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Clinical cystoisosporosis associated to porcine cytomegalovirus (PCMV, *Suid herpesvirus 2*) infection in fattening pigs

Walter Basso[a,b,c,*], Hanna Marti[d], Monika Hilbe[d], Titus Sydler[d], Anina Stahel[e], Esther Bürgi[a], Xaver Sidler[a]

[a] Department of Farm Animals, Division of Swine Medicine, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland
[b] Institute of Parasitology, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 266a, CH-3057 Zurich, Switzerland
[c] Institute of Parasitology, Vetsuisse-Faculty, University of Bern, Länggassstrasse 122, CH-3012 Bern, Switzerland
[d] Institute of Veterinary Pathology, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 268, CH-8057 Zurich, Switzerland
[e] Institute of Virology, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland

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ABSTRACT

Cystoisospora (syn. *Isospora*) *suis* is the causative agent of neonatal porcine coccidiosis and one of the main causes of diarrhoea in suckling piglets worldwide. Infection with porcine cytomegalovirus (PCMV, *Suid herpesvirus 2*) causes inclusion body rhinitis in pigs. In a Swiss pig herd (n = 2 boars, 7 sows, 2 gilts, 18 finishing pigs, 30 fattening pigs, 54 suckling piglets), an outbreak of PCMV infection with high morbidity in all age categories, characterized by fever, anorexia, reduced general condition, respiratory signs and increased piglet mortality, was diagnosed by histopathology and molecular methods. Five fattening pigs (age ~ 17 weeks) additionally showed diarrhoea, not typical for PCMV infections, and one fattener had to be euthanized due to poor condition. Histopathologically, severe fibrinopurulent jejunoileitis with extensive atrophy and fusion of intestinal villi, loss of goblet cells and crypt abscesses associated to *C. suis* infection were present. In the liver, herpesvirus intranuclear inclusion bodies were observed and PCMV was confirmed by PCR/sequencing. No further infectious causes of diarrhoea (i.e. Rotavirus A; TGEV; PEDV; PCV-2; enterotoxigenic *Escherichia coli* or *Lawsonia intracellularis*) were detected in the euthanized fattener. Coproscopically, *C. suis* oocysts were identified in the faeces from further fatteners with diarrhoea. While *C. suis* usually produces disease only in suckling piglets, its association with severe intestinal lesions and diarrhoea in ~17-week-old fatteners was surprising. It is supposed that the underlying PCMV infection might have contributed to the presentation of clinical cystoisosporosis in fattening pigs. The interaction mechanisms between these two pathogens are unknown.

1. Introduction

Cystoisospora (syn. Isospora) suis is the causative agent of cystoisosporosis or neonatal porcine coccidiosis. This protozoan parasite is regarded as one of the main causes of diarrhoea in suckling piglets worldwide and it is also widely distributed in Switzerland [1,2]. It is associated with big economic losses due to high morbidity, slower growth rates, reduced post-weaning performance and high costs of prophylaxis and treatment [3–5]. Piglets get infected after ingestion of sporulated oocysts from the contaminated environment. The parasite multiplies in the enterocytes of the small intestine, producing catarrhal to fibrinonecrotic enteritis associated with shortening and fusion of intestinal villi, non-haemorrhagic diarrhoea and decreased weight gain. Interestingly, while pigs of all age groups may excrete oocysts after infection, usually only suckling piglets during the first weeks of age develop the typical intestinal lesions and signs of disease [5,6]. It was hypothesized that this could be due to a functional immaturity of the immune system of the piglets during the first weeks of age [7]. Studies in gnotobiotic piglets suggested that *C. suis* is a primary pathogen [8,9], however, co-infections with bacterial (e.g. *E. coli*, *Clostridium*) or viral (e.g. Rota-, Coronavirus) agents may be associated with more severe clinical signs and higher mortality rates [10,11].

Porcine cytomegalovirus (PCMV, *Suid herpesvirus 2*, SuHV2) is a herpesvirus (Family *Herpesviridae*) belonging to the subfamily *Betaherpesvirinae*[12], extensively prevalent in pig populations worldwide. Infection with PCMV causes inclusion body rhinitis. This denomination was based on the typical basophilic intranuclear inclusion bodies (8–12 μm) that can be observed at the histopathological examination in cells of the nasal mucosa of infected pigs with rhinitis, associated to an enlargement of the infected cells (cytomegaly) and

* Corresponding author at: Institute of Parasitology, Vetsuisse-Faculty, University of Bern, Länggassstrasse 122, CH-3012 Bern, Switzerland.
E-mail address: walter.basso@vetsuisse.unibe.ch (W. Basso).

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their nuclei (karyomegaly), but inclusion bodies can be also present in other tissues [13,14]. PCMV is shed with nasal and ocular secretions, urine, and cervical fluids and is transmitted by the oronasal route and congenitally [15]. PCMV causes acute to subacute disease characterized by fever, reduced general condition, apathy, anorexy, respiratory (sneezing, coughing, dyspnoea), neurological signs and death in suckling piglets. The morbidity can reach 100% and the mortality is higher in very young piglets and in piglets receiving few or no maternal antibodies. In weaned and older pigs, the infection is usually subclinical to mild and self-limiting if uncomplicated, but it can lead to reproductive failure in pregnant sows [13–15]. A low level immunity against PCMV in the herd (e.g. naïve herds) could predispose to an outbreak and more severe clinical outcome [14,16].

2. Materials and methods

In a Swiss pig herd (n = 2 boars, 7 sows, 2 gilts, 18 finishing pigs, 30 fattening pigs and 54 suckling piglets: 15 born shortly before and 39 during the outbreak), a disease characterized by fever (up to 41 °C), anorexia and reduced general condition with very high morbidity was first detected in finishing pigs (weight ~ 100 kg), extending to all age categories within few days. The pigs were housed in indoor pens with straw bedding, access to an outdoor yard with concrete floor and very good hygiene (i.e., thorough disinfection of the pen before admission of new animals; daily removal of faeces and wet straw; daily washing of the outdoor paddock). Initially, four days after observing the first clinical signs, nasal swabs were taken from five finishing pigs with reduced general condition and analysed for Swine Influenza Virus (SIV) by real time PCR (ADIAVET SIV REAL TIME, ADIAGENE, Biomérieux, Saint-Brieuc, France), with negative results.

Three to four days after the first signs of fever, apathy and loss of appetite appeared in fattening pigs, five of the animals (aged 17–18 weeks) began to show severe diarrhoea and faecal samples were collected for parasitological analysis. One of the fattening pigs (breed: Large White × Swiss Landrace cross; age: 17 weeks; weight: 32 kg; length: 84 cm) that was presenting diarrhoea for two days was apathetic, dehydrated, had elevated rectal temperature (39.7 °C), pale mucous membranes, cold ears and sunken flanks. The animal was euthanized due to poor condition. At euthanasia, blood samples for routine haematological and biochemical analyses were collected. A standard necropsy was performed and samples from several organs (i.e. heart, lungs, kidneys, spleen, liver, brain, small and large intestines, inguinal and mediastinal lymph nodes and tonsils) were collected, fixed in 10% buffered-formalin, embedded in paraffin, cut at 5 µm sections and microscopically examined after staining with hematoxylin and eosin (H & E). Faeces from the euthanized pig and from further fatteners with diarrhoea were processed for coproscopy by both a combined sedimentation/flotation technique using zinc chloride solution (specific gravity 1.45) for detection of coccidian oocysts and helminth eggs [6] and by the SAFC technique, a sedimentation method using a sodium acetate - acetic acid - formalin solution, and diethyl-ether for fat extraction to detect vegetative and cystic stages of further protozoa (i.e. *Giardia duodenalis*, *Balantidium coli* and amoebae) [6]. Faecal samples collected at necropsy were also tested for Rotavirus A and porcine Coronaviruses (Transmissible Gastroenteritis Virus [TGEV] and Porcine Epidemic Diarrhoea [PED]) antigens by immunochromatography using commercial kits (FASTest® ROTA Strip Megacor, Hörbranz, Austria and Anigen Rapid TGE/PED Ag Test kit BioNote, Gyeonggi-do, Korea, respectively). Additionally, a bacteriological investigation mainly directed to detect enterotoxigenic *Escherichia coli* (ETEC) was performed as described by Schubnell et al. [2]. Ileum sections were examined histopathologically for *Lawsonia intracellularis* infection using the Warthin-Starry silver nitrate-based stain, and tonsils, mediastinal and mesenterial lymph nodes sections were analysed by immunohistochemistry for Porcine Circovirus Type 2 (PCV-2) infection using the monoclonal antibody F217 [17].

Liver sections with suggestive lesions were analysed for herpesvirus detection by nested-PCR [18]. For DNA extraction, 200 µl of xylene were added to a tube containing three (20 µm) formalin-fixed, paraffin-embedded (FFPE) liver sections. After vortexing for 1 min and centrifugation at 13,000 rpm for 5 min the supernatant was discarded. The samples were washed with 200 µl of absolute ethanol and after further centrifugation the supernatant was discarded. This step was repeated twice. The tubes were incubated open at 37 °C for 15 min to allow ethanol evaporation. Afterwards, the DNA extraction was performed with the QiAamp DNA Mini Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's instructions. The samples were tested by a nested-PCR with primers targeting highly conserved regions of the herpesviral DNA polymerase gene ("Panherpes PCR") [18]. The first PCR-round included three degenerate primers (DFA, ILK and KG1) in a multiplex format and the second PCR-round two degenerate primers (TGV and IYG) [18]. The 25 µl reaction mixtures included 1 µM of each primer, 2 U of Taq DNA Polymerase, 200 µM of each dNTP and 2.5 µl 10 × PCR Buffer containing 1.5 mM of $MgCl_2$. Five microliters of extracted DNA served as template for the first PCR round, 1 µl product was used for the second PCR. Cycling and analysis was performed as previously described [19]. Positive products were further sequenced using specific primers (TGVseq and IYGseq) [18] by an external laboratory (Microsynth, Switzerland). The obtained sequences were analysed by a BLAST search (NCBI BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

Coproscopically, a moderate amount of *Cystoisospora suis* oocysts and *Balantidium coli* cysts were detected in the faeces from the 17–18 week-old fattening pigs with diarrhoea. Blood analysis from the euthanized fattening pig showed neutrophilic leucocytosis with left shift and lymphopenia, increased serum levels of urea (14.8 mmol/l; normal: 1.9–6.5 mmol/l) creatinine (258 mmol/l; normal: 99–164 mmol/l), ASAT (GOT) (94 U/l; normal: 23–50 U/l) and GLDH (2.7 U/l; normal 0.3–2.1 U/l) and hypoalbuminemia (16.2 g/l; normal: 23–42 g/l; albumin/globulin 0.51; normal: 0.68–1.4). At necropsy, pale mucous membranes and subcutaneous tissues and a moderate body condition were noticed. Histologically, a severe acute fibrinopurulent jejunoileitis with extensive loss of intestinal villi, loss of goblet cells and crypt abscesses were observed and in some zones, fusion of intestinal villi was present (Fig. 1A). Abundant *C. suis* stages (mainly typical merozoite pairs within enterocytes) were associated with these severe lesions (Fig. 1B–C). In the liver, intranuclear inclusion bodies typical for a herpesvirus infection, moderate leukocyte stasis and a mixed-cell infiltration were detected (Fig. 1D). Additionally, a peracute neutrophilic interstitial pneumonia and leukocyte stasis were observed in lung sections. The lesions in lung and liver suggested the beginning of a septic process. The remaining examined organs did not show any histopathological alterations. Performed analyses for PCV-2, Rotavirus A, TGEV, PED and *L. intracellularis* detection yielded negative results. Bacteriological studies revealed growth of *E. coli* (non-haemolytic F4 and F5 negative), which was considered irrelevant. FFPE liver sections were tested for herpesvirus by nested-PCR and subsequent sequencing, which revealed 100% identity with PCMV GenBank sequences.

4. Discussion

Co-infection with *C. suis* and PCMV was diagnosed in a fattening pig with reduced body condition and severe diarrhoea by histopathological examination and molecular methods. Although many merozoites were identified in jejunum and ileum sections, no *C. suis* oocysts or further parasites could be detected in faecal samples from this animal by coproscopy. Histologically, mostly merozoite pairs typical for *C. suis* (and not for *Eimeria* spp.) [6], but no multinucleate meronts or oocysts were identified, suggesting a recent infection during the prepatent period. In