVA.

APPENDIX

PUBLISHED ARTICLE- CLINICAL MANIFESTATIONS OF *SENECAVIRUS A* INFECTION IN NEONATAL PIGS, BRAZIL, 2015

Clinical Manifestations of Senecavirus A Infection in Neonatal Pigs, Brazil, 2015

Raquel A. Leme,[1] Thalita E.S. Oliveira,[1] Brígida K. Alcântara, Selwyn A. Headley, Alice F. Alfieri, Ming Yang, Amauri A. Alfieri

We identified new clinical manifestations associated with Senecavirus A infection in neonatal piglets in Brazil in 2015. Immunohistochemical and molecular findings confirmed the association of Senecavirus A with these unusual clinical signs and more deaths. Other possible disease agents investigated were not associated with these illnesses.

Senecavirus A (SVA), formerly called Seneca Valley virus, is the single representative species of the genus *Senecavirus* (family *Picornaviridae*). SVA is a single-stranded, positive-sense, nonenveloped RNA virus with a genome size of ≈7.2 kb (*1*).

SVA infection was associated with porcine idiopathic vesicular disease (PIVD) in pigs in Canada (*2*), the United States (*1*), and Brazil (*3,4*). The clinical manifestations of PIVD are indistinguishable from those of other vesicular virus infections, including foot-and-mouth disease virus (FMDV), vesicular stomatitis virus, swine vesicular disease virus (SVDV), and vesicular exanthema of swine virus (*2,3*). These clinical signs include fluid-filled and ruptured vesicles and ulcerative lesions at the coronary band, hooves, and/or snout (*1–4*). In 2015, we identified new clinical manifestations associated with SVA infections in piglets in Brazil.

The Study

Since early 2015, increased numbers of deaths were recorded in pig herds from different geographic regions of Brazil. Piglets during their first week of life demonstrated clinical signs such as muscular weakness, lethargy, excessive salivation, cutaneous hyperemia, neurologic manifestations, and diarrhea; some died suddenly. Clinical signs lasted for 3–10 days and then disappeared in piglets that survived.

To determine the cause of these illnesses, we investigated 5 farms (A–E). Pig populations per farm varied from 10,000 to 23,000 animals, and piglet death rates during the first week of life ranged from 20% to 30%. Ten piglets that died spontaneously were examined (Table 1).

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Farms A, B, D, and E had gestating and/or farrowing sows with fluid-filled or ruptured vesicles at the coronary bands, hooves, or snouts; reproductive disorders were not observed. We had identified SVA RNA from sows at farms A and B (*3*) a week before the onset of clinical manifestations in these piglets.

Routine necropsies of all piglets were conducted soon after death. Tissues were fixed by immersion in 10% buffered formalin solution and processed for histopathologic evaluation. Selected tissue fragments were used in an immunohistochemical (IHC) assay designed with monoclonal antibodies to detect SVA (*5*). Duplicate sections of the organs and scrapings from oral vesicles and cutaneous lesions were collected for molecular diagnostics. From piglets at farms C, D, and E, we collected diarrheic fecal samples to investigate the possibility of enteric viruses. We analyzed 81 tissue samples and 6 diarrheic fecal samples during this study by a combination of pathologic and molecular diagnostic methods.

Molecular assays were conducted to identify viruses that might be associated with the reported clinical signs; these included SVA (*3*); FMDV, vesicular stomatitis virus, and SVDV (*6*); teschovirus A, sapelovirus A, and enterovirus G (*7*); porcine parvovirus (*8*); and porcine circovirus type 2 (*9*). Feces and fragments of the small intestine from piglets of farms C, D, and E were evaluated for porcine rotavirus species A, B, C (*10*), and H (*11*); porcine epidemic diarrhea virus (*12*); swine deltacoronavirus (*13*); and transmissible gastroenteritis virus (*12*).

Seventeen amplified products were submitted for sequencing. We conducted sequence identity matrix using BioEdit software version 7.1.11 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A phylogenetic tree based on nucleotide sequences was obtained using MEGA6 software (http://www.megasoftware.net).

The most frequent gross manifestations observed were petechial hemorrhages of the kidney (7 piglets) and ulcerative lesions at the tongue (6 piglets) and coronary bands (4 piglets) (Figure 1, panels A, B). Interstitial pneumonia, the predominant histopathologic alteration, occurred in all the piglets; other frequent lesions were diphtheric glossitis (6 piglets), lymphocytic myocarditis (6 piglets), ballooning degeneration of the transitional epithelium of the urinary bladder (Figure 1, panel C) and the ureters (4 piglets), and lymphoplasmacytic encephalitis (3 piglets).

Consistent SVA IHC staining occurred at the transitional epithelium of the renal pelvis and the urinary bladder

[1]These authors contributed equally to this article.

Table 1. Geographic locations and other charactereristics of pig farms affected by Senecavirus A, Brazil, 2015

Farm	State/region	Month of collection	Animal no.	Age, d	Principal clinical manifestations
A	Paraná/Southern Brazil	February	1	2	Weakness at birth, sudden death at 1–3 d of age
			2	1	
B	Paraná/Southern Brazil	February	3	2	Weakness at birth, sudden death at 1–3 d of age
			4	1	
C	Mato Grosso do Sul/Midwest Brazil	March	5	3	Cutaneous hyperemia, diarrhea, excessive salivation, lethargy, death
D	Santa Catarina/Southern Brazil	March	6	2	Acute diarrhea and/or wasting, death
E	Santa Catarina/Southern Brazil	July	7	2	Diarrhea, neurologic manifestations, sudden death
			8	2	
			9	4	
			10	5	

(Figure 1, panel D) of 4 piglets; within epithelial cells of the choroid plexus of the cerebrum (8 piglets) and the tongue (5 piglets); and at the ependymal cells of the choroid plexus, vascular endothelium, and the enterocytes of the villi of the small intestine (2 piglets) (Table 2, http://wwwnc.cdc.gov/EID/article/22/7/15-1583-T2.htm).

The expected SVA RNA fragment was amplified by reverse transcription PCR from 77.8% (63/81) of all organs; all tissues from piglet 4 were positive for SVA and only 1 tissue sample from 3 piglets (nos. 2, 3, and 10) yielded negative results. Moreover, the nucleic acids of all other viruses investigated during this study were not amplified.

Sequence analysis from the 17 amplicons showed 98.8%–100% nt and aa similarities between each other and other isolates from Brazil available in GenBank (accession nos. KR075677 and KR075678). The SVA isolates we identified had similarities that varied from 87.4% nt (GenBank accession no. EU271760) to 98.5% nt (GenBank

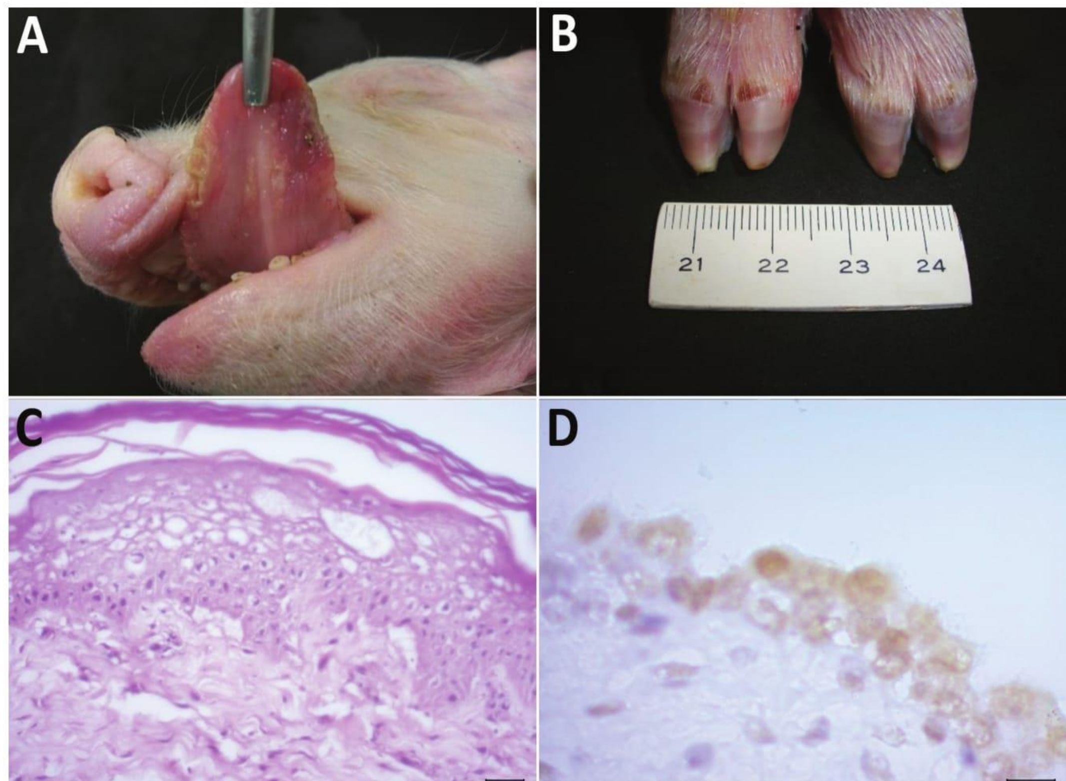

Figure 1. Pathologic alterations in piglets infected with Senecavirus A, Brazil, 2015. Gross examination shows multifocal diphtheric glossitis (A) and ulcerations of the coronary band (B). Histopathologic images demonstrate ballooning degeneration of the epithelium of the tongue (C) and positive immunoreactivity of the uroepithelium of the urinary bladder (D) to Senecavirus A. Panel B, scale shown in centimeters; panel C, hematoxylin and eosin stain; scale bar indicates 20 μm; panel D, immunoperoxidase; scale bar indicates 10 μm.

accession no. KC667560) and 94.4% aa (GenBank accession nos. EU271759 and EU271760) to 99.4% aa (GenBank accession no. KC667560) for isolates identified in North America. Phylogenetic analysis showed that the strains from this study (GenBank accession no. KT445973–KT445977) clustered with other known isolates of SVA and were distant from other picornaviruses associated with vesicular diseases (Figure 2).

Conclusions

SVA has been associated with PIVD in pigs with vesicular lesions at the snout, coronary band, and hooves (*1–3*). However, findings from our investigation suggest a new clinical syndrome associated with SVA infection that resulted in disease to multiple tissues and organs of these piglets.

The patterns of the cutaneous lesions identified in this study might be similar to those of other vesicular infections of picornavirus (FMDV and SVDV), in which ballooning degeneration of epithelial cells and the formation of microvesicles are hallmarks (*14*,*15*). In addition, FMDV and SVDV affect different organs of susceptible animals—the heart, lungs, lymph nodes, bone marrow, and central nervous system (*14*,*15*)—suggesting a wide organ tropism of these viruses.

An interesting feature during this study was the constant immunolabelling of SVA within epithelial cells of the choroid plexus of the brain and the surrounding endothelia of blood vessels in piglets with neurologic disease. On the basis of the IHC results and molecular findings in different tissues of the brain, we theorized that the neurologic manifestations of SVA observed during this investigation might be due to early infection of the choroid plexus through alteration of the integrity of the vascular epithelium and subsequent dissemination to the adjacent neuropil. The IHC detection of SVA within the urinary epithelium of all piglets suggests that urine might be a mode of dissemination and a possible source of contamination within affected pig farms.

Another unusual finding associated with SVA infection during this study was the occurrence of diarrhea in piglets. Molecular screening did not detect any of the common enteric viral pathogens of suckling piglets. However, the IHC and reverse transcription PCR identified SVA in the small intestine of piglets with diarrhea, demonstrating the ability of SVA to replicate within the enteric epithelium.

Our results suggest that SVA is a pantropic virus that produces a multisystemic disease entity in pigs infected at an early age. The constant immunolabelling of the uroepithelium of all piglets with SVA antigens might indicate that in-pen contamination, through urine, should be considered as a possible route for the dissemination of this virus.

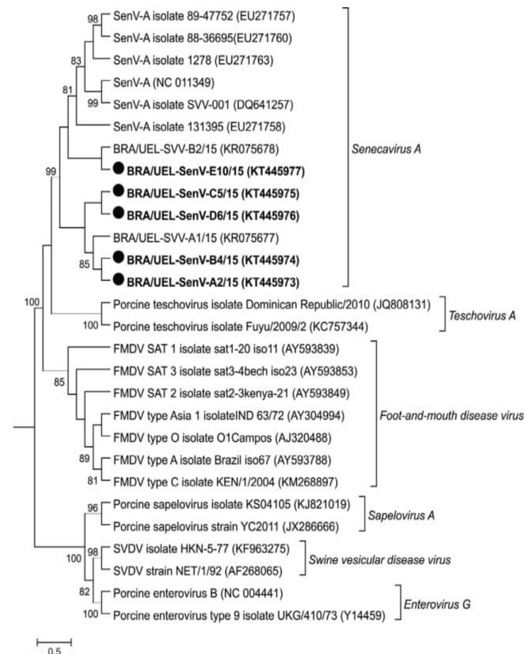

Figure 2. Phylogenetic relationship of strains of Senecavirus A identified in Brazil during 2015 (black circles) and other sequences available in GenBank derived from species of picornavirus associated with vesicular disease. Maximum-likelihood phylogenetic tree construction used the Kimura 2-parameter model with g distribution based on the partial viral protein (VP) 3/VP1 region of the Senecavirus A genome. GenBank accession numbers are given in parentheses. Bootstrap values determined in 1,000 replication. Scale bar indicates nucleotide substitutions per site.

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PUBLISHED ARTICLE - PATHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR FINDINGS ASSOCIATED WITH *SENECAVIRUS A* - INDUCED LESIONS IN NEONATAL PIGLETS

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INFECTIOUS DISEASE

Pathological, Immunohistochemical and Molecular Findings Associated with Senecavirus A-Induced Lesions in Neonatal Piglets

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Summary

This study investigated the cause of the mortality of piglets with cutaneous, enteric and neurological disorders from seven pig farms located in different geographical regions of Brazil. Twelve 1- to 5-day-old piglets were submitted for pathological evaluation. The principal gross findings included faint rib impressions on the pleural surface of the lungs ($n = 9$), diphtheritic glossitis ($n = 6$) and ulcerative lesions at the coronary band ($n = 5$). Histopathology revealed interstitial pneumonia ($n = 12$), myocarditis ($n = 6$), diphtheritic glossitis ($n = 3$), encephalitis ($n = 3$) and atrophy of intestinal villi with vacuolation of the superficial epithelial cells ($n = 6$). Immunohistochemistry with monoclonal antibodies specific for Senecavirus A (SenV-A) demonstrated immunoreactivity of the choroid plexus of the cerebrum, degenerate epithelium of ulcerative lesions of the tongue, the urothelium of the kidney and urinary bladder, and the superficial cells of the intestine. Reverse transcriptase polymerase chain reaction (PCR), PCR and/or quantitative PCR assays were used to investigate viral agents associated with vesicular and/or enteric diseases. Antigens and RNA of SenV-A were identified in multiple tissues of all piglets; molecular assays for all other viruses evaluated yielded negative results. These findings confirm the participation of SenV-A in the multiple lesions observed in these piglets. Several theories are proposed: SenV-A may be eliminated via the urinary system, neurological disease may occur due to initial invasion of choroid plexus, enteric disease may be related to atrophy and fusion of villi of the small intestine, and vertical transmission could be a form of dissemination.

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Keywords: pathogenesis; picornavirus infection; porcine idiopathic vesicular disease; Seneca Valley virus

Introduction

Senecavirus A (SenV-A), formerly referred to as Seneca Valley virus, is the single representative species of the *Senecavirus* genus, *Picornaviridae* family. SenV-A is a single-stranded, positive-sense, non-enveloped RNA virus with a genome size of approximately 7.2 kilobases (Hales *et al.*, 2008). This virus was first isolated as a cell culture contaminant in 2002 (Knowles, 2005), with the first complete genome sequence determined in 2005 (Hales *et al.*, 2008).

A vesicular disease of unknown aetiology, named porcine idiopathic vesicular disease (PIVD), was reported from pig herds of different countries, including Australia (Munday and Ryan, 1982), New Zealand (Montgomery *et al.*, 1987), the UK (ProMED-mail, 2007) and Italy (Sensi *et al.*, 2010). PIVD was also reported in pigs from Indiana, USA (Amass *et al.*, 2004; Singh *et al.*, 2012) and Manitoba, Canada (Pasma *et al.*, 2008). Recently, cases of SenV-A associated

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disease were described in pigs from distinct geographical regions of Brazil (Leme *et al.*, 2015) and the virus was also identified from the vesicular fluid and sera of sows with PIVD and in piglets from this country (Vannucci *et al.*, 2015; Leme *et al.*, 2016). In addition, the complete genome of SenV-A was obtained from an outbreak of piglet mortality in China (Wu *et al.*, 2016).

Although the genome of SenV-A virus is described by several groups (Hales *et al.*, 2008; Vannucci *et al.*, 2015), the pathological findings associated with this virus are poorly understood. This study describes the pathological, immunohistochemical and molecular findings associated with spontaneous SenV-A-induced infections in piglets, and supplements the results of a previous investigation (Leme *et al.*, 2016).

Materials and Methods

Clinical Information

The clinical history of the animals and the geographical distribution of the farms affected are given in Table 1. The affected piglets were 1–5 days old. The piglets originated from four States and three geographical regions of Brazil. Since January 2015, there have been reports of a vesicular disease of unknown aetiology affecting several pig herds from diverse geographical regions of Brazil. By the end of February 2015, two pig herds (A and B) from distinct cities of the State of Paraná, Southern Brazil, reported the birth of weak piglets and the sudden death of piglets at 1–3 days of age.

By March 2015, the death of piglets during the first week of age had been reported from two farms from the states of Mato Grosso do Sul, Midwest Brazil (farm C) and Santa Catarina (SC), Southern Brazil (farm D). Piglets from farm C demonstrated lethargy, excessive salivation, cutaneous hyperaemia and diarrhoea. The piglets from farm D presented with acute diarrhoea and/or wasting, with death occurring soon after.

In July 2015, four piglets (2- to 5-days-old) from a farm (E) located in SC, Southern Brazil, were submitted for necropsy examination. Piglets from this farm demonstrated acute diarrhoea and neurological signs. The clinical signs persisted for approximately 3–7 days and then disappeared in all piglets that

Table 1
Geographical locations of farms affected, biological data and clinical findings of piglets.

Farms affected	*Geographical locations (state/Brazilian region)*	*Piglet number*	*Age (days)*	*Principal clinical signs*
A	Paraná/Southern Brazil	1 2	2 1	Birth of weak piglets Sudden death of animals between 1 and 3 days of age
B	Paraná/Southern Brazil	3 4	2 1	Birth of weak piglets Sudden death of animals between 1 and 3 days of age
C	Mato Grosso do Sul/ Midwest Brazil	5[*]	3	Cutaneous hyperaemia Death Diarrhoea Excessive salivation Lethargy
D	Santa Catarina/ Southern Brazil	6[*]	2	Acute diarrhoea and/or wasting Death
E	Santa Catarina/ Southern Brazil	7 8 9 10	2 2 4 5	Diarrhoea Neurological signs Sudden death
F	São Paulo/Southeastern Brazil	11	3	Death Diarrhoea Neurological signs
G	Santa Catarina/ Southern Brazil	12	2	Death Diarrhoea Wasting

[*]Tissues of pigs received for histopathological evaluation.

recovered from this disease. In all of these farms, the total pig populations ranged from 10,000 to 23,000 animals, with mortality rates of piglets during the first week of age varying from 20% to 30%.

The consulting veterinarian at farm F (São Paulo, SP; Southeastern Brazil) indicated that diarrhoea affecting piglets began in late August 2015. At that time, there were 2,500 piglets with morbidity estimated at 60% and 15% mortality. In late September 2015, SenV-A was identified by reverse transcription-polymerase chain reaction (RT-PCR) assay of vesicular fluid and cutaneous lesions from gestating sows on farm G, Santa Catarina, Southern Brazil; there were 940 gestating sows with morbidity estimated at 30%, but no reported cases of mortality. This herd contained 150 piglets with diarrhoea, with 15% of the affected animals dying during the first week of age.

In addition, all farms, except farm C, reported that gestating and/or farrowing sows demonstrated fluid-filled and/or ruptured vesicles at the coronary band, hooves and/or snout; reproductive disorders were not observed. SenV-A RNA was identified in samples from the ruptured vesicles and ulcerative lesions of sows on farms A and B (Leme *et al.*, 2015).

Gross Pathology

Routine necropsy examination of 10 piglets was performed soon after death; tissue fragments were received from piglet number 5 (farm C) and piglet number 6 (farm D). Selected tissues from all animals subjected to necropsy examination, as well as the tissue fragments from the two piglets, were fixed in 10% neutral buffered formalin and processed routinely. Duplicate sections of the organs mentioned above, as well as swabs from oral vesicles and cutaneous lesion scrapings, were collected freshly during necropsy examination and maintained at −80°C until processed for molecular diagnostics.

In order to avoid cross contamination, equipment used during necropsy examination and sample collection was cleaned and immersed in a mixture of antiseptic and disinfectant solution between each collected sample. Faecal samples from piglets with diarrhoea from farms C, D, E, F and G were collected to investigate the possible participation of enteric viruses associated with diarrhoea.

Immunohistochemistry

Selected tissue fragments from each piglet, as well as the tissues from the piglets on farms C and D, were studied by immunohistochemistry (IHC) using monoclonal antibodies to detect SenV-A (Yang *et al.*, 2012). These reagents were kindly provided by Dr. M. Yang, National Centre for Foreign Animal Disease, Manitoba, Canada. The IHC protocol (Headley *et al.*, 2001) involved incubation with monoclonal antibody F60SVV-76 (1 in 50 dilution) overnight at 4°C. The secondary detection system was SuperPicture™ Polymer Detection kit (Invitrogen, Camarillo, California, USA).

Molecular Characterization of Senecavirus A

Tissue samples were disrupted with a MagNa Lyser™ (Roche Diagnostics, Mannheim, Germany), homogenized in 0.01 M phosphate buffered saline (PBS, pH 7.2) and clarified by centrifugation at 3,000 g for 10 min. Faecal suspensions were prepared at 10–20% (w/v) in 0.01 M PBS and centrifuged at 5,000 g for 3 min.

The supernatants were used for nucleic acid extraction. This was performed by using 400 μl of faecal homogenate (10–20% w/v) and proteinase K pretreated aliquots of tissue suspensions in a combination of phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate extraction methods (Boom *et al.*, 1990; Alfieri *et al.*, 2006). The extracted nucleic acid was eluted in 50 μl of ultrapure RNase-free di-ethylpyrocarbonate (DEPC)-treated sterile water (Invitrogen). Positive controls consisted of viral RNA from a previous study (Leme *et al.*, 2015); sterile ultrapure water (Invitrogen) was used as a negative control during all nucleic acid extractions and amplification procedures.

The possible participation of SenV-A RNA in these lesions was investigated by using a RT-PCR assay designed to target a 542 base pair sequence of the partial VP3/VP1 regions of the virus genome (Leme *et al.*, 2015).

Differential Molecular Diagnosis

Molecular assays were performed to identify the nucleic acids of viruses that might be associated with cutaneous and/or vesicular lesions, wasting, neurological or systemic disorders of pigs. These included SenV-A, foot-and-mouth disease virus (FMDV), vesicular stomatitis virus (VSV), swine vesicular disease virus (SVDV), *Teschovirus A, Sapelovirus A, and Enterovirus G*, porcine parvovirus (PPV) and porcine circovirus type 2 (PCV2). The participation of FMDV, VSV and SVDV were investigated as recommended by the OIE Terrestrial Manual (Hofner *et al.*, 1994; Núñez *et al.*, 1998; Reid *et al.*, 2000; OIE, 2015). The identification of teschovirus A, sapelovirus A and enterovirus G was investigated by targeting the partial 5′ non-translated region of the genome these

Table 2
Distribution of Senecavirus A antigen and nucleic acid in piglets.

Selected tissues/diagnostic techniques (IHC and RT-PCR)

Piglet number	*Tongue*		*Gingiva*		*Myocardium*		*Lung*		*Renal pelvis*		*Liver*		*Urinary bladder*		*Cerebrum*		*Cerebellum*		*Brainstem*		*Small intestine*	
	IHC	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*	*IHC*	*RT-PCR*
1[*]	+	+	+	+[‡]	–	+[‡]	–	+	+	+[‡]	–	–	NC	NC	+[¶]	–	–	+	–	–	–	–
2	+	+	NC	NC	–	+[‡, §]	–	+	+	+[‡]	–	+	NC	NC	+[¶]	–	–	+	–	+	–	+
3	+	+	NC	NC	–	+[‡, §]	–	+	+	+[‡]	–	+	NC	NC	+[¶]	–	–	+	–	+	–	+
4	+	+	+	+[‡]	–	+[‡]	–	+	+	+[‡]	–	+	NC	NC	+[¶]	+	–	+	–	+[‡]	–	+
5[†]					–	+	–	+[‡,§]			–	–									+	+
6[†]																					+	+[‡,§]
7	NC	NC	NC	NC	–	–	–	–	+	–	–	–	+	+	+[¶]	–	–	–	–	–	+	–
8	NC	NC	NC	NC	–	+	–	+[‡]	+	+	–	–	+	+	+[¶]	+	–	+	–	–	–	–
9	NC	NC	+	+	–	+	–	+	+	+	–	–	+	+[‡]	+[¶]	–	–	–	–	+	–	+
10	+	+[‡,§]	+	+	–	+	–	+	+	+	–	–	+	+	+[¶]	+	–	+	–	–	–	+
11	NC	NC	NC	NC	–	–	–	+	+	+	–	–	+	–	–	–	–	–	–	–	+	–
12	–	+	NC	NC	–	+	–	+	+	+	–	+	+	+	–	–	+[¶]	+	–	–	+	+

[*]Piglet with coronary band lesion RT-PCR positive.
[†]Tissues from piglets submitted for diagnostic evaluation, NC, not collected.
[‡]Sequenced sample.
[§]Isolate used in the phylogenetic analysis.
[¶]Choroid plexus.

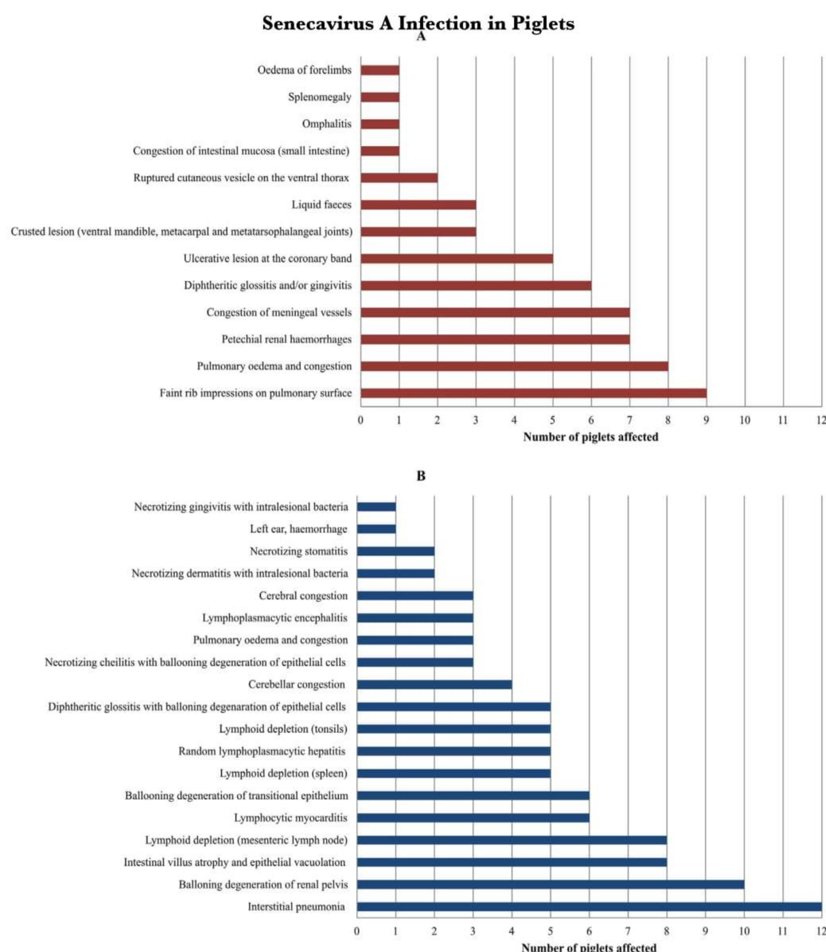

Fig. 1. Graphical demonstration of the distribution of the principal gross (A) and histopathological (B) findings in piglets infected with Senecavirus A.

viruses (Krumbholz *et al.*, 2003; Donin *et al.*, 2014). PCR and qPCR assays for PPV and PCV2, respectively, targeted the partial PPV NS1 (Soares *et al.*, 1999) and PCV2 ORF2 (McIntosh *et al.*, 2009) genomic regions.

All organs collected from each piglet were evaluated for all the viruses described above, except for PPV and PCV2, which were only tested for in samples of the heart and lungs. Additionally, faeces and fragments of the small intestine from piglets of farms C, D, E, F and G with clinical manifestations of diarrhoea were evaluated for porcine rotavirus species A (Gouvea *et al.*, 1990; Gentsch *et al.*, 1992), B (Marthaler *et al.*, 2012), C (Alfieri *et al.*, 1999) and H (Molinari *et al.*, 2014); porcine epidemic diarrhoea virus (PEDV), partial genes S and M (Kweon *et al.*, 1997; Kim *et al.*, 2001); swine deltacoronavirus (SDCoV), partial genes M and N (Wang *et al.*, 2014); and transmissible gastroenteritis virus (TGEV), partial gene S (Kim *et al.*, 2001). Positive controls consisted of nucleic acids of viral agents from a previous study (Leme *et al.*, 2016). Sterile water was used as negative control (Invitrogen). These controls were included in all PCR and RT-PCR assays.

Sequence Analysis

Seventeen amplified products from the SenV-A RT-PCR assay were submitted for sequencing. Table 2 presents the amplified products submitted for sequence analysis according to the tissue samples of each piglet. Amplicons were purified by the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) and quantified with a Qubit® fluorometer (Invitrogen). These were then sequenced in both directions with the forward and reverse primers in an ABI 3500 Genetic Analyzer with the BigDye® Terminator v3.1

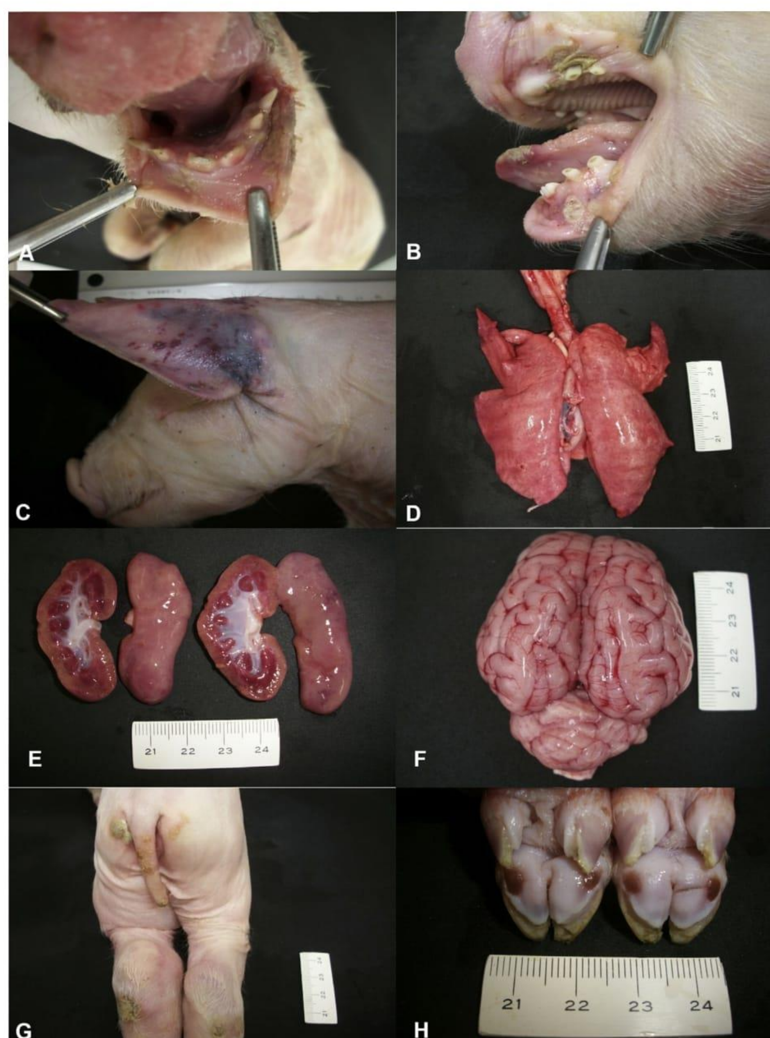

Fig. 2. Pathological changes in piglets infected with Senecavirus A. There is multifocal diphtheritic gingivitis (A) and glossitis (B), and petechial haemorrhages of the ear (C). Observe the faint rib impressions on the pulmonary surface (D), haemorrhagic nephritis (E), marked congestion of meningeal vessels of the brain (F), crusting cutaneous lesions at several anatomical locations (G) and ulceration of the plantar foot pad (H). Scale in cm.

Cycle Sequencing Kit (Applied Biosystems®, Foster City, California, USA).

The obtained sequences were examined for quality analysis of chromatogram readings by using the PHRED software (http://asparagin.cenargen.embrapa.br/phph); sequences were only accepted if base quality was equal to or greater than 20. Consensus sequences were then generated by the CAP3 program (http://asparagin.cenargen.embrapa.br/cgi-bin/phph/cap3.pl), after which the partial nucleotide sequences were initially compared by the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST) with similar sequences deposited in GenBank. Sequence identity matrix was performed using the BioEdit software version 7.1.11 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Results

Pathological and Immunohistochemical Findings

The pathological and immunohistochemical findings are summarized in Fig. 1 and Table 2. The most

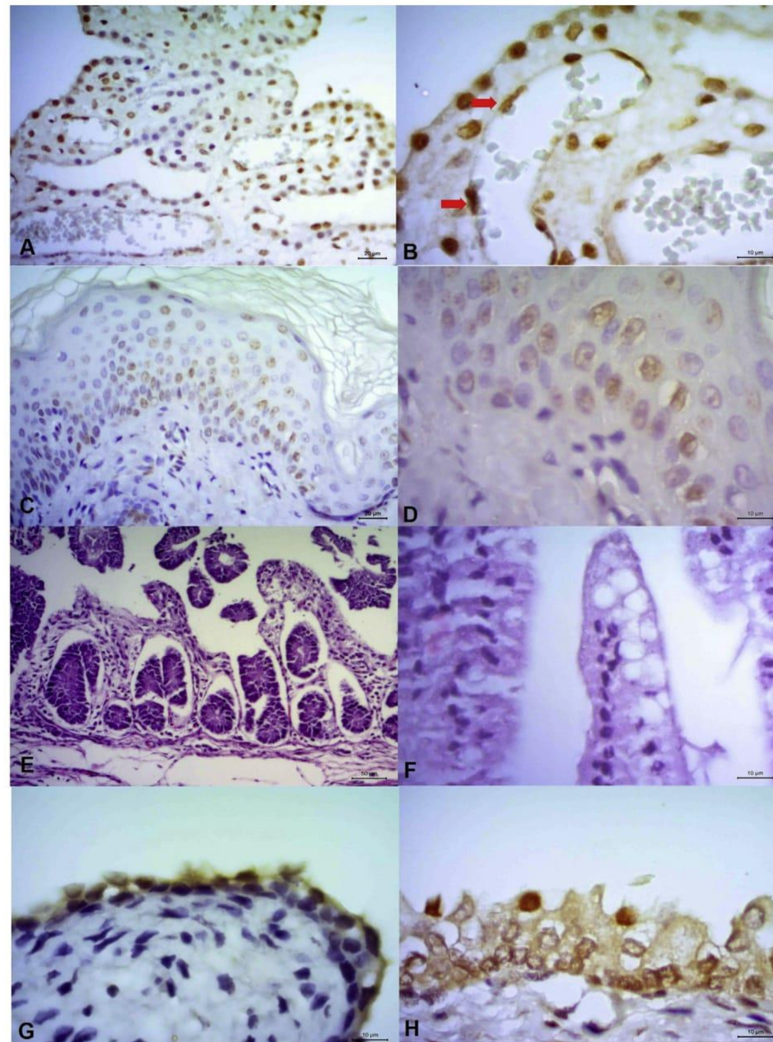

Fig. 3. Histopathological and immunohistochemical findings in piglets infected with Senecavirus A (SenV-A). (A) Cerebrum showing immunolabelling of epithelial cells of the choroid plexus. (B) The endothelial cells (arrows) of the capillaries are also immunoreactive to SenV-A. (C–D) Labelling of epithelial cells of the tongue for SenV-A antigen. (E) There is fusion of intestinal villi and (F) vacuolation of the superficial epithelial cells of the small intestine. (G) Labelling of the uroepithelium of the urinary bladder (G) and (H) the renal pelvis of the kidney for SenV-A antigen. IHC, A–D, G and H. HE, E and F.

frequent gross changes observed during necropsy examination of the 10 piglets were faint rib impressions on the pleural surface of the lungs ($n = 9$), pulmonary oedema ($n = 8$), congestion of meningeal vessels ($n = 8$), petechial haemorrhages of the kidney ($n = 7$), diphtheritic glossitis ($n = 6$) and ulcerative lesions at the coronary band ($n = 5$). Three piglets (numbers 1, 4 and 10) with multifocal cutaneous crusts at different anatomical locations (including the ventral region of the mandible, metacarpus, vulva and the metatarsophalangeal joint) also had diphtheritic glossitis. Liquid faeces, indicative of diarrhoea, were observed in the small intestine of piglets 8, 11 and 12, and were also reportedly seen in piglets 5 and 6. The gross lesions observed during this study are shown in Fig. 2. The stomachs of all piglets were filled with milk.

Interstitial pneumonia was the predominant histopathological alteration and occurred in all piglets examined by necropsy examination, as well as in the

pulmonary fragments of the other two piglets. Other frequent histopathological alterations included lymphoid depletion of the spleen, tonsils and/or lymph nodes ($n = 8$), lymphocytic myocarditis ($n = 6$), ballooning degeneration of the transitional epithelium of the urinary bladder and the ureters ($n = 6$) and lymphoplasmacytic encephalitis ($n = 3$). There was vacuolation of the superficial epithelial cells of the intestinal mucosa in six piglets (numbers 5, 6, 7, 8, 11 and 12) with clinical histories of diarrhoea, with atrophy of intestinal villi occurring in six piglets (numbers 5, 6, 9, 10, 11 and 12).

The distribution of SenV-A antigens in tissues from the piglets is given in Table 2. SenV-A antigens were identified in multiple tissues from all piglets. Consistent SenV-A immunohistochemical labelling occurred in the transitional epithelium of the renal pelvis and/or ureters ($n = 6$) of all piglets subjected to necropsy examination and in the urinary bladder (Fig. 3) collected from six piglets with ballooning degeneration of the epithelium. Consequently, there was concomitant positive immunolabelling of the epithelium of the urinary bladder and the renal pelvis in six piglets (numbers 7, 8, 9, 10, 11 and 12). Frequent labelling also occurred of the epithelial cells of the choroid plexus of the cerebrum ($n = 8$) and the affected tongue of most piglets ($n = 5$) with pathological alterations. In addition, labelling was observed in the vascular endothelium within the choroid plexus, and in the vacuolated superficial enterocytes of the small intestine of five piglets. Diarrhoea was observed in three of these animals (numbers 8, 11 and 12).

Molecular Characterization of Senecavirus A

SenV-A RNA of expected size was amplified successfully from the RT-PCR assays of most of the organs collected from the 12 piglets. The distribution of viral RNA in tissues/organs from each piglet is given in Table 2. SenV-A RNA was most frequently amplified from the lungs ($n = 10$), renal pelvis ($n = 9$), myocardium ($n = 9$), small intestine ($n = 8$) and cerebellum ($n = 7$). Viral RNA was amplified from the kidneys of most piglets ($n = 7$) that demonstrated antigens of SenV-A by IHC. This correlation also occurred for the urinary bladder of the six piglets that had viral antigens demonstrated by IHC. In addition, viral RNA was amplified from the intestine of most of the piglets with diarrhoea.

The RNA of teschovirus A, sapelovirus A and enterovirus G, that might be associated with cutaneous lesions and has been previously reported in pig herds from Brazil (Donin *et al.*, 2014, 2015), was not detected in any of the tissues from the piglets. Similarly, the targeted nucleic acid regions of viruses associated with vesicular disease, myocarditis and/or encephalomyelitis were not identified during this investigation. DNA of PPV and PCV2, viral agents related to early death of piglets, wasting, dermatitis syndrome, vesicular disease and/or systemic clinical signs, was not detected in the tissue samples evaluated. In addition, the RT-PCR assays for porcine RVA, RVB, RVC, RVH, PEDV, SDCoV and TGEV did not detect the genomes of these viruses in the faecal samples from piglets of farms C, D, E, F and G.

Phylogenetic Analysis

The SenV-A sequences derived from these piglets have been deposited in GenBank (Accession numbers KT445973, KT445974, KT445975, KT445976 and KT445977), and represent samples from most pig farms. Sequence analysis of the 17 amplicons from this study revealed 98.8%–100% nucleotide similarities between each other and with other isolates recently described in Brazil (KR075677 and KR075678). At the amino acid level, the isolates in this study were 100% identical with each other and with the two previously mentioned strains from Brazil, except for the amino acid sequences from piglets 5 and 10, which were 98.8% similar to each other and 99.4% similar to the other sequences in this study. Relative to the other SenV-A sequences available in GenBank (i.e. DQ641257, EU271757, EU271758, EU271759, EU271760, EU271761, EU271762, EU271763, KC667560 and NC_011349), the strains described here had homology ranging from 87.4% (EU271760) to 98.5% (KC667560) for nucleotide sequence and 94.4% (EU271759 and EU271760) to 99.4% (KC667560) for amino acid sequence. The diagrammatic representation of the phylogenetic relationship between the isolates from this study and similar strains of picornavirus associated with vesicular disease, deposited in GenBank, has been published elsewhere (Leme *et al.*, 2016).

Discussion

The results of these pathological and molecular investigations have demonstrated the participation of SenV-A in the multiple lesions observed in the 10 piglets subjected to necropsy examination and in the tissues of two piglets received for diagnosis from the Southern, Southeastern and Midwest regions of Brazil. Consequently, these findings extend the geographical distribution of SenV-A in swine to include Brazil, and correlate with other investigations from this country (Vannucci *et al.*, 2015; Leme *et al.*,

2015, 2016). The results suggest an early manifestation of SenV-A-induced infection in all piglets and demonstrate the susceptibility of young pigs to this virus, with resulting multisystemic disease (Leme *et al.*, 2016).

Currently, the known vesicular disease-associated picornaviruses might be considered as models to understand the forms of transmission, pathogenesis and infection associated with SenV-A, since these viruses have similar molecular features, are within the same family and produce indistinguishable clinical signs in pigs, suggesting similar viral biological properties in this host species. The ballooning degeneration observed in the cutaneous lesions induced by SenV-A during this study may have a similar pattern to that observed in the established diseases induced by FMDV and SVDV, in which ballooning degeneration of epithelial cells and the formation of microvesicles are the hallmarks of infection (Escribano-Romero *et al.*, 2000; Arzt *et al.*, 2011). Erosive lesions were also observed at the coronary bands of several piglets and in a study by another group (Vannucci *et al.*, 2015). These lesions are similar to those seen in cases associated with FMDV. Additionally, FMDV and SVDV affect different organs (e.g. myocardium, lungs, lymph nodes, bone marrow and central nervous system) of susceptible animals (Escribano-Romero *et al.*, 2000; Arzt *et al.*, 2011), suggesting a tropism of these viruses for different cells. Similar results were observed during this investigation, where multiple systems (i.e. nervous, respiratory, urinary, cutaneous and digestive) contained SenV-A antigens and/or nucleic acid as observed by IHC and RT-PCR, suggesting systemic dissemination of the virus in all piglets.

The atrophy and fusion of the villi of the small intestine with the vacuolation of superficial epithelial cells observed by histopathology in four piglets (numbers 5, 6, 11 and 12), associated with the positive immunolabelling of SenV-A antigens within these lesions, suggests that this infectious agent was associated with the clinical enteric manifestations that occurred on several farms. In addition, viral RNA was amplified from the intestinal content from three of these piglets. Although similar histopathological findings were described in suckling pigs infected experimentally with PEDV (Jung *et al.*, 2014), this virus was not amplified from any intestinal fragment and/or faeces from these piglets. The vacuolation of superficial epithelial cells observed in this and a previous study (Jung *et al.*, 2014) was considered to be ballooning degeneration, an early indication of virus-induced injury. The failure of molecular assays to identify other agents frequently associated with gastrointestinal disease in pigs supports the likely participation of SenV-A in the development of these enteric clinical manifestations and pathological findings.

Due to the consistent immunolabelling and amplification of SenV-A within epithelial cells of the choroid plexus, the urinary bladder and the renal pelvis, and the small intestine of several piglets, we propose three hypotheses. The first is that the neurological manifestations of this disease might be related to early infection of the choroid plexus via alteration of the integrity of the vascular epithelium and subsequent dissemination to the adjacent neuropil. The second suggests that urine from infected piglets may be a means by which the virus is disseminated and provides a possible source of contamination on affected pig farms (Leme *et al.*, 2016). Thirdly, the pathological, immunohistochemical and molecular results suggest that SenV-A can induce gastrointestinal disease in neonatal piglets. Experimental models are being designed to investigate these hypotheses.

During this study, and based on ongoing routine investigations (unpublished data) of the distribution of viral RNA and tissue antigens of SenV-A, we have observed that positive results for RT-PCR and IHC are more frequently and constantly obtained in specific tissues. RT-PCR for the detection of SenV-A RNA is more likely to be positive using pulmonary and myocardial tissues, and possibly also brainstem; while the tissues of choice for IHC are the renal pelvis and the urinary bladder. Consequently, we recommend these tissues for the individual detection (either by RT-PCR or IHC) of SenV-A; while the renal pelvis and urinary bladder is the tissue of choice for the combined molecular and immunohistochemical diagnosis of SenV-A infection.

FMDV crosses the placental barrier, particularly during the early stages of gestation (Ryan *et al.*, 2007). However, this study was done in sheep and it is not clear if FMDV or SVDV are able to cross the placental barrier resulting in intra-uterine infections in pigs. The detection of SenV-A by RT-PCR in 1- to 2-day-old piglets with pathological lesions and disseminated immunolabelling of viral antigens, suggests that the virus might have been vertically transmitted. We have previously identified SenV-A in multiple fluid-filled and/or ruptured vesicles and ulcerative lesions of the coronary band, hooves and/or snout from gestating sows of farms A and B (Leme *et al.*, 2015); however, it remains to be demonstrated experimentally that SenV-A can cross the placental barrier and infect the fetus.

In conclusion, the results from this investigation suggest that neonatal piglets are susceptible to infection by SenV-A, resulting in a multisystemic disease due to viral tropism for several tissues. The concomitant identification of SenV-A virus in sows and piglets

from three farms may suggest that vertical transmission is possible, while the consistent immunolabelling of the urothelium of all piglets for antigens of SenV-A might suggest that in-pen contamination, via urine, should be considered as a possible route for the dissemination of this virus. Furthermore, SenV-A appears to produce enteric manifestations and disease in neonatal piglets.

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