SCREENING FOR ENTERIC CORONAVIRUSES IN FECAL SAMPLES OF
FERAL PIGS OF CALIFORNIA, USA

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in
the Graduate School of The Ohio State University

By
Shristi Ghimire
Graduate Program in Comparative and Veterinary Medicine

The Ohio State University
2017

Master's Examination Committee:
Dr. Qiuhong Wang, Advisor
Dr. Renukaradhy J. Gourapura
Dr. Anastasia Vlasova
Abstract

Enteric coronaviruses (CoVs) of pigs include transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV) and porcine delta coronavirus (PDCoV). They cause significant economic losses in pig industry worldwide. In the US, PEDV emerged in 2013 and caused the death of over 7 million piglets within the first year. Feral pigs have contributed to the transmission of different viral, bacterial and parasitic infections to domestic pigs, other domestic animals and even to humans. However, their role in maintenance and transmission of porcine enteric CoVs is not well understood.

In this study, fecal samples of 44 feral pigs were collected from California, USA in early-mid 2016. RNA was extracted from the fecal content supernatant and subjected to conventional reverse transcription (RT)-PCR using two different panCoV primers to detect the presence or absence of porcine enteric CoVs. Quantitative RT-PCR (RT-qPCR) using PEDV N gene and PDCoV M gene specific primers and probes were also performed for amplification of specific viral genes. DNA sequencing of PCR products from likely positive sample was also performed for further confirmation.

None of the samples were clearly positive using conventional RT-PCR assays. Few samples were doubtful for PEDV or PDCoV genes and showed Ct values slightly above the detection limit. Repeated testing and/or DNA sequencing confirmed that those samples were negative for PEDV or PDCoV presence. Our results indicated that no
porcine enteric CoVs were circulating in the feral pigs in California in early-mid 2016. Similar studies from other states of USA with larger sample sizes will be helpful to ascertain whether wild pigs in USA are playing a role in the maintenance and transmission of porcine enteric CoV or not.
Acknowledgments

I would like to express my sincere gratitude to my honorable advisor Dr. Qiuhong Wang for her sustained encouragement, regular guidance, inspiration, valuable suggestions and meticulous supervision throughout the study period. My humble thanks go to my advisory committee members Dr. Renukardhya Gourapura and Dr. Anastasia Vlasova for their valuable suggestions and constructive criticisms. I am lucky to have very helpful lab members Chun-Ming Lin, Yixuan Hou, Xiaohong Wang, Patricia Boley, Xiang Gao and Susan Sommer-Wagner who are always ready to help each other. I am also grateful to Kathy Froilan, graduate program coordinator of Comparative and Veterinary Medicine and all the members of Food Animal Health Research Program who have made my each day easier. I owe my gratitude to my parents Dr. Narayan Pd. Ghimire and Niru Ghimire and my parents-in-law Bal Krishna Dhakal and Dambar Kumari Dhakal, for their continuous inspiration and blessings. I am always indebted to my sisters Shanta Pokharel, Dr. Pratikshya Ghimire, Dr. Srijana Dhakal and Dr. Sima Ghimire whose soothing words provided me courage to move each step ahead and my husband Santosh Dhakal for always supporting and encouraging me. I am also thankful to my friends from Nepal Mahesh KC, Dipak Kathayat, Nabaraj Baral and Pratikshya Neupane who have made my stay in the US memorable. At last I would like to thank everyone who has directly or indirectly helped me during my stay in the United States.
Vita

2007-2013................................. B. V. Sc. & A. H. Purbanchal University, Nepal
2013-2014................................. Veterinary Officer, National Zoonoses and Food
                                          Hygiene Research Center, Kathmandu, Nepal
2015 to present.......................... Graduate Research Assistant, Food Animal Health
                                          Research Program, The Ohio State University

Publications

Hou, Y., Lin, C-M., Yokoyama, M., Yount, B., Marthaler, D., Douglas, A., Ghimire, S.,
protein and attenuated porcine epidemic diarrhea virus in piglets. (Manuscript under
review)

Nepal. (Manuscript under review)

opportunities in Nepal. Veterinary world, 8(1), 61-65.

Fields of Study

Major Field: Comparative and Veterinary Medicine

Studies in Virology and Immunology
Table of Contents

Abstract .............................................................................................................................................. ii

Acknowledgments ............................................................................................................................ iv

Vita ..................................................................................................................................................... v

List of Tables ....................................................................................................................................... ix

List of Figures ..................................................................................................................................... x

Chapter 1: Literature Review ......................................................................................................... 1

Coronaviruses ....................................................................................................................................... 1

Human coronaviruses (HCoVs) .......................................................................................................... 2

Bat coronaviruses ............................................................................................................................... 3

Porcine Epidemic Diarrhea Virus (PEDV) ......................................................................................... 4

History and epidemiology .................................................................................................................. 4

PEDV in USA ..................................................................................................................................... 6

Structure of PEDV .............................................................................................................................. 7

Transmission ....................................................................................................................................... 8

Pathogenesis ..................................................................................................................................... 8
List of Tables

Table 1: Fecal samples collected from wild pigs from California, USA………………… 39
Table 2: Preliminary results of PEDV RT-qPCR assay of fecal samples .................. 41
Table 3: Preliminary results of PDCoV RT-qPCR assay of fecal samples……………….. 42
List of Figures

Figure 1. PEDV: cumulative confirmed and presumptive positive premises since June 5, 2014 in USA................................................................. 6

Figure 2. Feral swine populations in the US in 2015................................. 23

Figure 3. Map of California showing sample collection sites......................... 43

Figure 4. Sequence alignment of the conserved polymerase regions targeted by the two primer sets................................................................. 44

Figure 5. Gel images showing results of conventional RT-PCR assay using panCoV primer set 1................................................................. 45

Figure 6. Gel images showing results of conventional RT-PCR assay using panCoV primer set 2................................................................. 46

Figure 7. Representative graphs showing fluorescent profiles during PEDV and PDCoV specific RT-qPCR assays............................................. 47

Figure 8. Gel images of samples subjected to conventional RT-PCR assay using PDCoV M gene specific primers and with increase reaction volume........ 48
Chapter 1: Literature Review

Coronaviruses

Coronaviruses (CoVs) are enveloped, positive sense, single stranded RNA (ssRNA) viruses belonging to the family Coronaviridae in the order Nidovirales. Coronaviridae family has two subfamilies - Coronavirinae and Torovirinae [1]. Coronavirinae subfamily has four genera, viz., Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus. Alpha and beta CoVs primarily infect mammals, gamma CoVs infect birds and whales, and delta CoVs infect mammals, terrestrial and aquatic birds [2, 3]. A coronavirus particle is pleomorphic with diameters ranging from 120-160 nm [1]. The name 'corona' refers to the 'crown'-like (corona = crown in Latin) appearance of spike protein under an electron microscope [4]. Coronaviruses mainly cause respiratory and/or enteric infections [5, 6]. Coronaviruses have the largest known RNA genomes (26.4-31.7 kb) [1]. The open reading frame (ORF) 1a and 1b at the 5' end encodes 16 non-structural proteins and structural proteins, spike (S) protein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein, and accessory genes are encoded by ORFs at the 3' end [7]. Infectious bronchitis virus, isolated in late 1930s, was the first coronavirus to be isolated while human coronavirus was isolated only in mid 1960s [8].
Human coronaviruses (HCoVs)

Coronaviruses cause respiratory and gastrointestinal tract infections in humans. Human infections can range from mild and self-limiting disease to severe manifestations like bronchitis and pneumonia with renal involvement [9]. Human coronaviruses 229E and HCoV OC43 were the first to be isolated. In addition to these two strains, severe acute respiratory syndrome coronavirus (SARS-CoV), HCoV NL63, HCoV HKU1 and Middle East respiratory syndrome coronavirus (MERS-CoV) are the other major strains of HCoVs [10]. SARS-CoV was responsible for the first epidemic of 21st century[11]. The outbreak started in November 2002 from Guangdong province of southern China. Within months, it spread to multiple countries of Asia, North America and Europe leading to more than 8000 infections and approximately 800 deaths [12]. MERS-CoV was isolated first from Saudi Arabia in 2012. It is endemic in countries around the Arabian Peninsula [7]. By the end of 2015, over 1600 people were infected with MERS-CoV with approximately 600 deaths [13]. HCoVs are likely to have a zoonotic origin. SARS-CoV is believed to be maintained by horseshoe bats and was transmitted to palm civets in live animal markets of China before human transmission began [14]. Likewise, dromedary camels are found to be the major source of MERS-CoV transmission to humans [15]. Infection with SARS-CoV produces influenza-like syndrome characterized by high fever, malaise, rigor, fatigue and respiratory problems including pneumonia. Patients shed virus from nasal discharges and also through watery diarrhea [16]. MERS-CoV infects respiratory tract, kidney, intestines and liver leading to symptoms like fever, cough, shortening of breath, pneumonia and acute renal failure. MERS-CoV has broader tissue
tropism than any other HCoVs which might explain the reason behind high case fatality rate (>35%) of this virus infection in humans [17].

**Bat coronaviruses**

Bats are highly diverse mammals with more than 1200 species found throughout the world [18]. Due to their high diversity and biological and ecological characteristics, bats can maintain and transmit numerous pathogens to humans, animals and birds. Horseshoe bats are the likely source for origin of SARS-CoV as they are the natural host of coronaviruses closely related to those responsible for SARS outbreak [19]. Recent studies also revealed that bats harbor diverse species of alpha and beta coronaviruses. Similarly, coronaviruses phylogenetically related to MERS-CoV have also been identified in wide range of bat species in different countries suggesting that MERS-CoV also has bat origin [20]. Diverse Alpha- and Beta-CoVs have now been identified in a variety of bats globally [2, 21-23]. Studies on ecology of bat coronaviruses have further indicated that bats are possibly the natural host for all presently known coronavirus lineages [24].

**Porcine coronaviruses**

Transmissible gastroenteritis virus (TGEV) recognized in 1946 is the major cause of viral gastroenteritis and fatal diarrhea in neonatal pigs. It was first recognized in the United States by Doyle and Hutchings (1946) [25]. Porcine respiratory coronavirus (PRCV) is an attenuated variant of TGEV that infects the respiratory epithelia of pigs and mainly induces subclinical infections. PRCV causes interstitial pneumonia in pigs [26]. TGEV
and PRCV have over 90% genomic similarity. The differences in tissue tropism between these two viruses is due to the deletion mutation in spike protein of PRCV [26]. Porcine epidemic diarrhea virus (PEDV) is another enteric coronavirus of pigs. It appeared first in Europe in late 1970s [27]. It has caused significant economic losses in pig industries in Europe, Asia and North America [28, 29]. Porcine delta-coronavirus (PDCoV) is relatively new enteric coronavirus identified in 2012 in China [2]. Similarly, porcine hemagglutinating encephalomyelitis coronavirus (PHE) is the only known neurotropic CoV infecting pigs. This virus is antigenically different from other strains and produces neurological and digestive disorders [30]. It mainly infects piglets less than 3 weeks of age. The disease caused by PHE is also known as "vomiting and wasting disease" due to the clinical signs such as vomiting, anorexia, and depression [31]. This virus was reported first from Canada [32] but now is endemic in many other countries of Asia, Europe and North America where pig production activities are high [33-36].

**Porcine Epidemic Diarrhea Virus (PEDV)**

**History and epidemiology**

A new enteric disease was reported by British veterinarians in early 1971 and named as epidemic diarrhea (ED). This infection has subsequently spread to many other European countries. However, only in 1978, researchers from Belgium discovered the causative agent of ED as a coronavirus, CV777, distinct from other existing porcine CoV including TGEV and PHE, through experimental inoculation of pigs [37, 38]. After the discovery,
the virus was named porcine epidemic diarrhea virus (PEDV). In 1970s and 1980s PEDV affected many countries of Europe including The Czech Republic, United Kingdom, Belgium, Hungary, France, Germany, Switzerland and Italy and caused epidemics leading to severe losses in suckling pigs [39, 40]. PEDV outbreaks markedly decreased in European countries in 1990s and appeared only in the form of isolated outbreaks [40]. The herd immunity acquired after the disease became endemic might be responsible for the decline in severity and numbers of PEDV outbreaks [41]. Regarding PEDV in Asia, similar disease was reported early in 1973 from China [42] but the causative agent was isolated only in 1984 [43]. Japan reported PEDV outbreaks in early 1980s [44] while South Korea reported PEDV first in 1992 [45]. Similar PEDV outbreaks related to classical viral strains were reported from other Asian countries including Vietnam, Thailand, and The Philippines [46]. These Asian countries implemented one or another form of vaccination strategies which might have helped in preventing larger outbreaks related to classical strains of PEDV. For example, China started using inactivated or live attenuated PEDV CV777 vaccines or bivalent PEDV and TGEV vaccines from early 1990s which played a major role in controlling PEDV outbreaks before 2010 [42]. Similarly large PEDV outbreaks were controlled in Japan and South Korea through vaccination with different attenuated strains of PEDV [44, 46-48]. In Canada, though coronavirus-like particles were reported from swine herds in 1980s, no evidence of PEDV infection was available before 2014 [49]. Likewise, other American, African countries and Australia did not report any PEDV related outbreaks before 2013 [50, 51]. In 2010, highly virulent strain of PEDV emerged in China [28]. It caused high mortality
(50-100%) in neonates. The use of vaccines based on the classical PEDV strain CV777 did not inhibit the PEDV outbreaks and failed to alleviate the enormous economic losses associated with it [50]. Subsequently, this strain spread to other Asian countries, the Americas and also to European nations [52]. For example, Japan faced highly virulent PEDV outbreaks in 2013 and by the end of 2015, over 1000 outbreaks were reported [53]. Similarly, Taiwan reported PEDV outbreaks in late 2013 [54]. South Korea and Vietnam suffered PEDV outbreaks in 2013 [55, 56] while Philippines experienced those in 2014 [57]. In the US, PEDV first appeared in 2013 when it caused devastation in the US pig industry [58]. Presently, PEDV is one of the major health problems in pig population, globally. However, only Ukraine has reported highly virulent PEDV outbreaks among the European countries.

**PEDV in USA**

The highly virulent PEDV infection started on US swine farms in April, 2013 and the large outbreak ended in spring, 2014 [58, 59]. In its first year, PEDV killed around 10% (~7 million) of total pig population leading to huge economic losses in the US swine industry [29]. California was also one of the states where PEDV outbreak was recorded in 2014 [60]. This virus spread rapidly in the US, affecting 39 states (Figure. 1) including Hawaii by March, 2017 [60]. Soon, it also spread to other American countries including Canada, Mexico, Peru, the Dominican Republic, Columbia and Ecuador [61].
Figure 1: PEDV: cumulative confirmed and presumptive positive premises since June 5, 2014 in USA (https://www.aphis.usda.gov)

Structure of PEDV

PEDV belongs to *Alphacoronavirus* genus. It is an enveloped virus. The diameter including spike projection ranges between 95 to 190 nm where projection alone are approximately 18 to 23 nm in length [37]. Club shaped spike projections are radially spaced from the core. The genome is approximately 28 kb in length and encodes for seven ORFs [62]. The genome has 5' cap, 3' poly (A) tail and untranslated regions at both the ends. The 2/3\textsuperscript{rd} of the 5' end of the genome is occupied by two overlapping ORFs, ORF1a and ORF1b. ORFs 1a and 1b encode at least 16 non-structural proteins necessary for viral replication, transcription and translation. The remaining 1/3\textsuperscript{rd} of the genome has other five ORFs that encode the four structural proteins namely - spike, envelope, membrane and nucleocapsid and one accessory protein ORF3 [63].
Transmission

PEDV is a highly contagious pathogen mainly transmitted by the fecal-oral route. Some reports of aerosol transmission potential of PEDV also exist [64]. Transmission occurs after direct exposure of susceptible pigs to the diarrhea and vomitus of clinically or sub-clinically infected pigs or through ingestion of contaminated feed and water [65]. People involved in pig care (e.g. owner, workers, veterinarians), trailers used for transportation of farm animals, tools and other stuff (e.g. piglets, manures, feed), and/or wild animals and birds can also play a major role in dissemination of PEDV from one farm to another [40]. Transmission is facilitated by high viral shedding 11.5 to 13.7 log_{10} GE/mL in feces and requirement of low infectious dose of 7.35 log_{10} PDD_{50}/mL to set up infection [66]. Its environmental persistence also facilitates indirect transmission potential through different vehicles. Like other CoVs, PEDV is stable at lower temperatures. It withstands pH 5-9 at 4^0C and pH between 6.5 to 7.5 at 37^0C at least up to 6 hours [67]. It can survive up to 7 days in contaminated feed and up to 14 days in contaminated slurry even at 25^0C temperature [68]. Viral RNA was also detected in milk of PEDV infected sows suggesting for the possibility of vertical transmission of PEDV from sows to piglets [69, 70].

Pathogenesis

The cellular receptor for PEDV is not identified yet [71, 72]. Aminopeptidase N (APN) and sialic acid (N-acetylneuraminic acid) are the likely binding factors for PEDV [73]. APN is a 150 kDa glycosylated transmembrane protein which is highly expressed in
small intestinal mucosa [74]. PEDV S protein binds to cellular receptors to establish virus infection. It has S1 and S2 subunits [75]. S1 is responsible for receptor binding while S2 mediates membrane fusion during virus entry into the cells [71]. The virus replicates in the cytoplasm of villous epithelial cells and lyse the cells to release viruses. This leads to marked villous atrophy. This causes interruption in digestion and absorption of nutrients and electrolytes leading to malabsorptive watery diarrhea [40]. Grossly PEDV infected pigs have distended stomach filled with undigested milk curd and thin and transparent intestines filled with yellowish fluid [76]. Microscopically, atrophic enteritis, swelling of villous enterocytes and mild vacuolation in cytoplasm are observed. The villous height is reduced significantly. For example, the villous height to crypt depth ratio is reduced from $7.16 \pm 1.25$ of normal 4 days old cesarean-derived colostrum-deprived (CDCD) piglets to less than 2 [66].

**Symptoms**

Incubation period for PEDV infection ranges from 1-8 days. Virus shedding starts 2 days post-inoculation and can last up to 4 weeks. Symptoms are evident in young piglets up to a week involve severe watery diarrhea and vomiting for 3 to 4 days [37, 40, 76]. Excessive dehydration and electrolyte imbalance can lead to death. In newborn piglets infection with highly virulent PEDV strains can lead to mortality as high as 100%. In older pigs, clinical signs are self-limiting within a week but involve negative impact on growth performance due to anorexia and depression [77].
**Diagnosis**

Direct detection of PEDV RNA is possible by using conventional or real time RT-PCR in fecal samples [46]. PEDV M, N or S gene specific primers are used in PCR assays. Some antigen Enzyme-linked immunosorbent assay (ELISA) using PEDV specific monoclonal antibodies are also available to detect PEDV antigens which becomes useful in PEDV outbreaks in field conditions when viral antigen shedding is high [78]. Immunohistochemistry (IHC) and immunofluorescent (IF) assays are used to detect viral antigens in cell cultures and in intestinal sections [79]. Loop mediated isothermal amplification of PEDV RNA using specific primers (LAMP-RT-PCR) was developed recently and is also being used [80]. Indirect diagnosis of PEDV is done by antibody detection assays. Antibody ELISA, and viral neutralization (VN) assay are available to detect PEDV specific antibodies in serum and milk/colostrum samples [81]. Similarly, fluorescent focus neutralization (FFN) assay, indirect fluorescent antibody assay (IFA) and fluorescent microscope immunoassay (FMIA) can also be used in detection of PEDV specific antibodies [80].

**Treatment, control and prevention**

No specific treatment is available for piglets during PEDV infection. Since it causes dehydration, plenty of oral electrolytes should be supplied. Dry feed should be withdrawn and symptomatic treatments can be done [40]. Stringent biosecurity measures should be applied in the farm so as to avoid introduction of PEDV. This involves activities such as: (i) ban on entry of unauthorized vehicles, humans or wild animals/birds to the farm; (ii)
use of foot bath containing viricidal solution and proper cleaning and disinfection of vehicles and utensils being used in farm procedures; (iii) proper disposal of slurry and carcasses if any; and (iv) use of disinfectants such as phenol, quaternary ammonium compounds, glutaraldehyde and bleach for regular disinfection of farm premises [81]. Vaccination of sows is another strategy being used to prevent PEDV disease in piglets. The antibodies from immune sows are transferred passively to newborn piglets which can prevent severe outcome of PEDV infection [44]. Attenuated or inactivated vaccines are available in different countries. For example, historically China has been using CV777 inactivated/attenuated intramuscular (IM) vaccine, Japan has been using cell culture attenuated PEDV strain 83P-5 vaccine and South Korea has been using cell culture adapted PEDV strains SM98-1 (IM) and DR-13 (oral) for vaccination [82, 83]. In the US, inactivated whole virus vaccine and subunit vaccine based on S protein are conditionally licensed for use in pigs [84]. All these PEDV vaccines confer variable level of protection in the field conditions which is also evident from the fact that, despite the use of vaccines, virulent PEDV strain emerged and caused devastating impact in swine industry in China. Hence, further studies on PEDV vaccines are necessary to develop an effective and safe vaccine.

**Transmissible Gastroenteritis Virus (TGEV)**

TGEV is also a highly contagious enteric virus that causes up to 100% mortality in piglets. Mortality is relatively low in grower-finisher pigs [74]. TGEV was first described in the US in 1940s [25] and worldwide afterwards. Within two decades of its
identification the virus was spread to all the continents preferably countries where North American products were imported were the victim of disease at first [85]. In 1980’s TGEV was a great problem in the United States. Almost 26% of the case with diarrhea in piglets were due to TGEV, resulting in growth retardation, immune suppression and increased susceptibility to other diseases [85]. The disease mainly occurred in the winter time, which may be due to maintenance of the viral infectivity for longer period in cold weather. Major route of transmission of the virus is fecal-oral. However, transmission from milk of a sow to its piglets is also recorded [86]. The possible reasons for winter outbreaks of TGEV include farms with subclinical infection, host other than pigs and carrier pigs [40]. TGEV infects intestinal epithelial cells and mild infection also occurs in upper respiratory tract [26]. Virus can be recovered from macrophages, kupffer cells, mesenteric lymph nodes and Peyer’s patches. Similarly, the virus has also been isolated form tonsils, trachea and lungs [87]. However, porcine blood monocytes are not infected by TGEV [88]. Pigs of all age groups are susceptible to TGEV infection which is characterized by vomiting, severe diarrhea, dehydration and high mortality in piglets of age less than half month [89].

Like PEDV, TGEV is also enveloped and contains positive sense single stranded RNA genome of size 28.5 Kb. The overall diameter ranges from 60 to 160 nm. It also has a single layer of a club-shaped surface projection (spikes) ranging from 12 to 25 nm in length [90]. TGEV is very stable when stored frozen. Virus retains its infectivity in liquid manure for more than 8 weeks in 5°C but the infectivity decreases as temperature increases for example 2 weeks at 20°C and 24 hrs at 35°C. However TGEV was found to
be infectious for several days in 25°C water [40]. A deletion mutant of TGEV named as porcine respiratory CoV (PRCV) also circulates in pig population and causes respiratory infection. Due to the PRCV infection, impact of TGEV infection is thought to be reduced in North American and in European swine population. However, in PRCV seronegative North American pig population TGEV is still a major cause of severe diarrhea in newborn piglets [40]. In Asian countries, PEDV and TGEV occasionally co-circulate leading to an enormous economic loss in swine industry [91].

As in PEDV, TGEV also infects and replicates in the intestinal villi. It also leads to villous atrophy and loss of absorptive intestinal surface area. This results in severe watery diarrhea, and dehydration [92]. The ultimate cause of death is probably due to dehydration causing metabolic acidosis, and hyperkalemia leading to cardiac abnormalities [40]. Inactivated or modified live intramuscular vaccines are being used against TGEV since 1960s [93]. Since 1980s, oral vaccines are also developed and used [94]. Vaccination is generally practiced in sows before farrowing so as to transfer immunity to piglets through colostrum and milk. TGEV is inactivated by various disinfectants like formalin (0.03%), NaOH, quaternary ammonium compounds, ether, and chloroform [40].

**Porcine Deltacorona Virus (PDCoV)**

PDCoV is a member of *Deltacoronavirus* genus [2]. It also causes acute diarrhea, vomiting, dehydration and mortality in neonates but at lesser extent than PEDV and TGEV [95, 96]. PDCoV was first reported in Hong Kong in 2012 [2]. Delta CoVs were
detected in various mammalian and avian species. About 10% of the porcine fecal samples were tested positive for delta CoVs [2]. Recent studies show that in China PDCoV strains were present in specimens from diarrheic piglets dating back to 2004 [97]. In the US, PDCoV related diarrhea was first reported in 2014 and the isolated strains had nearly 99% nucleotide identity to the Hong Kong strains HKU15-44 and HKU15-155 [98]. PDCoV has been isolated from at least 18 states of the US (www.aphis.usda.gov/animal-health/secd), Canada, China, South Korea and Thailand. All these strains are highly related to the Hong Kong strains [99]. Recent reports suggest that PDCoV infection is quite common in concurrent infection with PEDV and rotavirus C especially in Midwestern US [100, 101]. The retrospective studies of fecal samples by RT-PCR and antibody assays in serum samples suggest that PDCoV should be circulating in US pig population at least from 2010 [102, 103]. The enveloped PDCoV is pleomorphic with diameter 60-180 nm. The single stranded genome has size around 25.4 kb and encodes the structural spike, envelope, membrane and nucleocapsid protein and sixteen nonstructural proteins [96]. Fecal-oral route may be the main means of PDCoV transmission, as in PEDV contaminated feed, water, farm utensils and clinically or sub-clinically infected pigs can be the source of PDCoV in farms [40]. It also causes villous atrophy due to its cytolytic nature as in PEDV and TGEV [95, 104]. APN is regarded as the receptor for TGEV [74] but whether APN serves as the receptor for PDCoV is not known. It also causes acute watery diarrhea in piglets and frequent vomiting followed by dehydration, loss of weight and death [95].
Wild boars and their interaction with domestic pigs and humans

Wild boars (*Sus scrofa*) are also known as wild pigs or Eurasian wild pigs. They mainly originated from European and Asian countries but are now found in many countries around the globe. As of 1990s, 16 subspecies of wild boars are recognized. Wild boars can adapt to different climatic conditions and reproduce very quickly. The domestic pigs (*Sus scrofa domesticus* or *Sus domesticus*) are subspecies of wild boars. Genetic studies revealed that domestication of wild boars started independently from European and Asian countries. Over time, these domesticated pigs were cross-bred leading to generation of hybrid forms [105, 106]. In the United States, wild pigs are referred to as feral pigs. The other terms used for describing the same wild boars include wild pig/hogs/swine; feral hogs/swine and piney woods rooters [106]. As with other wild animals, the interaction of humans and domestic pigs with wild pigs is also increasing owing to the facts such as deforestation, changes in land use pattern and global expansion of human population leading to increased suburban habitation [106]. Since wild pigs harbor several pathogens infectious to domestic pigs and even humans, increased interaction with them also increases the risk of disease transmission. In some part of the world, wild pigs are used in recreational hunting and for meat purposes, which further increases the risk of disease transmission especially in humans. Wild pigs can also transmit pathogens to other wild mammals and birds which in turn will infect domestic animals and then to the humans [107].
Wild pig population and role in disease maintenance and transmission

Wild pig population can be a potential threat for maintenance of viral, bacterial or parasitic pathogens as a reservoir and their transmission to domestic pigs and even humans. Therefore, epidemiological studies of different pathological agents of economic and public health importance are necessary in wild pigs. This can be carried out either directly by isolation of pathogenic agents in different specimens or indirectly by detection of pathogen specific antibodies. Previous epidemiological studies have shown that wild pigs can be a potential reservoir for different pathogens of vital importance in livestock and humans [108]. For any animals to be reservoir of a pathogen, there should be large number of susceptible hosts, they should be infected by the pathogen and be able to transmit pathogens to other susceptible animals [109, 110]. Since, this can happen in wild pigs for many pathogens including Classical swine fever virus (CSFV), African swine fever virus (ASFV) and Influenza A virus (IAV); they can play a significant role as a reservoir [111-113]. However, the risk factors, pathogenesis and epidemiology of various pathogens may differ in wilderness compared to domestic pigs and needs to be investigated in details.

Wild pig and viral infections

The wild boar population has helped in recent expansion of African Swine Fever Virus (ASFV) in different European countries and territories. Transmission of ASFV from wild boars to domestic pigs is contributed either by direct contacts in farms maintained under low biosecurity measures or indirectly through contaminated materials. Due to the
involvement of wild population in ASFV maintenance and transmission, its prevention and control has been a difficult task in European countries [114, 115]. Likewise, re-emergence of Classical Swine Fever Virus (CSFV) in Europe is also through wild pig exposures. For example, 60% of the CSF outbreaks in domestic pigs in Germany during 1993-1997 were attributed to direct or indirect exposure to wild pigs [111, 116]. In the US, CSF is eradicated from domestic pigs. A surveillance of 3661 wild pigs in the US during 2007 to 2008 could not detect CSF. No reports are available suggesting the existence of CSF in the US wild pigs so far [117].

Pseudorabies or Aujeszky's disease caused by Aujeszky disease virus (ADV) is also well established in the US wild pig population. [107]. Antibody surveillance from 10 sites in southeastern part of the US during 2001-2002 showed that 38% of the wild pigs were positive. Pseudorabies is eradicated from domestic pigs in the US [118]. Porcine circovirus 2 (PCV2) which causes PCV associated disease (PCVAD) characterized by multisystemic infection has also been reported in wild pigs in different countries [119, 120]. A serological surveillance of feral pigs conducted in 15 different states of the US during 2011-2012 showed that 25.3% of the pigs had antibodies against PCV 2 [121]. Similarly, prevalence of Porcine reproductive and respiratory syndrome virus (PRRSV) and parvovirus in the US wild pig population is found to be 2 to 2.5% and 14 to 17% respectively [121, 122].
Wild pigs and bacterial/parasitic infections

Like viral pathogens, different bacterial pathogens can infect wild pigs that form a potential source of disease transmission to domestic pig population. In Spain, 5.3% of wild boars tested had antibodies against *Erysipelothrix rhusiopathiae* [123]. In Italy 23.8% [124], in France, 37.5% [125] and in Spain 56.8% [126] of wild boars tested were showing antibodies against *Mycobacterium bovis*. In Croatia and Spain, brucellosis in domestic animals was likely to be linked to brucellosis in wild boars [127, 128]. Seroprevalence of antibodies of *Leptospira sp.* are reported to be 18% in Germany [129] and 2.6% in Italy [130]. Likewise, in Spain 26.6% wild boars were seropositive for *Mycoplasma hyopneumonia* and 11.3% for *Salmonella sp.* [123]. Similarly, wild pigs are also known to be infected with *Yersinia pestis, Coxiella burnetii, Francisella tularensis* and other bacterial pathogens [107]. In the US, *Salmonella* prevalence in domestic pigs ranges from 1.5 to 10.4% and *Campylobacter* prevalence ranges from 55.8 to 65 [131]. In wild pigs of the US, sero-prevalence of *Brucella* is 0 to 44% [107]. Similarly, prevalence of other bacteria like *Coxiella burnetii, Leptospira, Mycobacterium avium* and *Yersinia pestis* is 50%, 87%, 85% and 15% respectively [132].

Wild pigs are also infected with different parasites such as *Trichinella sp.* and *Toxoplasma gondii*. In Poland more than 88% of the human cases of *Trichinella* were linked to ingestion of wild boar meat [133]. Likewise, the seroprevalence of *T. gondii* in wild pigs in Spain were 43.5% [123] and 36.3% [127] in two separate studies. In the US, the prevalence of *Toxoplasma gondii* and *Trichinella* parasites in domestic pigs ranges from 0.6 to 24% and 0.6 to 0.7% respectively [131]. One study in wild pigs in the US
showed prevalence of *Trichinella spiralis* to be 39% [134]. Similarly, *Toxoplasma gondii* prevalence in wild pigs of the US was 49% in South Carolina, 0.9% in Georgia and 31% in Great Smoky Mountain National Park (GSMNP) [135].

**Wild pigs in zoonotic disease transmission**

Wild pigs also transmit viral, bacterial and parasitic pathogens of humans. The viral agents transmitted to humans through wild pigs are hepatitis E virus, swine influenza virus and Japanese encephalitis virus [107, 122, 136]. HEV was first isolated in pigs from USA [137]. HEV genotype 3 and 4 is now considered as a zoonotic disease and pigs are the likely reservoir for this agent [138]. Wild pigs have shown antibodies against HEV in different countries [139]. It may also be transmitted between wild pigs and deer [140]. In Japan, evidence of HEV transmission via consumption of wild boar liver was found [141]. The major bacterial pathogens found in wild pigs that can have zoonotic potential include *Mycobacterium bovis*, *Brucella sp.*, *Coxiella burnetii*, *Yersinia pestis*, and *Leptospira interrogans* [107]. A case of brucellosis in human was identified in Iowa State, USA. It was transmitted directly from the domesticated sows which contracted the disease from feral swine [142].

**Wild/feral pigs in USA**

Wild pigs are found throughout the Southern part of United States. The members of family *Suidae* were introduced into North America in late 14th century and in Florida and
Texas in mid-15th century by Spanish explorers [143]. They were raised under free-range practices and when such practice became illegal, pigs were released into the wilderness [143]. Such domesticated pigs once released into wilderness, they are referred as feral pigs. Till late 20th century they were confined to less number of states and were not as widespread as they are now. In recent years, wild pigs are expanding their habitat northward in the US. In 1982, 17 states reported wild pigs while in 2014 they were reported from 41 states. The population is estimated to be over 6 million heads [144]. Figure 2 shows the feral swine distribution in the US during the year 2015.

Previous studies have shown that wild pigs can be reservoir for viral, bacterial and parasitic diseases in the US [142]. For example, antibodies have been found against viral pathogens including pseudorabies and vesicular stomatitis virus. Similarly, antibodies for rickettsia and bacterial pathogens such as *Brucella* and *Leptospira* are also detected. Likewise, antibodies have been detected against multiple endo and ectoparasites in feral pigs [107]. Some of the previous experiments could not detect TGE virus neutralizing (VN) antibodies in wild pigs [122, 145]. However, this does not mean that wild pig population is resistant to this virus because experimentally infected wild pigs have developed clinical disease, induced VN antibodies and also transmitted virus to other susceptible pigs [145].
Figure 2: Feral swine populations in the US in 2015.
(Source: http://swine.vet.uga.edu/nfsms/information/map2015.htm)

Wild and feral pigs can also be the source for introduction of pathogens new to US domesticated pigs and other livestock population. Expanding wild pig population also makes it difficult to materialize the efforts to control a number of livestock diseases. For example, pseudorabies free status in livestock was achieved in the US after spending over 250 million dollars. However, if such diseases exist in wild pigs and get spread into the domesticated animals, all the efforts will be nulified [107]. In fact, US feral swine population was showing 0 to 46% prevalence rate against pseudorabies virus [146]. Similarly, for swine brucellosis the prevalence percentage was 0-53% indicating that feral swine can disseminate these pathogens to domesticated pigs once the opportunities favor [146]. Brucellosis is another disease in the US to which lots of money is being spent to
eliminate this from livestock population. Wild pigs are invasive species as they can multiply rapidly into the new area and get established. Pigs are found in all continents except Antarctica and are one of the most globally distributed mammals [146]. The cost of wild pig related crop damage and control initiatives in the US is over $1.5 billion [147].

**Wild pigs in California**

Wild pigs inhabit in Coastal regions of California since 17th century [148]. During early 1980s, 33 counties reported wild pigs while by 1990s 49 of 58 counties reported wild pig inhabitation [149]. At present, wild pigs are found in almost all counties of California. This shows that, feral pigs are expanding both inside the state and also from state to state. Some of the probable reasons for such expansion can be increased forage availability due to agricultural improvements and also due to release of wild pigs in private lands to populate them as game animals. Wild pigs are omnivores, primarily surviving on plant materials but also can consume other feed sources like earthworms, rodents, reptiles etc. [150] which also help them survive and spread to newer habitats. One study in 1996 in California showed that the cost of wild pig related crop and property damage was close to 2 million [151]. With expansion of wild pig population in many more counties, we can consider that the loss might have increased in recent years.
Chapter 2: Screening for enteric coronaviruses in fecal samples of feral pigs of California, USA

Introduction

Transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV) and porcine delta-coronavirus (PDCoV) are the known enteric coronaviruses (CoV) of pigs. They are enveloped positive sense, single stranded RNA viruses belonging to family Coronaviridae. These viruses are highly contagious and transmitted mainly by fecal-oral route. High viral shedding in feces is observed when pigs are infected [66, 152]. These viruses infect and replicate in the intestinal enterocytes causing life threatening acute enteritis characterized by malabsorptive and maldigestive diarrhea, vomiting and dehydration [40, 77]. TGEV, in particular, can also cause mild infection in upper respiratory tract [26]. Morbidity rates can go as high as 80-100% and mortality rates are 50-100% in TGEV and PEDV infected suckling piglets [40, 50]. PDCoV, however, appears to be milder showing 40-60% mortality rates in suckling pigs [152].

Enteric CoV infections cause significant economic loss in pig industry globally. In USA, TGEV was first described in 1940s and remained problematic till 1980s [25, 85]. However, after emergence of respiratory variant of TGEV known as porcine respiratory coronavirus (PRCV), the impact of TGEV subsided likely due to the fact that PRCV
infection induced cross protective immunity against TGEV [40]. Though PEDV was reported from 1970s in European countries, it emerged in USA only in 2013 [58, 59]. The highly virulent PEDV outbreaks of 2013/14 in the US killed 10% (~7 million) of total pig population [29]. By March 2017, PEDV was reported from 39 states of USA and also spread to neighboring countries including Canada and Mexico [61]. PDCoV was isolated first from Hong Kong in 2012 [2]. In the US, it was detected in 2014 and since then it has been reported from at least 18 states [98]. PDCoV is common in concurrent infections with PEDV and rotavirus C in Midwestern USA [100, 101].

Wild and feral pigs are found in many countries around the globe. Since they can adapt themselves to different climatic conditions and reproduce relatively fast, they can expand geographically and maintain their population [106]. Such pigs can maintain various viral, bacterial and parasitic pathogens and can transmit them to domestic pigs, other domestic animals and even to humans [107]. Increased interactions with wild and feral pigs due to deforestation, changes in land use pattern, increased suburban habitation and recreational hunting can further increase the risk of pathogen transmission from wilderness [106]. For an example, a study in Poland showed that wild boars got infected with African swine fever virus (ASFV) and helped in its dissemination to the territory as the infected boars can travel up to 25 km distance [112]. Likewise, the re-emergence of classical swine fever virus (CSFV) in European countries is linked to wild boar exposures [111, 116]. Similarly, bacterial pathogens such as *Brucella sp.*, *Erysipelothrix rhusiopathiae*, *Mycobacterium bovis* and *Leptospira* and parasitic pathogens such as *Trichinella sp.* and *Toxoplasma gondii* can also be transmitted to domestic pigs from wilderness [107].
Feral pigs are found throughout the southern part of US and are also expanding their habitat northward in recent years [144]. The cost of this invasive species related crop damage and control initiatives in the US is over a billion dollar per year [147]. Besides the property damage, feral pigs can also be the source for introduction of disease pathogens in domestic pig population. The fact that, infectious pathogens which are eradicated from domestic pigs are still circulating in US wild pigs, further increases the risk of pathogen re-introduction into domestic population [107]. Pseudorabies and brucellosis are the examples of such pathogens. US domestic pig population is declared free of both pseudorabies and brucellosis. Brucella exposure has been detected at least in 16 states with the prevalence of 0.3-52.6%, while pseudorabies exposure reported from at least 11 states with the prevalence of 0-46% indicating that they bear potential threat of pathogen transmission to domestic pigs [146, 153].

Though enteric CoVs can cause devastating impact in swine industry and wild/feral pigs might play a role in maintenance and transmission of enteric CoVs to domestic pigs, limited information is available in this context in the US. Since CoVs in particular PEDV is shed in large amount (11.5 to 13.7 log_{10} GE/mL) in feces [66], they survive in the environment with relative ease for longer period and can be transmitted easily, detailed study on ecology and epidemiology of porcine CoVs in wild pigs is necessary. Such studies can pave way towards better understanding of emergence and re-emergence of CoVs and formulating better prevention and control strategies in future. Hence, our aim in this study was to screen for porcine enteric coronaviruses in the fecal samples of feral pigs in the US. To start with, we used the fecal samples collected from California which
is one of the states affected by PEDV and PDCoV and also has feral pigs in almost all the counties.

Materials and methods

Sample collection

Fecal samples of feral pigs were collected by USDA-APHIS Wildlife Services and provided by Dr. Peng Tian (Produce Safety & Microbiology Research Unit, Western Regional Research Center, ARS, USDA, Albany, CA, USA). Samples were collected from 5 different counties of California in early-mid 2016. The map of California State indicating the sample collection sites is shown in Figure 3. In total, 44 samples were received. Among them, 12 were from male and 14 from female pigs and 9, 6 and 11 were from juvenile, sub-adult and adult pigs respectively. Age and sex information for 18 pigs were not available. The detailed sample characteristics are shown in table 1.

Sample processing

Fecal samples were diluted with phosphate buffered saline (PBS) at the ratio of 1:10. We have received pre-diluted from USDA ARS on ice packs. Samples were mixed thoroughly by vortexing and centrifuged at 18,407 x g for 5 min at 4°C. Supernatants were aliquoted and immediately subjected for RNA extraction. Remaining aliquots were stored at -80°C.

RNA extraction

26
Viral RNA was extracted from the supernatants of fecal samples by using 5X MagMAX-96 virus isolation kit (Ambion by Life Technologies, USA) and the RNA extraction robot MagMax Express (Applied Bio-systems, Foster City, CA) according to the manufacturer’s instructions. The viral RNA was eluted with 50 μL of elution buffer and used as the template for reverse transcription polymerase chain reaction (RT-PCR) and Taqman real-time RT-PCR (RT-qPCR).

**Conventional RT-PCR**

Two sets of pan-coronavirus primers designed previously to detect the highly conserved regions of polymerase genes of CoVs were used. Primer set 1 (forward primer IN-2deg, 5’-GGGDTGGGAYTAYCCHAARTGYGA-3’ and reverse primer IN-4deg, 5’-TARCAVACAACISYRTCRTCA-3’) targets on TGEV and PEDV and amplifies 452 bp fragment [154]. Similarly, primer set 2 (PanCoV- F2, 5’-AARTTYTAYGHHGYYTGG-3’ and PanCoV- R1, 5’-GARCARAATTCATGHGGDCC-3’) were designed to amplify TGEV, PEDV and PDCoV with an amplicon size of 668 bp (Hu and Saif, unpublished). Alignments of representative coronavirus sequences of conserved region of the polymerase genes applicable to each of the primer sets were performed using Clustal W method in Lasergene software (version 13) (DNASTAR, Inc., Madison, WI) and are shown in Figure 4.

RT-PCR was performed using Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, MA, USA). RT-PCR products were analyzed by 1.5% agarose gel electrophoresis. For primer set 1, a total reaction volume of 25 μL was prepared for one-
step RT-PCR consisting of 5 μL of 5x reaction buffer; 2.5 μL of 25 mM MgCl₂; 0.5 μL of 10 mM dNTPs; 0.5 μL of 100 pmole/μL forward primer; 0.5 μL of 100 pmole/μL reverse primer; 0.25 μL of 40 u/μL RNAsin; 0.25 μL of 10 u/μL AMV RT; 0.25 μL of 5u/μL Taq polymerase; 13.25 μL DEPC water and 2 μL of the sample RNA. The RT reaction was performed at 42°C for 60min. For PCR, initial denaturation was done at 95°C for 5min, followed by 35 cycles of 94°C for 1min, 50°C for 1min, 72°C for 1min and a final extension step at 72°C for 7min.

Similarly, for primer set 2, a total reaction volume of 20 μL was prepared for RT-PCR using QIAGEN Onestep RT-PCR kit (Qiagen Inc., Valencia, CA, USA) consisting of 4 μL of 5x reaction buffer; 0.8 μL of 10 mM dNTPs; 0.4 μL of 50 pmole/μL forward primer; 0.4 μL of 50 pmole/μL reverse primer; 0.2 μL of 40 u/μL RNAsin; 0.8 μL of enzyme mix; 11.4 μL DEPC water and 2 μL of sample RNA. The reverse transcription reaction was performed at 50°C for 30min. For PCR, initial denaturation was done at 95°C for 15min, followed by 35 cycles of 94°C for 40 sec, 52°C for 40 sec, 72°C for 1min and a final extension step at 72°C for 10min.

**Taqman real-time RT-PCR for detection of PEDV N gene**

Real time RT-PCR was done by using QIAGEN OneStep RT-PCR kit (Qiagen Inc., Valencia, CA, USA) as described previously [76]. Forward primer (PEDNFnew, 5’-CGCAAGACTGAACCCACTAAC-3’), reverse primer (PEDNR, 5’-TTGCCTCTGTGTTACTTGGAGAT-3’) and probe (PEDprobe, 5’- FAM-
TGYYACCAYYACCACGACTCCTGC-BHQ-3’) were used to detect 197 bp of PEDV N gene. A 20 μL reaction mix was prepared consisting of 11.4 μL water, 4 μL of 5x PCR buffer, 0.8 μL of 10 mM dNTPs, 0.4 μL of 50 μM forward primer PEDNFnew, 0.4 μL of 50μM reverse primer PEDNR, 0.4 μL of 5 μM PEDprobe, 0.2 μL of 40 u/μL RNAsin, 0.8 μL enzyme mix and 1.6 μL of RNA template. The reaction was carried out in a real-time thermocycler (RealPlex; Eppendorf, Germany). Reverse transcription reaction at 50°C for 30 min was followed by PCR with initial denaturation at 95°C for 15 min and 45 reaction cycles of 95°C for 15 sec and 56°C for 60 sec. The detection limit was 10 GE per 20 μL of reaction, corresponding to 4.8 log10 GE per mL of original feces. PEDV Iowa 106 RNA was used a positive control. RNA-free water was used as a negative control.

**Taqman real time RT-PCR for detection of PDCoV M gene**

RT-qPCR was conducted by using the QIAGEN OneStep RT-PCR kit (Qiagen Inc., Valencia, CA, USA) on a real-time thermocycler (RealPlex; Eppendorf, Germany). Forward primer (SDCVM-F, 5’-ATCGACCACATGGCTCCAA-3’), reverse primer (SDCVM-R1, 5’-CAGCTCTTGGCCCATGTAGCTT-3’) and probe (SDCVM-Probe, 5’-FAM-CACACCAGTCGTTAAGCATGGCAAGCT-IABkFQ-3’) were used to amplify 72 bp of PDCoV M gene. A 20 μL reaction was prepared consisting of 11 μL water, 4 μL of 5x PCR buffer, 0.8 μL of 10 mM dNTPs, 0.4 μL of 50 μM forward primer SDCV M-F, 0.4 μL of 50 μM reverse primer SDCV M-R1, 0.4 μL of 5 μM SDCV M-probe, 0.2 μL of 40u/ μL RNAsin, 0.8 μL enzyme mix and 1.6 μL RNA template.
The reaction was carried out in Eppendorf realPlex. Reverse transcription reaction at 50\(^{\circ}\)C for 30 min was followed by PCR with initial denaturation at 95\(^{\circ}\)C for 15 min and 45 reaction cycles of 95\(^{\circ}\)C for 15 sec and 56\(^{\circ}\)C for 60 sec. The results were analyzed by using the system software (Eppendorf realplex 2.2) and the RNA titers were calculated using standard curve. The detection limit of RT-qPCR was 10 GE per reaction, corresponding to 4.6 \(\log_{10}\) GE/mL. PDCoV OH-FD22 RNA was used as positive control. RNA-free water was used as a negative control.

**Sequencing**

The RT-PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) as described by the manufacturer. Briefly, DNA fragments were excised from the agarose gel and dissolved using buffer QG (3x of gel weight) at 50\(^{\circ}\)C. Once the gel dissolved, 1 gel volume of isopropanol was added and solution was transferred into Qiaquick spin column. Column was centrifuged for 1 min, flow through discarded, 500\(\mu\)L of buffer QG was added, centrifuged again and flow through was discarded. 750\(\mu\)L of buffer PE was added to each column, centrifuged 1 min and flow through was discarded. The spin column was transferred to a 1.5 ml micro centrifuge tube and the DNA attached on spin column was eluted using 50\(\mu\)L of elution buffer by centrifugation for 1 min. PCR product along with forward and reverse primers were sent to molecular and cellular imaging center (MCIC), OARDC for sequencing. Sanger sequencing by capillary electrophoresis using ABI Prism 3100xl genetic analyzer was performed. Reagents for sequencing included DNA template, a sequencing primer, thermal stable DNA
polymerase, nucleotides (dNTPs), fluorescently labeled 3’-BigDye dideoxynucleotides (ddNTPs) and buffer.

Data analysis
Data were presented quantitatively in percentage positive (no. of positive samples*100/total samples), if any samples detected positive. RT-qPCR data was analyzed using standard curve prepared by running samples of known virus titers. Once the sequence data was obtained, we viewed the result in chromatograph using Chromas software (http://technelysium.com.au/wp/chromas/). Raw sequence reads were trimmed to remove amplicon primer-linker and low-quality sequences. Sequence data were assembled and analyzed using Lasergene software package (version 13) (DNASTAR, Inc., Madison, WI). The Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov) was employed to find homologous hits.

Results
Coronaviruses were not detected by conventional RT-PCR.
RNA was extracted from all the fecal samples and conventional RT-PCR was performed using the two pan CoV primer sets as described in methodology. Positive control viruses were amplified with a band of 452 bp (for PEDV and TGEV) and 668 bp (for TGEV, PEDV and PDCoV) using primer set 1 and primer set 2, respectively. All the feral pig fecal samples were negative for CoVs in conventional RT-PCR using both primers. Gel
images for primer set 1 is shown in Figure 5 and gel images for primer set 2 is shown in Figure 6.

**PEDV was not detected by RT-qPCR**

We also performed RT-qPCR assay using PEDV N gene specific primers and a probe as mentioned in methodology. The PEDV RT-qPCR results are shown in Table 2. Most of the samples had no Ct values while 12 samples showed Ct values ranging from 34.30 to 43.39 which correspond to RNA titers of 6.58 and 3.75 log_{10} GE/mL. Since the detection limit of this assay was 4.8 log_{10} GE/mL, most of the Ct values of samples were in the border line of positive/negative.

We repeated RT-qPCR for these 12 samples in triplicate. Similar Ct values like earlier were obtained in one or two reactions but not in 3 for any of these samples. For further confirmation, we performed RNA extraction using fresh aliquots of samples and carried out RT-qPCR increasing the reaction volume to 50µL. This time, none of the samples showed Ct values suggesting that PEDV was not present on any of these samples. The initial positive results were false positive results. The representative fluorescent profiles of PEDV N gene specific RT-qPCR is shown in Figures 7A-7C.

**PDCoV was not detected by RT-qPCR.**

As for PEDV, we also performed RT-qPCR targeting PDCoV M gene as mentioned in methodology. The results are shown in Table 4. Ct values were not seen in 19 samples while rest 25 samples showed Ct values ranging from 35.91 to 37.34 with corresponding
titers of 5.5 and 4.9 log_{10} GE/mL. Representative fluorescent profiles of PDCoV M gene specific RT-qPCR is shown in Figure 7D.

Since the detection limit for this RT-qPCR assay was 4.6 log_{10} GE/mL and the observed titers in 25 samples were very close to that, we wanted to further confirm the results. For that, we selected 13 of 25 samples showing high titers, increased the reaction volume to 50µL instead of 20µL and performed conventional RT-PCR using PDCoV M gene specific primers. The RT-PCR products were then subjected to 2% agarose gel electrophoresis. Out of 13 samples, 5 showed bands, 4 of them were low intensity and one (sample number 10) had comparatively higher intensity band (Figure 8). DNA was extracted from the thicker band using gel extraction kit and sent for DNA sequencing. The sequence observed was analyzed using NCBI standard nucleotide blast software and turned out to be non-relevant to specific organisms. This result indicated that, these fecal samples were also negative for PDCoV. The initial positive results were false positive.

**Discussion**

Wild and feral pigs are tested positive against different viral pathogens all over the world. For example, pseudorabies seroprevalence in wild boars was 0.57% in Switzerland, 0.4-15.9% in Germany [155], 30% in Italy [130], 44% in Spain [156] and 54.5% in Croatia [157]. Similarly, porcine reproductive and respiratory syndrome virus (PRRSV) seroprevalence in wild boars was 3% in Spain [123], 11% in France [158] and 37.7% in Italy [130]. Likewise PCV 2 sero-positivity was 43% in Czech Republic and 51.8% in Spain [159]. Swine influenza virus, parvovirus, African swine fever virus and classical
swine fever virus are other viral pathogens for which the wild pigs are tested positive [107]. Evidence suggests that, wild pigs can play a crucial role in dissemination of these viral pathogens. For example, 60% of the classical swine fever outbreaks in domestic pigs in Germany during 1993-1997 were attributed to direct or indirect exposures to wild pigs [111, 116]. Similarly, recent emergence and expansion of African swine fever virus in European countries and territories are also contributed by wild boars which makes prevention and control of ASFV a difficult task in Europe [114, 115].

Like viral pathogens, different bacterial and parasitic pathogens can also infect wild pigs and form a potential source of infection to domestic animals [107]. Seroprevalence of brucellosis in wild boars was found to be 7.8% in Japan [160], 9.9% in Switzerland [161], 22% in Germany [162], 29.4% in Croatia [128] and 29.7% in Spain [127]. In Croatia and Spain, brucellosis in domestic animal was likely to be linked to brucellosis in wild boars [127, 128]. Prevalence of *Toxoplasma gondii* parasite was 43.5% in wild boars in Spain [123]. In Poland, more than 88% of human cases of *Trichinella* were linked to ingestion of wild boar meat [133].

All these information suggest that, wild pigs can play a major role in maintenance and transmission of viral, bacterial and parasitic pathogens to domestic pigs and even to humans causing zoonosis. However, limited studies are available on enteric CoV status in wild pigs globally and in particular in United States. In this study, we used fecal samples collected from California, USA; extracted RNA and analyzed by conventional RT-PCR with two pan CoV primer sets, RT-qPCR for PEDV and PDCoV, respectively, and DNA sequencing to make sure presence or absence of porcine enteric CoVs.
Feral pigs in United States are also shown to test positive against various pathogens [107]. One study carried out in 1996 in Oklahoma state in 120 wild pigs showed seroprevalence of \textit{Leptospira interrogans} (44%), porcine parvovirus (17%), PRRSV (1.7%) and swine influenza virus (11%) [122]. Similarly, the average sero-prevalence of brucellosis from 2007 to 2012 in nationwide feral pigs was 4.3% [150].

Feral pigs are also linked to human infections in the United States. Hunters from South Carolina and Pennsylvania contracted brucellosis from field dressing feral swine when they went for hunting in Florida [163]. Similarly, \textit{E. coli} outbreak of 2006 which made 200 people sick and killed 3 people nationwide was linked to the contamination of spinach with feral swine feces [164].

Regarding enteric coronavirus surveillance in the US, 560 wild pig serum samples were tested in 1990 but none of them had neutralizing antibodies against TGEV [145]. Likewise, sero-surveillance done in 117 wild pig sera in 1996 by indirect immunofluorescent assay (IFA) also did not detect TGEV antibodies [122]. In Czech Republic, however, 1/134 wild pigs were tested positive against TGEV during the hunting season from 1999-2005 by IFA in sera [165]. A recent study in 2011/12 in South Korea showed that the PEDV infection rate in wild boar was 9.75% [166]. In that study, RNA was extracted from fecal samples and conventional RT-PCR was carried out using PEDV S gene specific primers. Similar to this study, we also used fecal samples for detection of enteric CoVs. The primer set 1 we used was designed before PDCoV was identified from pigs [154] while primer set 2 was designed based on nucleotide alignments of all known CoV species including PDCoV (Hu and Saif, unpublished).
However, due to larger amplicon size the later one was less sensitive than the former. We used both of these primers in our assay to catch potentially all CoVs including the novel ones. Our conventional RT-PCR results showed that none of the test samples were positive for enteric CoVs. The dye used to stain PCR product in the gel could detect 6 ng of DNA for a fragment of 500 bp and 12 ng DNA for a 50 bp fragment. If the viral concentration was too low then the band might not be visualized clearly. Hence we also performed RT-qPCR targeting conserved genes of PEDV and PDCoV as they are much more sensitive than the conventional RT-PCR. Though, the initial RT-qPCR assay using PEDV N gene specific primer showed few likely positive samples with borderline Ct values, repeated analysis showed that those were false positive results. Hence, we conclude that all our samples were negative for PEDV.

In domestic pigs, the PDCoV is quite common in concurrent infection with PEDV and rotavirus C infection in Midwestern US [100]. To our knowledge, no epidemiological studies of PDCoV in wild pigs in the US or elsewhere is available so far. Since, the RT-qPCR assay performed using PDCoV M gene specific primer in our study also could not detect delta coronavirus in any of these samples; we conclude that these samples were negative for PDCoV.

Overall, our results suggested that the test fecal samples from wild pigs from California, USA were negative for enteric CoV RNA. In feces, PEDV in particular is known to be shed for more than a month [41]. Hence, while using fecal samples, the likeliness of detecting virus is higher when the infection is ongoing. In this study, we did not have serum samples which would otherwise have helped us detect antibodies to enteric CoVs.
in pigs due to previous infections. However, our results confirm that, feral pigs in California did not have ongoing enteric CoV infection. However, this does not rule out that feral pigs might be playing a role in enteric CoVs transmission in the US. Similar studies from other states of USA with larger sample sizes will be helpful to ascertain whether wild pigs in USA are playing a role in the maintenance and transmission of porcine enteric CoVs or not. Comparative study in feral pig population from PEDV endemic states and states without PEDV outbreak might also give better understanding of enteric CoV ecology and epidemiology in US wilderness.
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Date collected</th>
<th>Collection site (county)</th>
<th>Age</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>1/12/2016</td>
<td>San Luis Obispo</td>
<td>Adult</td>
<td>Male</td>
</tr>
<tr>
<td>6</td>
<td>1/12/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Female</td>
</tr>
<tr>
<td>7</td>
<td>1/12/2016</td>
<td>San Luis Obispo</td>
<td>Adult</td>
<td>Male</td>
</tr>
<tr>
<td>8</td>
<td>1/12/2016</td>
<td>San Luis Obispo</td>
<td>Adult</td>
<td>Female</td>
</tr>
<tr>
<td>9</td>
<td>1/12/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Female</td>
</tr>
<tr>
<td>10</td>
<td>1/12/2016</td>
<td>San Luis Obispo</td>
<td>Adult</td>
<td>Female</td>
</tr>
<tr>
<td>11</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Male</td>
</tr>
<tr>
<td>12</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Female</td>
</tr>
<tr>
<td>13</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Male</td>
</tr>
<tr>
<td>14</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Male</td>
</tr>
<tr>
<td>15</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Adult</td>
<td>Male</td>
</tr>
<tr>
<td>16</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Female</td>
</tr>
<tr>
<td>17</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Juvenile</td>
<td>Female</td>
</tr>
<tr>
<td>18</td>
<td>2/4/2016</td>
<td>San Luis Obispo</td>
<td>Adult</td>
<td>Female</td>
</tr>
<tr>
<td>19</td>
<td>3/2/2016</td>
<td>Kern County</td>
<td>Adult</td>
<td>Female</td>
</tr>
<tr>
<td>20</td>
<td>6/15/2016</td>
<td>Lake County</td>
<td>Adult</td>
<td>Female</td>
</tr>
<tr>
<td>21</td>
<td>6/15/2016</td>
<td>Lake County</td>
<td>Juvenile</td>
<td>Female</td>
</tr>
<tr>
<td>22</td>
<td>6/15/2016</td>
<td>Lake County</td>
<td>Adult</td>
<td>Female</td>
</tr>
<tr>
<td>23</td>
<td>6/15/2016</td>
<td>Lake County</td>
<td>Adult</td>
<td>Female</td>
</tr>
<tr>
<td>24</td>
<td>6/7/2016</td>
<td>Napa County</td>
<td>Sub-adult</td>
<td>Male</td>
</tr>
<tr>
<td>25</td>
<td>6/7/2016</td>
<td>Napa County</td>
<td>Sub-adult</td>
<td>Male</td>
</tr>
</tbody>
</table>

Table 1: Fecal samples collected from wild pigs from California, USA

*NA = not available
Table 1 continued

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Date collected</th>
<th>Collection site (county)</th>
<th>Age</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>6/16/2016</td>
<td>Kern County</td>
<td>Adult</td>
<td>Male</td>
</tr>
<tr>
<td>27</td>
<td>6/30/2016</td>
<td>Napa County</td>
<td>Sub-adult</td>
<td>Male</td>
</tr>
<tr>
<td>28</td>
<td>6/30/2016</td>
<td>Napa County</td>
<td>Sub-adult</td>
<td>Male</td>
</tr>
<tr>
<td>29</td>
<td>6/30/2016</td>
<td>Napa County</td>
<td>Sub-adult</td>
<td>Male</td>
</tr>
<tr>
<td>30</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>31</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>32</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>33</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>34</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>35</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>36</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>37</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>38</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>39</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>40</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>41</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>42</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>43</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>44</td>
<td>3/3/2016</td>
<td>Nevada County</td>
<td>Sub-adult</td>
<td>Female</td>
</tr>
<tr>
<td>Sample no.</td>
<td>Ct value</td>
<td>Log_{10}GE/mL</td>
<td>Sample no.</td>
<td>Ct value</td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>---------------</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>38.24</td>
</tr>
<tr>
<td>9</td>
<td>36.43</td>
<td>5.92</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>39.82</td>
<td>4.86</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>42.58</td>
<td>4</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>34.3</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>-</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>-</td>
<td>-</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>-</td>
<td>-</td>
<td>26</td>
<td>37.76</td>
</tr>
<tr>
<td>27</td>
<td>-</td>
<td>-</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>43.39</td>
<td>3.75</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>-</td>
<td>-</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>37.01</td>
<td>5.73</td>
<td>34</td>
<td>39.68</td>
</tr>
<tr>
<td>34</td>
<td>39.68</td>
<td>4.91</td>
<td>36</td>
<td>-</td>
</tr>
<tr>
<td>37</td>
<td>-</td>
<td>-</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>37.65</td>
</tr>
<tr>
<td>41</td>
<td>38.05</td>
<td>5.41</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>36.58</td>
<td>5.87</td>
<td>44</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2: Preliminary results of PEDV RT-qPCR assay of fecal samples.
<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Ct value</th>
<th>Log_{10} GE/mL</th>
<th>Sample no.</th>
<th>Ct value</th>
<th>Log_{10} GE/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35.82</td>
<td>5.31</td>
<td>23</td>
<td>36.64</td>
<td>5.09</td>
</tr>
<tr>
<td>2</td>
<td>37.24</td>
<td>4.92</td>
<td>24</td>
<td>36.82</td>
<td>5.04</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>35.84</td>
<td>5.30</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>26</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>36.91</td>
<td>5.01</td>
<td>27</td>
<td>36.68</td>
<td>5.08</td>
</tr>
<tr>
<td>6</td>
<td>37.19</td>
<td>4.94</td>
<td>28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>37.12</td>
<td>4.96</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>31</td>
<td>36.27</td>
<td>5.19</td>
</tr>
<tr>
<td>10</td>
<td>37.31</td>
<td>4.91</td>
<td>32</td>
<td>37.34</td>
<td>4.90</td>
</tr>
<tr>
<td>11</td>
<td>36.68</td>
<td>5.08</td>
<td>33</td>
<td>37.37</td>
<td>4.89</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>34</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>35</td>
<td>36.18</td>
<td>5.21</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>36</td>
<td>36.23</td>
<td>5.20</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>37</td>
<td>35.69</td>
<td>5.35</td>
</tr>
<tr>
<td>16</td>
<td>35.07</td>
<td>5.51</td>
<td>38</td>
<td>36.19</td>
<td>5.21</td>
</tr>
<tr>
<td>17</td>
<td>35.80</td>
<td>5.32</td>
<td>39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>35.28</td>
<td>5.46</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
<td>41</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>36.23</td>
<td>5.20</td>
<td>42</td>
<td>37.21</td>
<td>4.93</td>
</tr>
<tr>
<td>21</td>
<td>35.74</td>
<td>5.33</td>
<td>43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>-</td>
<td>44</td>
<td>37.18</td>
<td>4.94</td>
</tr>
</tbody>
</table>

Table 3: Preliminary results of PDCoV RT-qPCR assay of fecal samples.
Figure 3: Map of California showing sample collection sites
Figure 4: Sequence alignments of the conserved polymerase regions targeted by the two primer sets

“*” Indicates consensus sequence
Figure 5: Gel images showing results of conventional RT-PCR assay using panCoV primer set 1. Primer set was tested using TGEV, PEDV and PDCoV positive samples (5A); gel images for samples: (5B) 1 to 6; (5C) 7 to 11; (5D) 12 to 17; (5E) 18 to 23; (5F) 24 to 29; (5G) 30 to 34; (5H) 35 to 39 and (5I) 40 to 44. PEDV strain Iowa106 was used as positive control while running samples. M refers to marker.
Figure 6: Gel images showing results of conventional RT-PCR assay using panCoV primer set 2. Primer set was tested using PEDV, TGEV and PDCoV positive samples (6A); gel images for samples: (6B) 1 to 14; (6C) 15 to 28 and (6D) 29 to 40 are shown. PEDV strain Iowa106 was used as positive control while running samples. M refers to marker.
Figure 7: Representative graphs showing fluorescent profiles during PEDV and PDCoV specific RT-qPCR assays. Fluorescent profile of PEDV N gene specific RT-qPCR assays: (7A) initial screening; (7B) repetition of selected samples in triplicate and (7C) repetition of selected samples after increasing the reaction volume. (7D) Fluorescent profile of PDCoV M gene specific RT-qPCR.
Figure 8: Gel images of samples subjected to conventional RT-PCR assay using PDCoV M gene specific primers and with increase reaction volume. Gel image for samples: (8A) 1, 6, 10, 11, 16, 18 and 20 and (8B) 21, 24, 25, 27, 31, 35. M refers to marker, PDCoV strain OH-FD22 was used as a positive control.
References


151. Frederick, J.M., Overview of wild pig damage in California. 1998.


