SEROPREVALENCE OF BVD AND MOLECULAR DETECTION OF THE VIRUS IN CATTLE AND BUFFALOES AND ITS RISK FACTORS IN SELECTED FARMS IN SELANGOR AND SABAH, MALAYSIA

By

NURULHIDAYAH BTE KHALID

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Veterinary Science

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Bovine viral diarrhoea virus (BVDV) is a single stranded plus sense RNA virus of Pestivirus genus under *Flaviviridae* family. Bovine viral diarrhoea (BVD) disease is manifested by diarrhoea and immunosuppression that exacerbates other respiratory diseases. BVDV seroprevalence in cattle was reported in Malaysia, but no BVDV antigen detected and isolated. Furthermore, there was no BVDV study conducted in buffaloes in this country. The objectives of this study were to determine the seroprevalence of BVDV, to isolate, identify, and molecularly characterized local BVDV, and to determine the risk factors of BVDV in cattle and buffaloes in the selected farms. Seroprevalence of BVDV was determined using commercial kit LSIVet™ Ruminant BVD/BD p80 kit-Serum/Milk (ThermoFischer Scientific, USA). The overall seroprevalence of BVDV was 28.2% (95% CI=22.1-35) and was significantly different between farms ($P<0.01$, $\chi^2=67.172$). Farm G, A, D, H and C have seroprevalence of 61.8%, 51.9%, 41.7%, 10.2%, and 10.0% respectively. Farm B and Farm F were seronegative. All serum samples of buffaloes were seronegative. The BVDV seroprevalence were significantly different between species ($P=0.01$, $\chi^2=10.504$). Seroprevalence of BVDV for both cattle and buffaloes were 24.8% (95% CI=19.3-30.9). The individual and management risk factors were determined by logistic regression analyses. Potential risk factors for BVDV seroprevalence were lactating animals (OR=2.244, 95% CI=1.163-4.331), intensive system (OR=5.914, 95% CI=2.147-16.295), the use of AI (OR=17.723, 95% CI=7.101-44.234), has milking parlour (OR=2.151, 95% CI=1.096-4.224), shared pen for feeding (OR=7.729, 95% CI=2.065-28.926), did not have employee in the farm (OR=3.958, 95% CI=1.718-9.120), have the employee staying inside the farm (OR=6.469, 95% CI=2.555-16.375), did not have separate vehicle drop off area (OR=5.957, 95% CI=1.938-18.32), did not restrict visitor access (OR=5.957, 95% CI=1.938-18.32). Only one sample positive (1/253) following RT-PCR targeting conserved 5'UTR region of BVDV. Thus, BVDV antigen prevalence was 0.40% (95% CI=0.0-2.2). UPM/MAL/BVDV/D17 was classified under subgenotype BVDV-1a as determined by the hypervariable E2 region of BVDV. Adaptation in bovine turbinate (BT) cell cultures produced cytopathic effects (cpe) but

it was not specific to BVDV. In conclusion, BVDV is present and circulating in cattle but not in buffaloes. Since BVDV exists in many subgenotypes, it is imperative for Malaysia to control the entry of more BVDV subgenotypes by screening all the incoming cattle at all borders.

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SEROPREVALENS BVD DAN PENGESANAN MOLEKUL VIRUS PADA LEMBU DAN KERBAU DAN FAKTOR RISIKONYA DI LADANG TERPILIH DI SELANGOR DAN SABAH, MALAYSIA

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Virus cirit-birit viral bovin (BVDV) adalah virus RNA bebenang tunggal dengan kekutuban positif dari genus Pestivirus dan keluarga *Flaviviridae*. Penyakit cirit-birit viral bovin (BVD) dimanifestasikan oleh cirit-birit dan imunotindasan yang menyumbang kepada penyakit pernafasan. Seroprevalens BVDV pada lembu dilaporkan di Malaysia, tetapi tiada antigen BVDV dikesan dan diasingkan. Tambahan pula, tiada kajian BVDV dijalankan terhadap kerbau di negara ini. Objektif kajian ini adalah untuk menentukan seroprevalens BVDV, untuk mengasing, mengenal pasti, dan mencirikan secara molekul BVDV tempatan, dan menentukan faktor risiko BVDV pada lembu dan kerbau di ladang terpilih. Seroprevalens BVDV ditentukan menggunakan kit komersial LSIVet™ Ruminant BVD/BD p80 kit-Serum/Milk (ThermoFischer Scientific, USA). Keseluruhan seroprevalens BVDV ialah 28.2% (95% CI=22.1-35) dan berbeza dengan ketara antara ladang ($P<0.01$, $\chi^2=67.172$). Ladang G, A, D, H dan C mempunyai seroprevalens masing-masing 61.8%, 51.9%, 41.7%, 10.2%, dan 10.0%. Ladang B dan Ladang F adalah seronegatif. Semua sampel serum kerbau adalah seronegatif. Seroprevalens BVDV adalah berbeza dengan ketara antara spesies ($P=0.01$, $\chi^2=10.504$). Seroprevalens BVDV untuk kedua-dua lembu dan kerbau adalah 24.8% (95% CI=19.3-30.9). Faktor risiko individu dan pengurusan ditentukan oleh analisis regresi logistik. Faktor risiko yang berpotensi untuk seroprevalens BVDV ialah haiwan menyusu (OR=2.244, 95% CI=1.163-4.331), sistem intensif (OR=5.914, 95% CI=2.147-16.295), penggunaan permanian beradas (AI) (OR=17.723, 95% CI=7.101-44.234), mempunyai tempat memerah susu (OR=2.151, 95% CI=1.096-4.224), berkongsi bekas makan (OR=7.729, 95% CI=2.065-28.926), tidak mempunyai pekerja di ladang (OR=3.958, 95% CI=1.718-9.120), pekerja tinggal di dalam ladang (OR=6.469, 95% CI=2.555-16.375), tidak mempunyai kawasan memunggah yang berasingan (OR=5.957, 95% CI=1.938-18.32), tiada sekatan akses pelawat (OR=5.957, 95% CI=1.938-18.32). Hanya satu sampel positif (1/253) berikutan penyasaran RT-PCR ke kawasan terpelihara 5'UTR BVDV. Oleh itu, prevalens antigen BVDV ialah 0.40% (95% CI=0.0-2.2). UPM/MAL/BVDV/D17 dikelaskan di bawah subgenotip BVDV-1a seperti yang ditentukan oleh kawasan hipervariasi E2 BVDV. Adaptasi dalam kultur sel turbinat

lembu (BT) menghasilkan kesan sitopatik (cpe) tetapi ia tidak khusus untuk BVDV. Kesimpulannya, BVDV hadir dan beredar dalam lembu tetapi tidak pada kerbau. Memandangkan BVDV wujud dalam banyak subgenotip, adalah penting bagi Malaysia untuk mengawal kemasukan lebih banyak subgenotip BVDV dengan menyaring semua lembu yang masuk di semua sempadan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

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3'UTR	3' untranslated regions
5'UTR	5' untranslated regions
Ag-ELISA	Antigen enzyme-linked immunosorbent assay
AGID	Gel immunodiffusion
AI	Artificial insemination
AUD	Australian Dollar
BAV-3	Bovine adenovirus type 3
BDV	Border's disease virus
BoHV-1	bovine herpesvirus type 1
BPIV-3	Bovine parainfluenza virus type 3
BRSV	Bovine respiratory syncytial virus
BT	Bovine turbinate, turbinat lembu
BVD	Bovine viral diarrhoea, cirit-birit viral bovin
BVDV	Bovine viral diarrhoea virus, virus cirit-birit viral bovin
CD46	Cluster of differentiation 46
CI	Confidence interval
CO_2	Carbon dioxide
CP	Cytopathic
cpe	Cytopathic effects, kesan sitopatik
CSF	Cerebrospinal fluid
CSFV	Classical swine fever virus
DB772	2-(2- benzimidazolyl)-5-[4-(2-imidazolino) phenyl] furan dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
dpi	Day post infection

DPX	Distyrene, plasticizer, and xylene
DVS	Department of Veterinary Services
EDTA	Ethyledeniaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
FA	Fluorescent antibody
g	Gravity
H & E	Haematoxylin and eosin
IACUC	Institutional Animal Care and Use Committee
IBR	Infectious bovine rhinotracheitis
ICTV	International Committee on Taxonomy of Viruses
ID	Identification
IHC	Immunohistochemistry
IRES	Internal ribosome entry site
JEV	Japanese encephalitis virus
kDa	Kilodalton
KK	Kedah-Kelantan
Lao PDR	Lao People's Democratic Republic
LDL	Low-density lipoprotein
LID	Local Indian Dairy
MAFI	Ministry of Agriculture and Food Industries
MD	mucosal disease
mg/kg	Milligram/kilogram
MHC I	Major histocompatibility class I
MHC II	Major histocompatibility class II
mL	Milliliter

MLV	Modified live virus
mRNAs	Messenger RNAs
NCP	Non-cytopathic
nm	Nanometer
nt	Nucleotide
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
P25	25^{th} passage
P5	5^{th} passage
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PI	Persistently infected
RNA	Ribonucleic acid
RNAse	Ribonuclease
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEA	Southeast Asian
SNT	Serum neutralization test
SPSS	Statistical Product and Service Solutions
ssRNA	Single stranded RNA
TI	Transiently infected
UK	United Kingdom
USD	Unites States Dollar
WHO	World Health Organization
WNV	West Nile virus
µL	Microliter

CHAPTER 1

INTRODUCTION

1.1 Research Background

The production of beef and milk in Malaysia are insufficient to meet the population's demand (Sim and Suntharalingam, 2015). The main problems faced by both sectors include low productivity, low quantity of forages, low quality of supplements, less cost-effective feed, and low beef price to feed cost ratio (Mohamed et al., 2013; Sim and Suntharalingam, 2015; Abdulla et al., 2016). Therefore, importation of breeder animals mainly from Australia, New Zealand, and Thailand has been the best policy to meet the local demand (Ariff et al., 2015; Abdulla et al., 2016).

Both beef and dairy cattle in Malaysia are from the temperate breeds (*Bos taurus*) and the tropical breeds (*Bos indicus*) or the crossbreed of both species. The beef cattle are reared in integrated system under oil palm plantation, large farms, traditional farms, and commercial feedlots. This type of production system usually consists of Kedah-Kelantan (KK), Bali, and Brahman cattle breed (Jamaludin et al., 2014). The dairy cattle typically consist of Friesian, Sahiwal, Jersey, Local Indian Dairy (LID) breed and/or crossbreed between those breeds (Sim and Suntharalingam, 2015). They are usually reared by large farms or smallholder farmers. The smallholder farmers characterized by small herd size with an average number of ten cows and lack of investment in quality breeding stock practiced extensive production system, scattered and poorly organized farm (Gabdo, 2014; Abdulla et al., 2016). As in 2021, total cattle population in Malaysia was 701,117 and is mainly centered in Peninsular Malaysia states namely Pahang (155,686), Johor (99,091), Terengganu (86,152), Kelantan (79,546), Perak (57,461), and Kedah (51,230) (DVS and MAFI, 2021).

The Asian water buffaloes (*Bubalus bubalis*) in Malaysia are of the swamp type. It is mainly used as a draft and secondarily for meat (Villanueva et al., 2018). The majority of these buffaloes are reared extensively by tethering or grazing system in communal pastureland (Ariff et al., 2015; Hamid et al., 2016). Meanwhile, dairy buffaloes (*Bubalus bubalis*) are less popular than beef buffaloes. They are riverine type mainly Murrah and Nili-Ravi breed and are reared primarily for milk and secondarily for meat. The dairy buffaloes are kept in confinement and fed with cut and carry forages and other feed materials while occasionally left for grazing (Hamid et al., 2016). The average number of buffaloes in the smallholder scale is 1 to 5 while on a large-scale farm, the population can be up to 400 buffaloes (Cruz et al., 2007). The total buffalo population in Malaysia in 2021 was 63,587 concentrated in Sabah (11,759), Pahang (11,511), Sarawak (6,947), Perak (6,746), and Terengganu (5,117) (DVS and MAFI, 2021).

Bovine viral diarrhoea (BVD) is caused by bovine viral diarrhoea virus (BVDV) and is among the important disease of cattle (Lanyon, 2014; Khezri, 2015; Moennig and Becher, 2018). However, several BVD cases also reported in other ruminant livestock

such as sheep, goat, mithuns (*Bos frontalis*) and free-ranging wild ruminant species namely white-tailed deer, red deer, chamois, ibex, as well as roe deer (Casaubon et al., 2012; Shi et al., 2016; Nelson et al., 2016; Singh et al., 2017). As BVD can occur in all stages of production, the virus may lead to significant economic losses to the farming industry due to its effect on the reproductive performance of the infected animals, poor performance of immunotolerant animal, and disease occurred due to BVD-related immunosuppression (Lanyon and Reichel, 2013). The animals suffer from acute infection, repeat breeding, abortions, congenital defects, increased neo-natal mortality, and increased death among young stock (Houe, 2003). Furthermore, BVDV was detected in the lungs of 15 out of 102 (15%) cattle with histologic evidence of pneumonia during long haul voyages from Australia to China, Middle East, and Russia (Moore et al., 2014). According to Lanyon and Reichel (2014), the cost of BVD to the national industry of Australia is also estimated to be AUD 7.9 million. The estimated direct financial loss due to BVD were USD 0.50-687.60 per animal in the United States and some European countries according to Richter et al. (2017).

BVDV is a member of the Pestivirus genus and *Flaviviridae* family. Other members of the Pestivirus genus are Border's disease virus (BDV) and classical swine fever virus (CSFV). Sequence analysis of the genomic ribonucleic acids (RNAs) of these viruses and certain antigenic characterization categorized BVDV-1 and BVDV-2 as two different species (Neill, 2013; Smith et al., 2017). Furthermore, these four viruses differ from the genus of Flavivirus because they encode two special proteins specifically N^{pro} and E^{rns} while harbouring the same feature of the *Flaviviridae* (Callens et al., 2016). The open reading frame (ORF) carrying genes for five structural proteins and seven nonstructural proteins which arranged the genome are as follows: NH_2-N^{pro}, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B-COOH (Sato et al., 2016; Bazzucchi et al., 2017). The five structural proteins are capsid (C), three enveloped glycoproteins (E^{rns}, E1, and E2), and a very small viral protein (p7) (Callens et al., 2016). Meanwhile, the seven non-structural proteins are N^{pro}, NS2, NS3, NS4A, NS4B, NS5A, NS5B (Sato et al., 2016; Bazzucchi et al., 2017). Strains of BVDV also exist as different biotypes; non-cytopathic (NCP) and cytopathic (CP) (Fulton, 2009; Pinchuk et al., 2015; Neill et al., 2013). The CP BVDV causes damage in cell culture by vacuolation and cell lysis while NCP BVDV does not cause any changes (Wang et al., 2014; Khodakaram-Tafti and Farjanikish, 2017). These biotypes are independent of genotypes (Brodersen, 2014; Wuryastuti et al., 2018).

BVD is an endemic disease in cattle populations in many parts of the world (Reichel et al., 2018; Qi et al., 2019). BVDV infection also increasingly reported in other ruminant species. In Malaysia, a study in Selangor demonstrated an overall 33.2% prevalence of BVDV antibody, with seropositivity range 0%-75.9% in four dairy cattle farms and a mixed cattle farm (Daves et al., 2016). BVDV antibody has been detected in sheep and goat in three government farms in Malaysia with 0%, 8%, and 23% seroprevalence (Maizan et al., 1998). Furthermore, Saidi et al. (2018) detected BVDV-2 from a pooled swine tonsils in Sabah, Malaysia. The prevalence of BVDV in cattle herds among Southeast Asian (SEA) countries were reported to be ranging from 2.1% to 82% (Duong et al., 2008; Aye et al., 2017). According to Kampa et al. (2004) 73% bulk milk sample (160/220) were seropositive BVDV in Thailand. There was also a report of 4.9% BVDV seropositivity in buffaloes in Lao PDR (Olmo et al., 2018). The dairy cattle in Ethiopia,

Brazil, Bangladesh, Colombia, Kenya, and China, and were found to have 32.6%, 51.1%,75.17%, 79.15%, and 89.49% BVDV antibody respectively (Deng et al., 2015; Ramirez-Vazquez et al., 2016; Uddin et al., 2017; Aragaw et al., 2018; Okumu et al., 2019). Meanwhile, cattle in Brazil and Botswana were found to have 40.1% and 53.3% BVDV antibody (Marques et al., 2016; Lysholm et al., 2016). The prevalence of BVDV exposed cattle herds were reported to be varying from 82% to 100% in Australia (Lanyon and Reichel, 2014; McGowan et al., 2020). Although reports vary across states, a seroprevalence of 75% to 85% of the BVDV antibody in adult cows was also documented in Australia (Taylor et al., 2006).

Based on all of the published data, 31.6% (2193/6939) and 20.8% (1443/6939) of the BVDV isolates studies involved BVDV1b and BVDV1a respectively. BVDV1b is the predominant subgenotype in Americas 53.0% (738/1392) and Asia 39.7% (703/1770) while BVDV1a is predominant in Europe 22.7% (732/3220). In contrast, 95.9% (425/443) of the field isolates in Australia classified as BVDV1c. There are limited data and may not represent for the whole Africa continent, but BVDV1a has been detected more frequently than other subgenotypes in South Africa (Yesilbag et al., 2017).

BVDV can be transmitted either vertically within a herd or horizontally from cow to foetus (Nelson et al. 2016). Transmission via vertical route is through in utero and horizontal route is by direct contact with acute transiently infected (TI) animal or persistently infected (PI) animal (Nelson et al., 2016; Morarie-Kane et al., 2018). Meanwhile, in wild ruminants, BVDV is transmitted horizontally via oral and nasopharyngeal secretions or iatrogenic routes. Iatrogenic routes include rectal examinations, contaminated live vaccines, and injections with contaminated needles (Palomares et al., 2013; Nelson et al, 2016).

There are several risk factors of BVDV infection. In Poland, there were correlations between BVDV infection and pasture ($p=0.004$), the number of grazing animals ($p<0.001$), and purchase of animals for replacement ($p=0.004$) (Rypula et al., 2020). Study by Kumar et al. (2018) in cattle in Tamil Nadu, India concluded that urban location, tail to tail housing pattern, galvanized iron roof, more than 1 km distance between farm, and within 500, distance between farm and manure were significantly associated with BVDV serological status ($p<0.05$). Furthermore, significant higher odds were found in dairy herds (OR=1.63, 95% CI=1.06-2.50); for larger herds (OR=1.04 for every 10 extra animals in the herd, 95% CI=1.02-1.06); for herds that participate in shows or markets (OR=1.45, 95% CI=1.10-1.91); for herds that introduced cattle into the herd (OR=1.41, 95% CI=1.18-1.69); for herd that share pasture or have direct contact with cattle of other herds at pasture (OR=1.32, 95% CI=1.07-1.63) (Van Roon et al., 2020). Other than that, cattle with seropositive infectious bovine rhinotracheitis (IBR) (OR=2.38, p=0.0479) or Neosporosis (OR=3.15, p=0.00122) were more likely to be seropositive BVDV while burning the dead animals (OR=0.17, p=0.014) was identified as protective factor (Ortega et al., 2020). According to Marques et al. (2016) the risk factors identified were as follows: area ≤ 120 hectares (OR=3.06, 95% CI=1.43-6.53), high animal density (OR=3.48, 95% CI=1.24-9.79), weaning age ≤ 60 days (OR=10.99, 95% CI=1.31-91.9), exchange of animals (OR=4.95, 95% CI=2.08-11.8), calf mortality >5% (OR=2.33, 95% CI=1.07-5.11), and use of natural breeding and artificial insemination (AI) (OR=3.06, 95% CI=1.22-7.67).

Virus isolation, antigen enzyme-linked immunosorbent assay (Ag-ELISA), immunohistochemistry (IHC), nucleic acid probe hybridization, and reverse transcriptase-polymerase chain reaction (RT-PCR) are commonly used for BVDV antigen detection (Dubovi, 2013; Lanyon et al., 2014). Although virus isolation is the gold standard for BVDV diagnosis, RT-PCR is often preferred due to less time consuming, less expensive, independent to cell culture facilities, and highly sensitive (Dubovi, 2013). A variety of samples like blood, milk, follicular fluid, saliva, and tissue samples can be tested by RT-PCR (Goktuna et al., 2017). The use of primers specific to the 5 untranslated regions (5'UTR) revealed that it can identify BVDV-1 and BVDV-2 (Larska et al., 2013). The Ag-ELISA is simple, rapid, and useful for high throughput application especially during the detection of PI animal and herd screening (Larska et al., 2013; Humphry et al., 2018). Unlike RT-PCR, Ag-ELISA cannot return useful results on pooled serum samples. Meanwhile, IHC is one of the most popular methods in BVDV antigen detection but restricted to tissue samples commonly ear notch samples (Lanyon et al., 2013; Scharnbock et al., 2018). Detection of BVDV antibodies provides a valuable way to determine individual immune status and any exposure to BVDV previously (Lanyon et al., 2013). Several antibodies detection methods available are dot-blot enzyme immunoassay, agarose gel immunodiffusion (AGID) test, microsphere-based immunoassay, serum neutralization test (SNT), and enzyme-linked immunosorbent assay (ELISA) (Dubovi, 2013; Lanyon et al., 2013).

1.2 Research Questions

Despite reports from various parts of the world, there has been limited study on BVD in Malaysia. No molecular study was conducted on BVDV in cattle in this country. There was also limited data on the management factors as compared to animal factors that posed risk to BVDV infection in cattle. A previous study has been reported seroprevalence of BVDV in sheep, goat, and cattle, but in a small niche. Hence, this is a pilot study of seroprevalence, molecular detection, and risk factors of BVDV infection in buffalo in Malaysia. Furthermore, cattle and buffaloes in Malaysia are at risk of contracting the disease with regards to the endemic situation in the importation country namely Australia and Thailand. This is supported by the evidence that BVDV was detected in the long-haul voyage of the imported cattle from Australia.

1.3 The Objectives of the Study

i. To determine the prevalence of BVDV antibodies in cattle and buffaloes in the selected farms.
ii. To isolate, identify, and molecularly characterized BVDV from the sampled animals.
iii. To determine the risk factors of seroprevalence BVDV.

1.3.1 Research Hypotheses

The hypotheses of this study were as follow: -

i. The prevalence of BVDV antibodies varies between cattle and buffaloes.
ii. The BVDV antigen is present in cattle and buffaloes.
iii. The BVDV infection is associated with multiple risk factors.

1.4 Significance of the Study

The BVDV risk factors in this study provided information on the variables associated with the increased risk of disease. Moreover, with the advance of highly sensitive molecular techniques such as polymerase chain reaction (PCR), it is desirable to explore the potential of this method to determine BVDV infection, status of cattle and buffaloes in Malaysia as well as to explore the genetic characteristics and phylogeny of local BVDV isolates. The results of this study provided useful information on the existing herd health programmes, future national strategies in ruminant importation, biosecurity measures, BVD vaccination and control programmes. Furthermore, this was the first BVDV study conducted in buffaloes in this country.

1.4.1 Scope of the Study

In general, the scope of this study was to assess the seroprevalence of BVDV in cattle in selected farms in Selangor and buffaloes in a farm in Sabah. Then, serological test was carried out and the seroprevalence of BVDV was determined. Then, the statistical analysis was carried out to determine the risk factors of BVDV. Antigen detection was also carried out to determine the prevalence of BVDV antigen in the farm. Previous study conducted revealed that BVDV was circulated in Selangor. Therefore, further step in BVDV detection was carried out. Meanwhile, Sabah was selected for this study because this state has the highest population of buffaloes in Malaysia.

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