Original Article

Reproductive Loss due to Pestivirus Infection in Dairy Cattle Herds in Thailand

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Abstract

Infection with bovine pestivirus, Bovine Viral Diarrhoea Virus (BVDV), causes significant loss in cattle production worldwide. A new type of bovine pestivirus, atypical pestivirus, was identified in natural infected calf firstly in Khon Kaen, Thailand in 2004. The epidemiological studies have been done in the area, but loss in reproduction due to the infection has never been accessed. This study aims to investigate losses in herd's reproduction and update epidemiological data in the population where atypical pestivirus was found. During 2008-2009, 420 dairy herds in the area were classified according to infective status into high-positive and non-infective herds by antibody analysis in bulk tank milk samples. Data of reproduction of cows in both groups, at the same proportion, with 4 indices, i.e. calving to first service interval (CFS), calving to conception interval (CCI), calving interval (CI) and overall pregnancy rate, were compared by t-test. The CSF, CCI, CI and overall pregnancy rate of the high-positive herds were significantly (p<0.05) poorer than the non-infective herds; 121 vs 89 days, 170 vs 127 days, 450 vs 406 days and 49% vs 57%, respectively. Moreover, the proportion of the high-positive herds did not change from the 4-year earlier investigation, indicating that the virus still circulates in the area and may cause losses in Thai dairy production in the near future.

Keywords: bovine viral diarrhea virus, cattle, pestivirus, reproductive loss

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บทคัดย่อ

การสูญเสียทางการสืบพันธุ์ในโคนมไทย จากการติดเชื้อไวรัสเพสติ

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การติดเชื้อเพสติไวรัส (pestivirus) ในโคหรือไวรัสโบวายไวรัลไดอะเรียส่งผลให้เกิดความเสียหายอย่างยิ่งต่อการผลิตโค การศึกษาในประเทศไทยปี พ.ศ. 2547 พบการติดเชื้อเพสติไวรัสสายพันธุ์ใหม่ เป็นเพสติไวรัสชนิดพิเศษ (atypical pestivirus) ในลูกโคนมในเขตจังหวัดขอนแก่น แต่ยังไม่มีการศึกษาผลต่อการสืบพันธุ์ในโค การศึกษาในครั้งนี้มุ่งแสดงผลของไวรัสต่อการสืบพันธุ์และศึกษาด้านระบาดวิทยาของไวรัสนี้ในพื้นที่ที่พบเพสติไวรัสชนิดพิเศษนี้ในปีพ.ศ. 2551-2552 น้ำนมถังรวมจากฟาร์มโคนมจำนวน 420 ฟาร์มได้ใช้เป็นตัวแทนฟาร์มเพื่อประเมินภาวการณ์ติดเชื้อ โดยแบ่งฟาร์มเป็นกลุ่มที่มีการติดเชื้อในระดับสูงและกลุ่มที่ไม่ติดเชื้อ จากนั้นเก็บข้อมูลด้านสมรรถนะการสืบพันธุ์ในแม่โคทั้งสองกลุ่ม 4 ดัชนี ได้แก่ ระยะห่างวันคลอด-ผสมครั้งแรกระยะห่างวันคลอด-ผสมติด ระยะห่างวันคลอดและร้อยละการผสมติด ผลการศึกษาพบว่าดัชนีการสืบพันธุ์ทั้ง 4 ดัชนีในแม่โคของฟาร์มที่มีการติดเชื้อในระดับสูงและที่ไม่ติดเชื้อ มีค่าที่แตกต่างกันอย่างมีนัยยะสำคัญ ($p<0.05$) ได้แก่ระยะห่างวันคลอด-ผสมครั้งแรก121 และ 89 วัน ระยะห่างวันคลอด-ผสมติด 170 และ 127 วัน ระยะห่างวันคลอด 450 และ 406 วัน และ ร้อยละการผสมติดเท่ากับ 49 และ 57 ตามลำดับ นอกจากนี้พบว่าอัตราส่วนของฟาร์มที่มีการติดเชื้อในระดับสูงนี้ไม่ลดลงจากการศึกษาเมื่อปี พ.ศ. 2547 แสดงถึงการมีไวรัสนี้อยู่ในประชากรโคในพื้นที่และอาจก่อความเสียหายมากขึ้นในอนาคต

คำสำคัญ: โบวาย ไวรัลไดอะเรีย โค เพสติไวรัส การสูญเสียทางการสืบพันธุ์

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Introduction

Bovine viral diarrhea virus (BVDV) is one of the common causes of losses in cattle reproduction worldwide. Many studies indicate that the estimated cost of losses are high (Houe, 2003; Fourichon et al., 2005; Valle et al., 2005; Lee et al., 2008). There are two well-known genotypes of Pestivirus in cattle; BVDV-1 and BVDV-2, which differ in both antigenic and genetic properties and could be identified in cattle population in many countries. However, a new group of pestivirus in cattle was found in Brazil, Thailand and recently, Italy and they were classified into atypical pestivirus or, unofficially, BVDV-3 (Schirrmeier et al., 2004; Cortez et al., 2006; Ståhl et al., 2007; Decaro et al., 2011). The Thai atypical pestivirus isolate, Th04/KhonKaen, was the first atypical pestivirus isolate that identified in a live calf of a dairy cattle herd in Khon Kaen province (Ståhl et al., 2007; Kampa et al., 2010).

In general, cattle infected with BVDV usually undetected by clinical appearances. However, the infected cattle may show signs of secondary infections due to the immunosuppressive effects of BVDV especially in calf (Peterhans et al., 2003; Burciaga-Robles et al., 2010). In pregnant animal, the virus always seems to cross the placenta and may cause a variety of effects to embryo and/or fetuses, depending on the cow's immune status and age of gestation (Fray et al., 2000). In susceptible herds, infection may result in a consequence of reproductive failure such as embryonic death, repeat breeding, abortions, malformed or stillborn calves. One of the most important consequences is a production of life-long virus transmitter, persistently infected (PI) animal which do not have any immune response to the persisting virus. PI resulted from a vertical transmission during the first trimester of pregnancy. Thus, as a key measurement of the virus control, PI must be indentified and removed. Still, an effective control measure will also require a continuing surveillance protocol such as gathering herd's status by pooled sample, testing regularly and preventing the viral-introduction into free-virus herds (Alenius et al., 1997; Lindberg and Houe, 2005).

In Thailand, failure on cattle reproduction is one of the most concerned problems of farmers. The overall pregnancy rate of dairy cows in Thailand is less than 55% (Bureau of Biotechnology in Livestock Production, 2010). Although epidemiological data indicated a wide-spread of BVDV in dairy cattle in

Thailand (Virakul et al., 1997; Kampa et al., 2004; Kampa et al., 2009) and the atypical pestivirus was identified in Khon Kaen province in 2004, reproductive losses due to the BVDV infection have never been studied. Furthermore, since there has been no implementation of strict BVDV control in any cattle population of Thailand, thus epidemiological data at present status are also needed. The purposes of the study were to gain further knowledge on the epidemiological data of BVDV infections among dairy herds in a population where atypical pestivirus was found. A loss in herd's reproduction was also studied to determine the significance of the infection in dairy population that cattle were free to trade without control of BVDV.

Materials and Methods

Study population and classification of herds: In October 2008-May 2009, a cross-sectional study was carried out in small-medium sizes dairy cattle herds Khon Kaen province. In this area, vaccination of BVDV has never been practiced. Most of the milk production in this province which has about 420 dairy herds, is commercialized through 3 public milk collection centers (MC 1, 2 and 3) which are located nearby. Bulk tank milk (BTM) samples, from all 420 herds, were used as herd's representative for its BVDV status. By BTM analyses with an indirect ELISA (SVANOVIR® BVDV-Ab kit, SVANOVA Biotech AB, Sweden) herds were classified into class 0-3 BVDV status according to the Scandinavian BVDV control programme (Niskanen, 1993; Jalali et al., 2005). In the programme, class 0 herds have a very low or low level of antibodies in the bulk milk because of no infection of the virus. Herds in classes 2 or 3 have a moderate or high of level of antibodies which probably result from a previous infection. Class 3 herds have a very high antibody level in BTM because of an active infection and are likely to have PI which generally expresses itself as viral source and causes seroconversion in the surrounding animals.

Comparison of herd's reproductive performance in class 0 vs. class 3 herds: Herds in class 0 and 3, at the same proportion in each MC, their cows were data collected by using individual heath and reproductive records retrospectively from 2007 until the date of individual sample collection. The reproductive data comprise four reproductive index, i.e. calving to first service interval (CFS), calving to conception interval (CCI), calving interval (CI) and overall pregnancy rate. Associations of the reproductive index with infective status (class 0 *vs.* class 3) were analyzed by Independent sample *t*–test or Two-independent sample *t*-test depending on distribution of the data. P-values ≤ 0.05 were considered statistically significant.

Identification of PI in class 3 herds: Milk or serum samples were individually collected from all cattle in class 3 herds for identification of infective status (free-/ acutely-/ persistently infected). Antibody of were firstly detected by an indirect ELISA (IDEXX BVDV total Ab test, IDEXX Laboratories, Inc). The seronegative samples were tested further with a commercial E^{rns} capture ELISA (Herd Check BVDV Ag/Serum plus, IDEXX Laboratories, INC.) for detection of BVDV antigen. All ELISA procedures were done according to the manufacturers' instructions. If any cattle had BVDV antigen in their samples, a new sampling was taken on the next 4-5 months to certify PI status of the cattle. Record of semen used in the PI's mother was also investigated.

Sample collection, treatment and storage: After collection, milk and blood samples were transported at 4-8^{0}C to the laboratory of the Faculty of Veterinary Medicine, Khon Kaen University on the day of the sampling. Fat in milk was discarded after 1000x g centrifugation and the skim milk samples were kept at -20^{0}C until analyzed with the indirect ELISA. Serum samples were centrifuged at 1000x g, and then were divided into 2 parts. The first part was heat-inactivated at 56^{0}C for 30 min and stored at -20^{0}C in 2.0-ml vials until analysed with the antibody and antigen capture ELISAs. The second part was stored at -20^{0}C until identified of atypical pestivirus with RT-PCR.

Identification of the atypical pestivirus by One-step RT-PCR: The viral RNA was extracted directly from the antigen positive samples using TRIzol Reagent (Invitrogen, Carlsbad, USA), following the manufacturer's instructions. A specific fragment of atypical pestivirus was amplified with 2 atypical pestivirus primers, designed by Liu et al. (2008). The One-step RT-PCR assay was performed using SuperScript ™One-step RT-PCR with Platinum *Taq* (Invitrogen ™) with the following mixture; 7 µl of template RNA, 10 µM of each primers, 1 µl of RT/Platinum *taq* mix, and 25 µl of reaction mix and autoclaved distilled water. The assay was run in Techne TC 512 (Techne Inc) with the following thermal steps: reverse transcription at 45^{0}C for 25 min, then the synthesized cDNA was initially denatured at 95^{0}C for 2 min. The PCR step was followed by 35 cycles of denaturation at 94^{0}C for 15 sec, annealing at 55^{0}C for 30 sec and extension at 72^{0}C for 60 sec. The PCR products, size 86 bp, were detected by gel electrophoresis method. RNA of BVDV-2 strain 178003 and double distilled water were used as negative controls.

Results

Prevalence of BVDV infection in 420 dairy cattle herds in Khon Kaen: By an indirect ELISA test, prevalence of BVDV-antibody positive herds, class 1-3, among the 420 herds was 35%. Results from BVDV analyses of the 420 BTM samples are given in Table 1. Among the 148 seropositive herds (65%), 41 herds (10%) were classified as class 3 or herds in active infection.

Prevalence of PI: By farmer's allowance, all cattle in 39 out of 41 class-3 BVDV herds were individually sampled. The total number of samples was 975. There were 503 samples (52%) that had specific antibodies to BVDV. All seronegative samples were further analyzed by E^{rns} antigen ELISA. The antigen detection test revealed 5 antigen positive sera from

Table 1 Bovine viral diarrhoea virus antibody levels in bulk tank milk samples from 420 dairy herds, collected from 3 milk centres (MC) in Khon Kaen, Thailand on October 2008, classified into 4 BVDV infection levels.

MC	Number of herds	Number of herds in BVDV class (%)			
		0	1	2	3
1	193	114 (59%)	46 (24%)	8 (4%)	25 (13%)
2	132	83 (63%)	32 (24%)	3 (2%)	14 (11%)
3	95	75 (79%)	15 (16%)	3 (3%)	2 (2%)
Total	**420**	**272 (65%)**	**93 (22%)**	**14 (3%)**	**41 (10%)**

4 dairy cattle herds; 3 herds in MC1 and another herd in MC2. A second serum sampling in all the cattle of 4 herds was done in 4-5 months after the first herd visit. Before the second sampling was done, a calf of herd number 2 died. Thus, there were 4 confirmed PI cattle of 4 dairy herds. Herd 1 and 2 were neighbors. Cattle of both herds could contact each other over their fence.

Effect of BVDV infection on herd's reproductive performance: Among the visited 39 BVDV class-3 herds, 2 herds had poor records, but samples were individually collected from all cattle. The other two herds were not allowed for research. Thus, the total number of herds in the study of reproductive performance was 37 herds of class-3 BVDV. Data on reproduction were collected from 37 herds of class 0 which was located close to class 3 herds. The overall data were collected from records of 366 cows of class 0 (37 herds) and 357 cows of class 3 (37 herds) BVDV herds. By statistical analysis the mean values of each reproductive index of cows in each group were significantly different (p<0.05) (Table 2). During the past 2 years, there were no records of introducing new animals into the antigen positive herds. All antigen positive calves were born by different semen ID.

Circulating of atypical pestivirus in dairy cattle in Khon Kaen: Four persistently infected animals were confirmed their status by second samplings. A one-month old calf died (calf number 2 of herd 2) before a new blood sample was taken. Thus, the calf was identified as a virus-positive animal. Serum of the virus-positive calf and 4 PI animals were RNA extracted and diagnosed by RT-PCR for searching of atypical pestivirus. In Table 3 results from diagnosis by RT-PCR indicated a persistent infection with mixed BVDV strains in the studied area. In MC 1, there were 3 herds (herd number 1-3) of PI presentation. PI animals in herd 1 and 3 were not infected with atypical pestivirus as well as the virus-positive calf of herd 2. Another calf of herd 2 and a calf in herd 4, which were situated in MC 2, were infected with atypical pestivirus. By cow's records, all PI-mothers used frozen semen from various bulls. None of the PI calf was born by the same bull.

Table 2 Comparison of herd's reproductive index between 37 BVDV class-3 and 37 BVDV class-0 dairy cattle herds in Khon Kaen province.

Reproductive index	Herd's BVDV status		p- Value
	Class 3	Class 0	
Calving to first service interval (mean±SD; days)	121.59±34.08	89.05 ±23.97	p<0.001
Calving to conception interval (mean±SD; days)	170.69±39.87	127.03±30.11	p<0.001
Calving interval (mean±SD; days)	450.42±38.42	406.60±30.85	p<0.001
Overall pregnancy rate (%)	48.54	56.82	p<0.05

Table 3 Identification of persistently infected animals in 39 class 3-BVDV infected herds in Khon Kaen province, 2009.

MC	Herd No.	Calf No.	Age of animal at each antigen detection (months)		Atypical pestivirus
			1st	2nd	
1	1	1	2	6	No
	2	2	1	death	No
		3	6	11	Yes
	3	4	10	15	No
2	4	5	7	12	Yes

Discussion

Bulk tank milk investigation showed a low proportion of infected BVDV herds, 35%, in Khon Kaen province. The proportion of the free-BVDV herds increased noticeably from 33% in year 2004 to 65% in year 2008 (Kampa et al., 2009). Without intervention, the population could be free from the BVDV infection by natural process and common herd practice; this is called 'self-clearance' process which occurs when PI animals are removed from the herd because of death, trade or culling of poor performance and the herd must not be re-introduced of the virus. Many epidemiological studies confirm the process among cattle population in many part of the world, including Thailand (Viltro et al., 2002; Ståhl et al.,

2006; Kampa et al., 2009). This evidence of dairy herds in Khon Kaen province has obviously been seen from 2000 to the present study. By bulk tank milk studies, prevalence of BVDV infections decreased from 89% in year 2000 to 35% in year 2008 without any actively control efforts taken against the infection. Even in the recent years, the regulations concerning BVDV are officially controlled only at bull stations, but other stations are not involved in this control. Cattle trade within the country has no regulations concerning BVDV. Furthermore, vaccines against BVDV have not been used in this area of Thailand. Thus, our study confirmed the self-clearance process of BVDV infection in Khon Kaen dairy population. However, 10% of the dairy herds were still in class 3 which remained unchanged from the investigation 4 years earlier, which gave a critical starting point of new transmission and/or endemic circulation of the virus in a restrict group. Furthermore class-3 BVDV herds were recently found in MC 2 and MC 3, supporting a circulating BVDV in a small group in the area. Although a direct transmission of BVDV mostly occurs via direct contact with PI animals, which is possible in a neighboring herd or co-pasture herds, it merely happens in a different population like MC. Because each MC had their service persons and distance between farms was far, transmission from MC to MC is unlikely to happen.

Infection of BVDV caused considerable effects on Thai dairy herd's reproductive performance. Herds in class 3 had obviously poorer reproductive index than class 0. Moreover, the overall pregnancy rate of cows in class 3 herd at 48.54% was lower than the dairy cattle herds both at province level, 54.5%, and at national level, 54.8% (Bureau of Biotechnology in Livestock Production, 2010). The effects indicate an outcome of trans-uterine infection that causes embryonic loss, abortion, mummy and stillbirth (Bielefeldt-Ohmann 1995; BonDurant, 2007). In Brazil, the finding of atypical pestivirus isolates from aborted bovine fetuses suggested that pestivirus circulated in the cattle population of Brazil and also caused reproductive problems as well (Cortez et al., 2006).Although herd management may affect the investigated index, class 0 herds were selected by their locations that were close to the class 3 herds. Thus, herd management did not apparently affect the index.

Five virus positive calves were found in 4 dairy herds, and 4 of them were confirmed as PI. The PI calves functioned as a herd source of viral spreading since the large amount of virus shed thereby produced a high proportion of seropositive cattle including the lactating cows, thus had high antibody levels in BTM. All PI and another virus positive calf were younger than 2 years. No PI was found in groups of heifers or cows. Studies by Houe (1993) and Baker (1995) suggested that most PI had poor conditions, i.e. malformation, weakness and growth retardation, thus they usually died at young age. Studies by Duffel (1985) and Muñoz-Zanzi et al. (2003) also reported a high mortality rate of PI in the first year. Furthermore, the poor condition calf were usually culled, hence low number of adult PI were identified.

Results from RT-PCR revealed at least 2 genotypes of BVDV circulating in the dairy cattle in Khon Kaen. Interestingly, herd 1 and 2 are neighbors, cattle enabling to the contact between a loose fence. But a PI calf of herd 2 carried atypical pestivirus while the PI of herd 1 did not. In addition, the virus positive calf of herd 2 was not infected with atypical pestivirus, but was infected with different genogroup from its companions. Although virus mutation may occur, as point mutation, a difference at genotype level is uncommon by direct transmission via herd's PI. The most common route of BVDV transmission is direct contact between animals; from PI to susceptible animal. In our present study, it was most likely that the different strains had been introduced to the cattle population by some means rather than direct contact via PI. Because all dams that gave birth to all 4 PI and a virus-positive calf were not PI and they were raised in herds. In addition, there was no record of introduction of new animals into the herds. Thus, the viruses were introduced individually to each cow. Cryo-preserved semen is suggested as one of the potential sources of trans-boundary infection (Kirkland et al., 1994; Rikula et al., 2008). The other contaminated biological products such as multi-dose drug/vaccine in large vial can be responsible for the (re)introduction of the virus as well (Niskanen and Lindberg, 2003). Although the most probable factor was indirect transmission via semen and insemination, none of the PI was born by the same semen ID. Thus, other routes of indirect transmission are needed to be investigated further.

Conclusion

The study presented losses in reproduction due to BVDV infection in dairy cattle in Thailand, which affect from difference genotypes of bovine pestivirus. Evidence of an ongoing self-clearance process of the infection is confirmed, but with a caution of the virus circulation in the area because of an unchanging class-3 proportion. More studies in other dairy raising areas in Thailand by means of BTM investigation, antigen detection and molecular studies are therefore warranted for more data on the spreading of the atypical pestivirus and other genotypes, to gain the whole picture of the infection in Thailand and to plan a proper country-control measure.

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