RESEARCH REPORT
SWINE HEALTH

Title: Investigating the pathogenicity of pestiviruses or pesti-like viruses isolated from recent swine epidemics – **NPB #05-023**

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Abstract

Bovine viral diarrhea virus (BVDV) is a virus usually found in domestic and wild ruminants. However, a BVDV was recently isolated from a case of severe mortality (about 55%) in finishing swine. This finding raised a question about what role this virus might have played in the field case. In addition to the BVDV, others agents were detected in affected pigs from this case. One of these was a bacteria that is not commonly found in pigs, Haemophilus parasuis serotype 13. Although different serotypes of H. parasuis can cause significant illness and death in pigs, the bacteria are not typically associated with severe mortality as in the field case. One idea to explain the high mortality observed in this case is that there was an interaction between the BVDV and the H. parasuis resulting in the high mortality.

A series of studies were conducted to 1) characterize the BVDV isolate, 2) test the clinical effects of the BVDV on pigs, and 3) test the potential interaction between the BVDV and the H. parasuis isolates from this field case. At the genetic level the BVDV isolate is most similar to the cattle BVDV type 1b strains. In contrast to type 1b strains, the swine BVDV isolate grows very well in porcine cell lines suggesting it has adapted to swine. Experimental infection of pigs with the BVDV isolate had no negative clinical effect. Experimental infection of pigs with the H. parasuis isolate made them very sick and 4 of the 12 pigs were euthanized for humane reasons. Pigs infected with the BVDV and H. parasuis isolate responded similarly. Collectively, the results of these studies suggest the BVDV isolate did not have a direct role in the high mortality field case, and a significant amount of the mortality could be attributed to infection with this serotype 13 H. parasuis.

Introduction

This report describes studies testing the virulence of a unique virus and bacteria isolated in 2004 from a severe epidemic of mortality in 12-13 week-old-pigs. The onset of disease was recognized several weeks post placement in the finisher. Affected pigs developed lethargy, rear limb weakness, central nervous system disease, and sudden death. Within a few weeks more than 80% of the 966 pigs were affected with about 530 deaths. Live and dead pigs were submitted to a state veterinary diagnostic laboratory for necropsy. Significant findings included swollen hock joints that appeared to be infected with bacteria; lungs of dead pigs were diffusely heavy, red, and wet; lungs of live pigs had random small areas of consolidation affecting 5-10% of the lung; and the tissue lining the brain had a cloudy white appearance as opposed to a normal clear and shiny appearance. Microscopic examination of tissues revealed acute inflammation in multiple organs and joints. This inflammation was compatible with a bacterial infection. The following bacteria, Haemophilus parasuis, Pasteurella multocida, and Arcanobacterium pyogenes, were isolated from one or more samples (joints, lungs, central nervous system) collected from the live and dead pigs. Porcine circovirus type 2 and bovine virus diarrhea virus (BVDV) were identified by PCR, a molecular diagnostic method. Follow up testing isolated the BVDV from pig tissues. Although BVDV has been isolated from swine before, this finding is relatively unique. Likewise, when the H. parasuis was serotyped, it was determined to be a serotype 13, an uncommon serotype in the U.S. The severe outbreak of disease with high mortality in this case raised questions about what role the BVDV might have played in the epidemic, and if there was a synergistic interaction between the BVDV and the H. parasuis serotype 13 detected in this case.

To address these questions, the studies reported here were conducted to characterize the BVDV field isolate, test the clinical effects of this virus on pigs, test the clinical effects of the H. parasuis serotype 13 bacteria on pigs, and determine if there is a synergistic interaction between BVDV and H. parasuis.

Objectives

1) Characterize the antigenic and genetic properties of the BVDV-1 field isolate.

2) Evaluate the pathogenicity of various pestiviruses or pesti-like viruses isolated from field cases of swine disease epidemics.

Materials and Methods:

Source of BVDV and H. parasuis:

The BVDV and H. parasuis isolates were recovered by the University of Minnesota Veterinary Diagnostic Laboratory staff as part of the diagnostic investigation described above. The isolates were provided to NADC as part of the collaborative studies and will be referred to as MN-BVDV and MN-H, respectively. The MN-BVDV was grown in Porcine Kidney cell line (PK-15) to a titer of about 3×10^7 cell culture infectious dose 50%. The MN-H was grown to a titer of 2×10^7 colony forming units (cfu).

Characterization of pestivirus:

The genetic makeup of the virus can be determined by nucleotide sequencing of part or all of the virus genome, the part of the virus that holds all of the genetic information for the virus. For the purposes of this study, a part of the MN-BVDV genome was sequenced and this data was compared to other BVDV strains.

Viruses can only be grown within a cell. A routine laboratory procedure is to grow viruses in cell cultures that have been prepared from various organ tissues from different animal species. Sometimes the ability of a virus to grow, or not grow, in different cell lines can be used to characterize the virus. The ability of the MN-BVDV to grow in various cell cultures was tested.

The antigenic makeup of a virus can be determined by mixing a virus isolate with different monoclonal antibodies. Monoclonal antibodies are important because a monoclonal antibody reacts or binds to only one specific part of a viral protein. The binding of a monoclonal antibody to a viral protein can be expressed as a yes or no event. If a virus is reacted with a number of monoclonal antibodies that are known to bind to different sites, then a pattern of yes or no reactions is observed. These yes and no reactions can be summarized as a virus-specific pattern. The monoclonal antibody pattern for one virus can then be compared to other viruses in an effort to characterize the virus. The MN-BVDV was reacted with a panel of monoclonal antibodies to determine the similarity of this BVDV to other known BVDV-like viruses from other species.

Clinical effects of BVDV in pigs:

A preliminary study was conducted with pigs that were derived by cesarean section and were colostrum deprived. These pigs are referred to as CDCD pigs. The rationale for using CDCD pigs to study the effects of a virus is that the pigs are free from any influence of typical pig diseases. This is because 1) the pig is free from swine pathogens because it was derived by a sterile surgery and is raised in a clean room, and 2) the pig is free from any maternal influence on its immune system because the pig has not suckled the sow and it has not ingested colostrum. The CDCD pig model provides an opportunity to study the direct effect of a virus on the host that is not hindered by other diseases. However, CDCD pigs are not normal and they can be very susceptible to bacterial infections. This issue can be minimized through strict hygiene and the use of antibiotics.

Three separate CDCD pig trials were conducted. Trials 1 and 2 had 10 pigs each that were equally divided into control and principle pigs. Trial 3 had 4 controls and 12 principle pigs. Principle pigs were given an intranasal inoculation of MN-BVDV containing approximately 5×10^6 infectious virions on day 0 of the experiment. Pigs were monitored for clinical effect and rectal temperatures were taken in trial 3. In each trial blood samples were collected on or about experiment days -3, 0 3, 6, 9, 12, 15 and at the post-mortem exam or necropsy. A complete blood cell count (CBC) was performed on each blood sample. The pigs were euthanized with an intravenous injection of pentobarbital and necropsied. Internal organs of pigs were examined for visible signs of tissue damage or lesions.

Clinical effect of dual infection of BVDV and H. parasuis in pigs:

Conventionally-farrowed pigs were used for this study. Sows were purchased from a high-health herd and transported to NADC about 2 weeks prior to farrowing. On gestation day 113 each sow received an intramuscular injection (IM) of ceftiofur crystalline free acid[a] per label instructions. Each pig received 10 mg of ceftiofur crystalline free acid[a] given IM within 12 hours of birth and at weaning (3-4 days-of-age). Weaned pigs were moved into different isolation rooms and fed a milk replacer 4 times per day for 5 days at which time a dry creep feed was mixed with the milk replacer to form gruel. Over the next 11 days the milk replacer was reduced to zero as dry feed was introduced to the pigs. Fresh water was provided at all times.

At 3 weeks of age 45 pigs were randomly divided into Group 1 (non-challenge controls), Group 2 (MN-BVDV challenge), Group 3 (MN-H challenge), and Group 4 (MN-BVDV/ MN-H challenge) consisting of 9, 12, 12, and 12 pigs, respectively. At 10 weeks of age the pigs in groups 2 and 4 received a MN-BVDV challenge on day 0 of the experiment with approximately 5×10^6 infectious virions. On day 7, pigs in groups 3 and 4 received MN-H challenge with approximately 2×10^7 cfu. One-half of the pigs in each group were euthanized on day 10 and the other half on day 14. Blood samples were collected on days -4, 0, 3, 7, 10, and 14 from groups 1 and 2 and from groups 3 and 4 on day 0 and at necropsy. A complete blood cell count was performed on all blood samples. Nasal swabs were collected on day 0, and at necropsy swabs were collected from the nasal cavity, thoracic cavity, abdominal cavity, from both hock joints, and from the surfaces of the brain for bacterial isolation.

Results:

Characterization of pestivirus:

Genetic testing of the MN-BVDV indicated this virus was most similar to the BVDV group 1b viruses, the predominant BVDV subgenotype currently circulating in the U.S. cattle population (Figure 1). The MN-BVDV isolate grew equally well in bovine and porcine cell lines to a virus titer of about 10^7 TCID/ml. Monoclonal antibodies commonly used to detect BVDV group 1b laboratory strains or BVDV1b strains isolated from field cases did not bind with the MN-BVDV isolate. The monoclonal antibody binding pattern was similar to that seen with a BVDV1b strain isolated from a persistently infected deer.

Clinical effects of BVDV in pigs:

When compared to the controls, no significant clinical effect was observed in the MN-BVDV inoculated CDCD pigs. However, several days post-inoculation the character of the feces for some MN-BVDV inoculated pigs changed from a soft loose stool to a dry, formed stool. Constipation was not noted in any of the virus-inoculated or control pigs. At necropsy mild tissue changes or lesions were observed in the MN-BVDV inoculated pigs consisting of a slight increase in the size of the lymph nodes associated with the lungs. No other lesions were noted. No significant differences in the CBC counts were found between the control and the BVDV-challenge group.

Clinical effect of dual infection of BVDV and H. parasuis in pigs:

When compared to the control pigs (group 1), no obvious clinical effect was observed in the MN-BVDV inoculated pigs (group 2). In contrast, severe clinical effects occurred within 48 hours of inoculation in both groups that received MN-H (Table 2). They consisted of acute lameness in one or more legs, listlessness, loss of appetite (anorexia), and loss of coordination (ataxia). The severity of the disorders varied between pigs and in some pigs they became moribund or unable to rise combined with a lack of awareness. However, some pigs appeared relatively normal at the time of necropsy. At necropsy, lame pigs generally had one or more swollen joints that contained excessive amounts of fluid that ranged from clear and thin in appearance to cloudy and thick with extensive swelling in the tissues surrounding the joint. Some of the pigs had severe inflammation of the chest and abdominal cavities resulting in deposition of fibrin, a protein that can accumulate in the body following severe inflammation. As in the previous study, no significant differences in the CBC counts were found between the control and the MN-BVDV-challenge group. A significant increase in total white blood cells and neutrophils was detected in the MN-H challenge groups (data not shown). No significant differences were detected in the lymphocyte populations tested following MN-BVDV challenge. No H. parasuis was isolated from the control pigs or from the day 0 nasal swabs (data not shown). H. parasuis was isolated from one or more swab samples collected at the time of necropsy from all 24 pigs experimentally infected with MN-H (Table 2). A systemic infection was detected in 8 and 4 of the pigs in groups 3 and 4 respectively.

Discussion:

The MN-BVDV isolated from the field case is unique. Based on genetic analysis this virus is most closely related to a cattle BVDV virus known as BVDV type 1b. However, the ability of this MN-BVDV virus to grow readily in swine cell lines suggests the virus may be pig adapted. This is in contrast to BVDV type 1b isolates that grow poorly, or not at all in porcine cell lines. Antigenic testing was interesting because monoclonal antibodies commonly used to detect BVDV1b laboratory strains or BVDV1b strains isolated from field cases did not bind with the MN-BVDV isolate. Surprisingly, the monoclonal binding pattern was similar to that seen with a BVDV1b strain isolated from a persistently infected deer. While the binding panels for the porcine and deer isolates are similar, genetic sequence analysis suggests these two viruses are not closely related to each other. The significance of these observations is not clear at this time.

CDCD pigs were chosen to test the effects of the MN-BVDV isolate because they should be free of BVDV, and they do not have a highly adapted immune system. Because of this, CDCD pigs are generally more susceptible to pathogens than conventionally-raised pigs making CDCD pigs a sensitive model for studying the effects of a viral disease. Some of the CDCD pigs had a change in the consistency of their stool 4-5 days after inoculation when the stool became drier and firmer when compared to the controls. We are not sure of the significance of this observation, but it is interesting because constipation has been observed in pigs exposed to classical swine fever virus, a virus that infects swine and is closely related to BVDV. No other clinical signs were noted following BVDV infection of the CDCD pigs. Likewise, no significant changes in the CBC for the BVDV-infected pigs were found.

Based on these studies the MN-BVDV isolate does not appear to be highly pathogenic in CDCD pigs and probably not in conventionally-raised pigs as well. The lack of clinical effect following an experimental BVDV infection of pigs has been reported by others.(Carbrey et al., 1976; Walz et al., 1999) Thus, it seems unlikely the MN-BVDV isolate was the primary cause of the high mortality reported in the field case. However, infection with this virus may have predisposed pigs to an infection with one or more other agents, specifically, the H. parasuis serotype 13 isolate identified in a number of pigs that died during the early finishing stage of production.

The second pig study was designed to test the effect a MN-BVDV infection might have on a subsequent H. parasuis infection. Based on the work of others, CDCD pigs might be too sensitive to an H. parasuis infection resulting in death.(Blanco et al., 2004; Vahle et al., 1997) If the H. parasuis pig model was too sensitive, then it would not be possible to design an experiment that would demonstrate a synergy between the MN-BVDV and MN-H. For example, if all of the MN-H inoculated pigs died, then it would not be possible to determine if the MN-BVDV infection increased the severity of the MN-H infection. A less sensitive H. parasuis infection model, when compared to CDCD pigs, utilizes conventionally-farrowed pigs given an oronasal inoculation.(Blanco et al., 2004; Brockmeier, 2004; Oliveira et al., 2003) In this scenario, the oronasal inoculation route will colonize the pigs, but not necessarily make them sick. This was not the case in the second pig study. The MN-H isolate given oronasally by itself was very pathogenic with 4 of 6 Group 3 pigs having a systemic H. parasuis infection by 3 dpi. Three of these pigs were severely affected. Interestingly, this is in contrast to Group 4 pigs at 3 dpi. At this time point, 0 of 6 Group 4 pigs has a systemic infection and none of them were severely affected. However, by 7 dpi 4 of the 6 remaining Group 4 pigs had a systemic infection and all 4 were severely affected. Based on this study testing the interactions of MN-BVDV and MN-H, it appears 1) the H. parasuis serotype 13 isolated from the field case is very pathogenic and probably played a significant role in the high mortality reported in the field case; and 2) any interaction between the MN-BVDV and MN-H was limited. As would be expected, the CBC count

was significantly elevated with a dramatic neutrophillia following the MN-H challenge. There were no significant differences in the CBC counts between groups 3 and 4.

During the last year a syndrome of an acute onset of high mortality in finishing-age swine has been reported in the United States. These cases are indistinguishable from cases in Canada that were first reported about 2 years ago. Investigations into the U.S. epidemics have consistently identified porcine circovirus type 2 (PCV2) in tissues collected from affected pigs. Preliminary studies have identified a PCV2 genotype that has not been recognized previously in North America (unpublished observations). This genotype is referred to as PCV2 group 1, and the PCV2 that is ubiquitous in North America is referred to as PCV2 group 2. There is speculation about the role of PCV2 group 1 in the high mortality syndrome. For example, Could this PCV2 genotype play a significant role in causing death in finishing-age swine? The case from which the MN-BVDV and MN-H were isolated involved pigs imported from Canada early in 2004. Perhaps this case of high mortality in finishing-age swine in Minnesota involving pigs was related to the PCV2 genotype 1 virus. PCV2 was detected in tissues from this case and preliminary analysis indicates this PCV2 is the group 2 virus, the virus that is endemic in North America. The hypothesis that the PCV2 group 1 virus may have played a role in this epidemic is not supported; however, PCV2 group 2 may have had a role.

In summary, the MN-H isolate was very pathogenic in our study, we found no clinical effect attributed to the MN-BVDV, and we did not demonstrate an increase in disease with a dual infection of MN-H and MN-BVDV.

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[a] EXCEDE™ for Swine, Pfizer Animal Health.

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Lay Interpretation.

Bovine viral diarrhea virus (BVDV) is a virus usually found in domestic and wild ruminants. However, a BVDV was recently isolated from a case of severe mortality (about 55%) in finishing swine. This finding raised a question about what role this virus might have played in the field case. In addition to the BVDV, others agents were detected in affected pigs from this case. One of these was a bacteria that is not commonly found in pigs, Haemophilus parasuis serotype 13. Although different serotypes of H. parasuis can cause significant illness and death in pigs, the bacteria are not typically associated with severe mortality as in the field case. One idea to explain the high mortality observed in this case is that there was an interaction between the BVDV and the H. parasuis resulting in the high mortality.

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Table 1: Monoclonal antibody (Mab) binding pattern for BVDV strains based on reaction with a panel of 5 Mab.

	BVDV Strains							
Mab	NY-1	TGAN	Gill93	Hess9804	NMSU925	Hess135-89	Deer	Pig
CA-1	Y	Y	Y	Y	Y	Y	N	N
CA-3	Y	N	N	Y	Y	Y	N	N
CA-34	Y	Y	Y	Y	Y	Y	Y	Y
CA-36	Y	Y	Y	Y	Y	Y	N	N
CA-82	Y	Y	Y	Y	Y	Y	N	N

BVDV type 1b viruses: NY-1 and TGAN – Laboratory reference strains; Gill93, Hess9804, NMSU925, Hess135-89 – Field Reference Strains; Deer, Pig - BVDV isolated from deer and pig.

Y = Yes, the Mab reacted with the specific virus strain.
N = No, the Mab did not react with the specific virus strain.

Table 2: H. parasuis bacterial isolation on swabs and lung lavage collected from pigs 3 and 7 days-post-inoculation (dpi) with MN-H.

	Group	Nasal	Trachea	Chest	Abdomen	Joint	Brain	Lung
3 dpi	3	6*	5	3	3	3	3	4
	4	6	6	0	0	0	0	6
7 dpi	3	6	5	1	2	0	2	4
	4	6	5	1	1	4	1	6

Group 3 = Pigs inoculated with MN-H on day 7 of the experiment and euthanized 3 and 7 dpi with MN-H.
Group 4 = Pigs inoculated with day 0 of the experiment with MN-BVDV and with MN-H on day 7 of the experiment and euthanized 3 and 7 dpi.
* Number of pigs positive for H. parasuis out of 6 pigs tested at that time.

Figure 1: Genetic comparison of the MN-BVDV (goyal pesti) with swine pestivirus isolates (CB5c, CSFV-Alfort) and cattle pestivirus isolates (BDVDtype 2-890 strain, BVDVtype 1a-NADL strain, BVDVtype 1b-NY-1 strain).