First report of variant pseudorabies virus infection in goats in China: a neglected infectious disease to ruminants

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Abstract

Pseudorabies virus (PRV), the cause agent of Aujeszky's disease, is an infectious pathogen which greatly affects the heathy development of pig industry worldwide. The low specific host tropism of PRV allows this virus to infect a variety of animals, such as pigs, cattle, minks, dogs, and even possible humans. However, the occurrence of PRV natural infection in goats has never been documented. Herein we provided robust evidences demonstrating the first case of a variant PRV infection leading to the acute goat death in Yunnan Province, China, which might be resulted from mixed feeding with PRV-infected fattening pigs. Therefore, this report not only highlights the potential threat of newly emerging variant PRV strain(s) to goat industry, but also appeals the development of effective and safe vaccines against PRV variants for goats/ruminants in future.

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Running title: Pseudorabies virus infection in goats

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Summary

Pseudorabies virus (PRV), the cause agent of Aujeszky's disease, is an infectious pathogen which greatly affects the heathy development of pig industry worldwide. The low specific host tropism of PRV allows this virus to infect a variety of animals, such as pigs, cattle, minks, dogs, and even possible humans. However, the occurrence of PRV natural infection in goats has never been documented. Herein we provided robust evidences demonstrating the first case of a variant PRV infection leading to the acute goat death in Yunnan Province, China, which might be resulted from mixed feeding with PRV-infected fattening pigs. Therefore, this report not only highlights the potential threat of newly emerging variant PRV strain(s) to goat industry, but also appeals the development of effective and safe vaccines against PRV variants for goats/ruminants in future.

Keywords: pseudorabies virus variant, goat, natural infection, China

Introduction

Pseudorabies virus (PRV), also called as Suid alphaherpesvirus 1 that belongs to the genus *Varicellovirus* of the sufamily*Alphaherpesviridae* , within the family *Herpesviridae* , is an enveloped virus with a length of nearly 143 kb linear double-stranded DNA genome encoding more than 72 genes (Sun et al., 2016; Szpara et al., 2011). The unique natural and reservoir host for PRV are pigs (including wild boars), however the first suspected infection of this pathogen was recorded in American cattle with clinical signs characterized by intense itching in 1813 (Laval & Enquist, 2020). Since the initial isolation of PRV in 1902, the presence of this pathogen was widely documented almost in all pig raising countries, indicating that PRV has quickly swept across the world and posed a huge threat to various animal species, especially to the development of pig industry (Lee & Wilson, 1979).

Pseudorabies (PR) is a highly contagious disease that causes severe clinical symptoms in the infected pigs, thereby leading high morbidity and mortality especially to the sucking piglets. The infected piglets usually present clinical signs characterized by diarrhea, vomiting, nervous system disorders (such as tremor, dyskinesia, and lethargy) (Sun et al., 2016). Vaccination is regarded as the most effective approach for the eradication of PR. Though the infectious disease has successfully been eradicated in some developed countries via the wide application of virulent gene-deleted live vaccines, PRV remains a fatal pathogen circulating in Chinese pig population (Freuling, Müller, & Mettenleiter, 2017; Sun et al., 2018). In this regard, at least two great PR outbreaks have been witnessed in China, mainly resulting from PRV classical strains prevalent in the 1990s and variant strains circulating after 2011, respectively.

PRV has a broad species tropism, which facilitates it to infect a variety of mammals, including carnivores (Jin, Gao, Liu, Zhang, & Hu, 2016; H. Liu et al., 2017; Serena et al., 2018), ruminants (Cheng et al., 2020), and rodents. Even a case of human encephalitis caused by a PRV variant strain has been reported recently (Q. Liu et al., 2020). However, naturally occurring PRV infection in goats has not been reported yet. In current study, we provided the first case of a variant PRV infection causing the acute goat death in Yunnan province, China. Meanwhile, the genetic characteristics of this PRV strain YNG were characterized in detail.

Materials and methods

2.1 Clinical symptoms and specimen collection

A total of 136 black goats showing clinical signs of possible PRV infection were included in this study. The clinical symptoms in these goats were carefully observed and characterized. Splanchnic (including lymphonodus, lung, kidney, and liver) and brain tissues from the dead goats were collected for pathogenic diagnosis by polymerase chain reaction (PCR) or reverse transcriptase-coupled polymerase chain reaction (RT-PCR), and virus isolation. Meanwhile, 10 serum and 16 fecal samples from fattening pigs in this farm were collected for the detection of antibodies and pathogens, respectively. Additionally, related information including feeding and housing of these goats was surveyed and documented.

2.2 Identification of PRV in collected specimens

The homogenates of each tissue specimen from goats or fecal samples from pigs living nearby were mixed

with sterile phosphate-buffered saline (PBS) and undergone repeated freezing and thawing cycles, then centrifuged at 12000×g for 10 min. The viral nucleic acids in the supernatants were extracted using a commercial DNA/RNA extraction kit (Takara, Dalian, China), and stored at -80 .

Prepared DNA samples were used for detecting the presence of PRV by PCR method with a pair of primers listed in **Tab. 1** , PCR reaction (25 μL) mixture included 12.5 μL of 2×Taq Plus PCR Master mix (Takara, Dalian, China), 1 μL of each primer (10 pmol), 4 μL of DNA template, and 6.5 μL of DPEC treated water, the cycling parameters were performed as described previously (Tan et al., 2020). Prepared RNA samples were exploited to detect Rabies virus (RV) and Caprine arthritis encephalitis virus (GAEV), respectively, as previously established methods (Li et al., 2013). PCR products were visualized in 1% agarose gel electrophoresis.

2.3 Virus isolation

Cerebrum specimens from the dead goat were handled as describe above, the supernatants were filtrated through a 0.22 um filter (Millipore, USA) and incubated with monolayer PK15 cells. The cells were cultured in Dulbecco modified eagle medium (DMEM) supplied with 5% newborn bovine serum (NBCS) and 1% streptomycin/penicillin. After one-hour incubation, the supernatants were removed and replaced with maintenance medium (DMEM with 2% NBCS and 1% streptomycin/penicillin). When obvious cytopathic effects (CPEs) were observed, the cells and supernatants were harvested. After three cycles of thawing and refreezing, PCR was performed to detect the presence of PRV nucleic acid in the supernatants. Isolated and identified PRV virus was designated as YNG strain.

2.4 Indirect immunofluorescence assay (IFA)

PK15 cells infected with YNG strain were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 for 10 min at room temperature, respectively. Then the cells were blocked with 3% bovine serum albumin (BSA) for 1h, and probed with mouse monoclonal antibodies against the *gB* and *gE* proteins of PRV (1:1000) for 4 h. Following the removal of the supernatants, the cells were washed with PBS three times, the second antibodies (goat anti-mouse-FITC) (1:2500) were applied and incubated for 45 min in dark environment. The images were taken using a fluorescent microscope (Olympus, Tokyo, Japan).

2.5 One-step growth curve experiment

To investigate the growth characteristics of YNG strain in vitro, monolayer Vero and PK15 cells seeded in 24-well plates were incubated with different strains (YNG and YNP, an isolation from pig farms where was close to this PRV-outbreaking goat farm) at multiplicity of infection (MOI) of 0.01 for 1 h. Both the cell debris and supernatant were collected after repeated freezing and thawing cycles, the viral copies and titers were determined via real-time PCR after the DNA extraction and $TCID_{50}$ assay, respectively.

2.6 Sequence alignment and phylogenetic analysis

To analyze the genetic characteristics of PRV strain isolated from goat in the present study, the major viral immune (*gB* , *gC* , and*gD*) and virulence genes (*gE* and *TK*) of YNG strain were amplified by PCR with specific primers (**Tab. 1**). Positive PCR products were purified, sequenced by Tsingke company (Changsha, China), these sequences were submitted to the Genbank database. These newly sequences, together with their corresponding reference sequences downloaded from GenBank database, were aligned using DNAStar version 7.10 (Lasergene) software for comparison. Phylogenetic trees were also re-constructed using the neighbor-joining (NJ) method in MEGA 7.0 software with 1000 bootstrap replicates based on the *gC* and*gE* gene, respectively.

2.7 Enzyme-linked immunosorbent assay (ELISA)

16 serum specimens from pigs were collected to examine the presence of*gE* antibodies using a commercial ELISA kit (IDEXX Laboratories, USA).

2.8 Experimental infection of mice

Twenty-four 5-week-old female Kunming mice purchased from Hunan SJA laboratory animal CO., LTD were randomly divided into four groups, six mice in each group were challenged with 10^4TCID$_{50}$ of various PRV strains (YNG, YNP, and another classical PRV strain genetically close to Ea strain, HNLY) via hind footpad injection, respectively, while the mice in the control group were inoculated with equal volume of DMEM. These mice in distinct groups were maintained in the same environment, and their health conditions were monitored three times per day. The survival curve of mice in each group was produced by using the GraphPad Prism 8.0 software (GraphPad software, La Jolla, CA, USA).

2.9 Statistical analysis

All experiments received more than three replicates, the statistical significances of data obtained from different groups were analyzed by GraphPad Prism version 8.0 (GraphPad software, La Jolla, CA, USA), and ns represents $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, respectively.

Results

3.1 Detection of the causative agent(s) causing the death of goats

In November 2015, most of black goats showing clinical signs of possible PRV infection were reported in a farm in Yunnan, China. Subsequently, a detailed survey was conducted, the symptoms in these goats included high fever, appetite loss, skin itch and fidgety with no ages and sexes limitation (**Fig. 1A-B**). Additionally, some of the ill goats displayed severe diarrhea (**Fig. 1C**), and all of these goats died eventually. The pathological examination showed the occurrence of pneumonia hemorrhage, lymphadenopathy syndrome with hemorrhage, brain hyperemia and hemorrhage in dead goats (**Fig. 1D**).

Further investigation revealed that these goats were fed together with fatted pigs only by the separation of a wall, while all pigs in this farm had been immunized with the PRV attenuated live vaccine (Bartha-K61 strain) at the development stage of piglets. Furthermore, all animals were fed by the same breeder.

To explore the possible causative agent(s) leading to the death of goats in this case, the viral nucleic acids were extracted from brain tissues of the dead goats, PCR and RT-PCR methods were performed to detect the presence of pathogens such as PRV, RV, and GEAV. Interestingly, the specimens presented PRV positive while RV and GEAV negative. Furthermore, the treatment of these diseased goats with antibacterial drugs, such as Cefatriaxone and Sulfadiazine, had poor therapeutic effect. Meanwhile, 10 serum specimens from the fattening pigs raised near the goats were positive for PRV *gE* antibody, while only 1 of 16 fecal samples from these pigs were tested positive for PRV by PCR method (data not shown). Thus, we concluded that PRV infection was the leading cause resulting in the death of goats.

3.2 Isolation and identification of PRV strain from the dead goat

To characterize the features of PRV strain from goat, the supernatants of cerebrum specimen from the dead goat were incubated into monolayer PK15 cells for 1 h, and the cells were then harvested upon observing the appearance of over 80% CPEs. Obvious CPEs could be observed less than 16 h post-infection (**Fig. 2A**), furthermore, the presence of PRV nucleic acids (namely, PRV-positive) was confirmed by PCR in the supernatants of the first, second, third, fifth, and seventh passages of cells incubated with YNG strain (**Fig. 2B**). Additionally, the infectious virions of YNG and YNP strains in Vero cells were specially recognized by mouse monoclonal antibodies against the *gB* and*gE* proteins of PRV via IFA (**Fig. 2C**).

3.3 Growth characteristics of YNG strain comparing with other variant strains

One-step growth curve experiment, CPE observation, and TCID50 assay were conducted to analyze the growth characteristics of these PRV strains aforementioned in PK15 and Vero cells, with YNG strain as the control. The viral copies were determined according to the standard curve: lg [virus copies] = -0.2293x+9.832, r^2=0.9963. As shown in **Fig. 3** , YNG strain exhibited a similar growth curve as that of YNP strain both in PK15 and Vero cells. The virus titers of YNG and YNP strains in PK15 and Vero cells at the 36 hpi were $10^{7.14}$, $10^{6.88}$ and $10^{6.80}$, $10^{6.75}$TCID$_{50}$/mL, respectively (**Fig. 3**). Likewise, there was no obvious difference

of CPEs in PK15 and Vero cells triggered by distinct PRV strains, suggesting that YNG strain should be a PRV variant.

3.4 Analysis of sequence variation in the newly goat PRV strain

To further address the genetic characteristics of YNG strain, the major viral immune (*gB* , *gC* , and *gD*) and virulence gene (*gE* and *TK*) sequences were successfully amplified and sequenced. The findings revealed that the lengths of *gB* ,*gC* , *gD* , *gE* and *TK* genes of YNG strain were 2745 bp, 1464 bp, 1209 bp, 1737 bp, and 1023 bp, respectively (GenBank accession numbers: MW458931, MW392295, MW458932, MW458933, MW458934). Sequence analysis results also showed that there existed continuous nucleotides insertion encoding^{65}AASTPAA^{71} in the *gC* gene in comparison to referring PRV strains in genotype I, such as Bartha and Becker strains (**Fig. 4A**). Similarly, a 6-nucleotide deletion (encoding ^{278}SP^{279}) in the *gD*gene was identified in YNG strain relative to classical PRV strains (eg. Fa and Ea) prevalent in Chinese pig population before 2011, indicating that YNG strain is a PRV variant (**Fig. 4B**). Compared with other referring variants, only few amino acid variations in *gB* gene of YNG strain were identified, while no amino acid variations of *TK* ,*gC* , and *gD* genes were found. Notably, compared to other PRV strains, a 3-nt deletion (encoding D at the position of 490) was found in the *gE* gene of YNG strain and another variant PRV strain (JX/CH/2016, Genbank number accession: MK806387) isolated in Guangdong province, China (**Fig. 4C**).

To define the evolutionary position of YNG strain among numerous referring strains from different regions or hosts, *gC* and*gE* gene based phylogenetic trees were constructed, respectively (**Fig. 4D&E**). The results demonstrated that all PRV strains prevalent in the world could be divided into two genotypes (genotype I and II), and the genotype II was composed of classical PRV strains prevalent before 2011 and variant PRV strains circulated after 2011 in China. Notably, most of PRV strains isolated from different hosts (pigs, dogs, wolves, sheep, foxes, human, etc.) prevalent in China, including YNG strain, belonging to the genotype II, presented a random distribution. Additionally, the phylogenetic position of YNG strain was closer to these of variant PRV strains than classical PRV strains, and far away from the traditional vaccine strains (such as Bartha) belonging to the genotype I.

3.5 The pathogenicity of YNG strain in vivo

In order to evaluate the pathogenicity of YNG strain in vivo, Kunming mice (six-week-old) were chosen as animal models and inoculated with 0.1 mL of 10^5 $TCID_{50}$/mL of YNG, YNP, or HNLY strain via intramuscular injection at the hind leg, respectively. The clinical signs and death time of the mice in different groups were carefully observed. The results revealed that all of the mice challenged with YNG or YNP strain displayed severe itching before 42 hpi, and died before 72 hpi, while the mice inoculated with HNLY strain only exhibited slightly clinical signs before 72 hpi (**Fig. 5A**). Additionally, the brains of mice infected with YNG and YNP strains showed similar pathological changes, such as severe hemorrhage, while those challenged with HNLY strain only presented slight hemorrhage lesion when compared with the control group (**Fig. 5B**). Accordingly, higher viral copies in the cerebrum, kidney, and spleen of YNG or YNP strain-infected mice were found, relative to those in HNLY strain-infected mice (**Fig. 5C**), indicating a similar pathogenicity of YNG strain as PRV variant to mice.

DiscussionThough extensive efforts have been devoted to the eradication of the disease caused by this pathogen, PRV remains pandemic in some countries/regions, mainly in Asia and Europe (Q. Liu et al., 2020). For instance, the latest researches revealed that the average positive rate of PRV nucleic acid among 16256 tissue specimens collected from the mainland China between 2012 and 2017 was 8.3% (Sun et al., 2018), while the sero and molecular prevalence rates of wild PRV in pigs in Shandong province of China from 2015 to 2018 even reached to 52.7% (8667/16457), 15.7% (257/1638), respectively (Ma et al., 2020), indicating the still severe status of wild PRV in Chinese pig populations. The current study reported the first case of natural PRV infection in goats, thereby further expanding the list of non-natural hosts challenged by this pathogen. Thus, our new finding, together with previously confirmed species including humans and its lethality to these susceptible animals (Ai et al., 2018; Cheng et al., 2020; Jin et al., 2016; H. Liu et al., 2017)

and multiple infection ways (Q. Liu et al., 2020), collectively suggest that PRV may be a zoonotic pathogen (Ai et al., 2018; Yang et al., 2019) and more attention should be paid to it in the future.

Large numbers of ruminants raised in China, severe prevalence of PRV in Chinese pig population and frequent transportation of various animals (pigs, goats/sheep, cattle, etc.,) among different regions greatly enhance the possibility of the contact between pigs and these PRV susceptible animals, thereby leading to the transmission of PRV from host pigs to non-host animals (Tan et al., 2021). Consistent with this, our study for the first time confirmed the PRV transmission from pigs to goats, mainly due to the close contact of raising places for those two species. This conclusion was stemmed from a series of evidences: a) serum or fecal specimens from pigs raised nearby presented PRV antibody or nucleic acid positive; b) PRV viruses were confirmed and isolated from infected or died goats; c) isolated YNG strain exhibited highly genetic sequence homology with PRV variant strain YNP, moreover, its pathogenicity was successfully reproduced in mice as its counterpart YNP. These highlight the really threat of PRV to goats or even ruminants, as partially supported by a previous report (Kong et al., 2013). As a precaution measure, far physical isolation of raising places for different animal species is therefore suggested.

Genetically, PRV strains prevalent in the world are divided into two genotypes, most of PRV strains in China belong to the genotype II (He et al., 2019), while the latter one can be further classified into two sub-genotypes (PRV classical and variant strains). Furthermore, PRV variant strains emerged since 2011 (e.g. JS-2012, HN1201, hSD-1/2019) in China showed higher pathogenicity to animal models than classical strains (i.e. Ea and Fa) (Luo et al., 2014; Tong et al., 2015). More direct evidences substantiating the conclusion were that vaccination with PRV Bartha-K61 strain could prevent the occurrence of classical PRV infection but fail to provide complete protection against the newly emerging PRV variants (8, 9). Importantly, the attenuated live vaccine (Bartha-K61 strain) instead led to high pathogenicity to sheep (Kong et al., 2013). Likewise, immunization with Bartha-K61 strain or HB98 strain (another PRV attenuated live vaccine designed based on Ea strain) caused histopathological lesions in dogs (Lin et al., 2019; Willemse, Rondhuis, Goedegebuure, & Maas, 1977). It is worth noting that the recombinant PRV variant strain with US7&US8&UL23 gene deletion is highly lethal to dogs, while the US7&US8&UL23&US3 gene-deleted recombinant PRV strain is safe to this species. Of course, whether this genetically modified PRV strain can be developed for vaccine candidates against variant PRV infection in dogs needs further investigations (Yin et al., 2020). Taken together, these facts described above suggest that currently commercial vaccines might be not suitable for the eradication of PR in ruminants (eg. sheep/goats and cattle), accordingly, safer vaccines with efficient protection should be developed for this purpose in the future.

In summary, we reported the first case of the occurrence of PR in goats and confirmed that the goat-originated PRV strain was a Chinese variant strain by genetic sequence analysis. These suggest that variant PRV strains should receive sufficient attention in terms of the healthy development of goat industry in China. Considering the confirmed PRV infection to humans (Q. Liu et al., 2020), the potential transmission of PRV from goats to humans cannot be ruled out.

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Conflict of interests

The authors declare that they have no competing interests.

Ethical approval

All animal experiments were conducted according to the rules of Animal Ethics Committee of the Hunan Agricultural University, Changsha, China (43321503), and the mice were housing in the animal facility of

Hunan Agricultural University (Changsha, Hunan, China).

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Figure captions

Figure 1 :Typical clinical signs and pathological changes were observed in PRV-infected goats, such as skin itch and fidgety (**A&B**), diarrhea (**C**). Also, severe hyperemia and hemorrhage were shown on the cerebrum from the infected goat (**D**).

Figure 2 : Isolation and identification of YNG strain from goat. (**A**) Obvious the cytopathic effects (CPE) of PK15 cells induced by the infection of the third passages of YNG strain, the CPE of cells in the left were characterized by being rounded and floated. The right cells were set as negative group, Scale bar = 200 um. (**B**) The existence of infectious PRV virions in the cell cultures of the first, second, third, fifth, and seventh passages were confirmed via PCR, which could yielded the expected DNA bands (˜450 bp) of PRV gE in 1% agarose gel electrophoresis. DNA+ and DNA– were regarded as positive and negative controls during DNA extraction, respectively. And PCR- was the negative control during PCR amplification. (**C**) Indirect immunofluorescent assay (IFA) for the detection of PRV in Vero cells using anti-PRV gE and gB primary antibodies. Scale bar = 100 um

Figure 3 : The proliferation characteristics of YNG strains in vitro. (**A**) One-step growth curves of YNG and YNP strains on PK15 and Vero cells at MOI of 0.01, the total PRV copies from the supernatant and cells in triplicates were measured via real-time PCR. (**B**) The viral titers of YNG and YNP at 36 hpi were determined in PK15 cells by $TCID_{50}$.

Figure 4: Analysis of sequence variation in YNG strain. (**A**) A 7-aa insertion (^{64}AASTPAA^{70}) in the gC gene of PRV strains in genotype II compared with that in genotype I. (**B**) A 2-aa deletion (^{278}SP^{279}) in the gD gene of PRV variants in genotype II and Becker strain compared with that of classical PRV strains in genotype II. (**C**) A 3-nt deletion (^{1469}ACG^{1471}) encoding D at the position of 490 in the *gE* gene of YNG strain and another variant PRV strain prevalent in China (GenBank no. MK806387 (JX/CH/2016)

compared with that of the other strains. (**D&E**) Phylogenetic trees based on *gC* and *gE* gene sequences of YNG strain and other reference strains generated by the neighbor-joining method in MEGA 7.0 software. The red and black prismatic represented YNG strain obtained in this study and PRV strain isolated from human, respectively.

Figure 5 : The pathogenicity of YNG strain in mouse model. (**A**) Survival curve (n=6/group) in Kunming mice after intramuscular injection with different PRV strains (10^4 $TCID_{50}$/mouse). (**B**) Pathological changes of cerebrum from mouse in different groups. (**C**) The viral copies in different organs from mice infected with different PRV strains were determined according to the standard curve constructed by the threshold cycle (CQ) values of the serial 10-fold dilution of standard plasmid with known copies via real-time PCR method. Values are means+-standard deviation (SD) = 3, significant differences were determined when P>0.05 (ns), P<0.05(*), P<0.01(**), and P<0.001(***).

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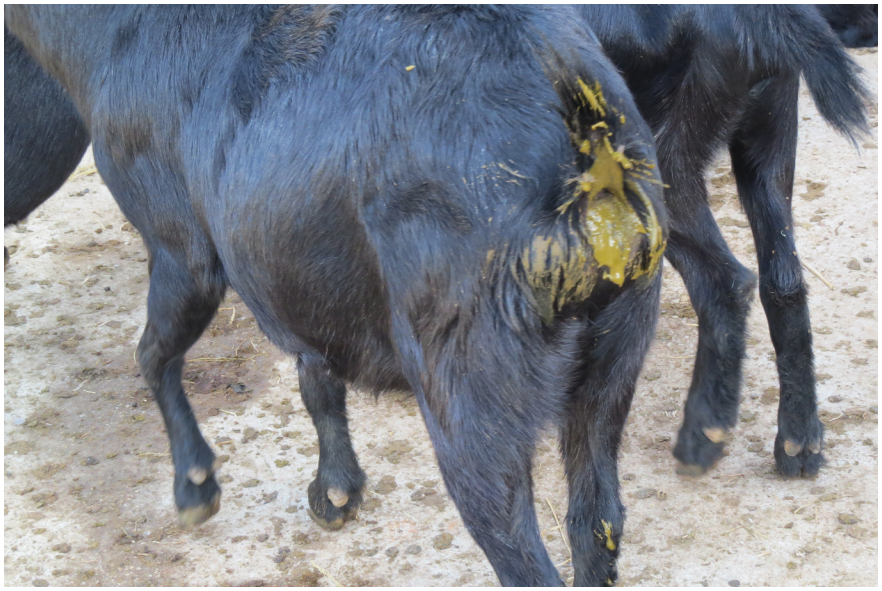

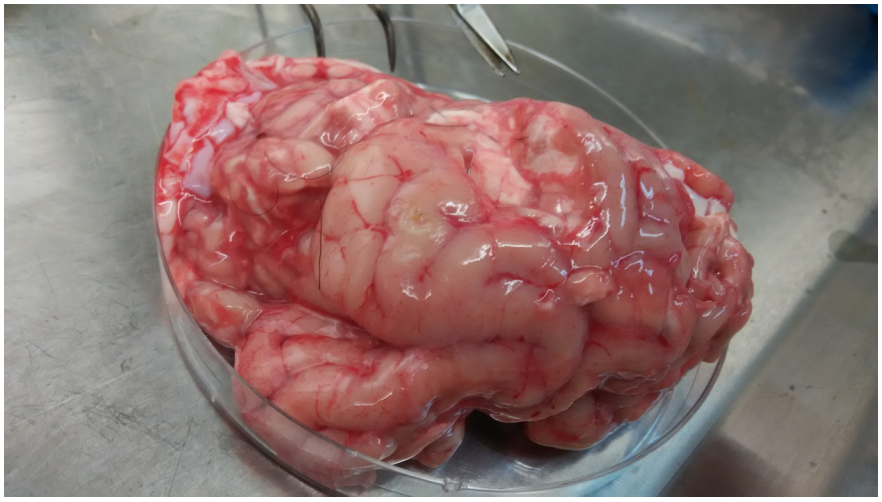

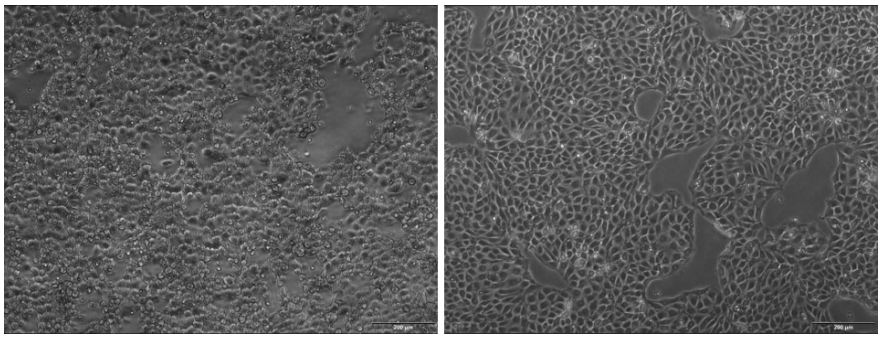

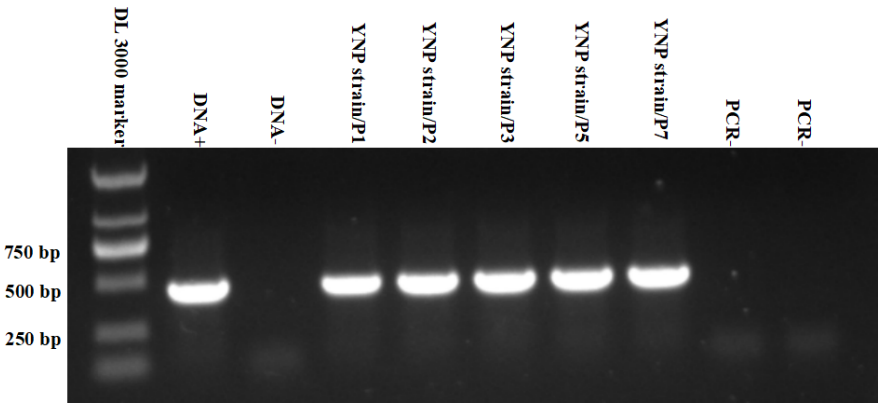

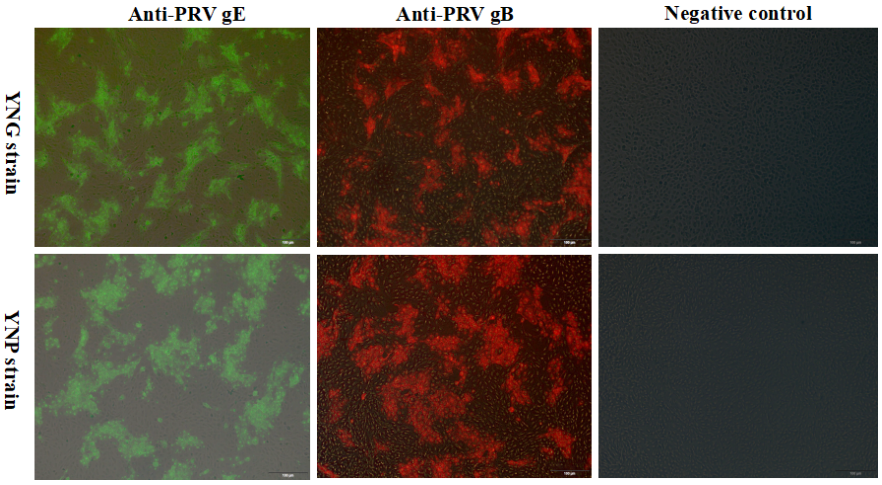

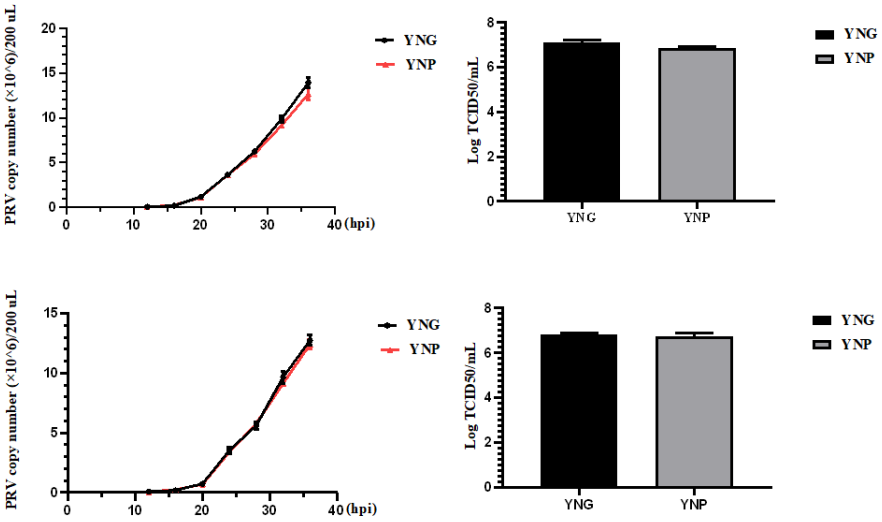


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