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MODERN ADVANCES ON THE DIAGNOSIS OF BOVINE VIRAL DIARRHOEA VIRUS IN CAMELIDS

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) is one of the most leading causes of diarrhoea in many species of animals including camelids. Special attention is being paid to studying various aspects of the BVDV infection, pathogenesis, epidemiology, diagnosis and vaccines in the bovine species. Little is still known about these aspects in camelids especially dromedaries. The development of accurate diagnostic tests is a critical step for the identification of infected animals, especially persistently infected and for the control and eradication of BVDV. Although adaptation of the currently used diagnostic assays for the virus in the bovine and ovine species for the diagnosis of BVDV in the camelids, it is highly recommended to develop novel specific diagnostic assays of the virus in camels. There are several strategies for the diagnosis of BVDV including virus isolation, detection of viral (antigens, antibodies and nucleic acids) and identification of the circulating strains of the virus by doing sequencing and phylogenetic analysis. Identification of the persistently infected animals is one of the main challenges facing the diagnosis and control of the virus. Our main objectives are to highlight the most recent advances in the field of BVDV diagnosis, currently used diagnostic assays and suggested some specific approaches for the diagnosis of BVDV in camelids. Fine-tuning the currently used diagnostic assays and development of other novel specific assays to detect BVDV infection in dromedary camels will have a substantial positive impact on the control of BVDV infections in camels.

Key words: Assays, BVDV, camels, detection, diagnosis, isolation, molecular

The bovine viral diarrhoea virus (BVDV) was previously known as a mucosal disease that is responsible for high economic losses among the affected animals in most countries around the world (Fray *et al,* 2000). BVDV represents one of the common causes of immunosuppression, mucosal disease and reproductive failures among various species of animals such as cattle, sheep, goats and other heterologous species of animals such as camelids (Passler and Walz, 2010).

BVDV is a member of the Pestiviruses causing serious problems in the affected species of animal such as reproductive failure, decrease in milk production and abortion (Ostachuk, 2016, Passler and Walz, 2010). Classification of BVDV based on the ability to grow on cell culture and induce cytopathology revealed two genotypes called BVDV-1 and BVDV-2 (Al-Kubati *et al,* 2021).

There are several approaches for the diagnosis of BVDV in various species of animals. Isolation and identification of the virus and its antigens, antibodies and nucleic acids are among the main methods for virus diagnosis. Although BVDV was detected a while ago, most of the research on the virus is mainly focused on various aspects of the viral infection in some domestic animals such as cattle, sheep, goats and pigs (Passler and Walz, 2010). Recently, several studies reported the prevalence of the BVDV in some of the family camelids (Gao *et al,* 2011, Topliff *et al,* 2009). Some experimental studies were carried out to investigate the possibility of BVDV infection in the new world camels including llamas and alpacas (Wentz *et al,* 2003). This study showed the possibility of BVDV infection under experimental conditions however, low seroprevalence was reported in these animals suggesting that cattle is the main source of BVDV infection in camels (Wentz *et al,* 2003). BVDV infection of the cattle during the duration of their pregnancy results in the development of some important phenomena called persistently infected newborn animal (PI) (Grooms, 2004). These PI animals

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are considered as good virus shedders and contribute high risk for the rest of the herd and must be culled (Hilbe *et al,* 2007).

Isolation of BVDV

Virus isolation remains the gold standard technique for the diagnosis of BVDV infection in various species of animals (Gao *et al,* 2011; Peterhans *et al,* 2010; Topliff *et al,* 2009). BVDV isolation can be done from various clinical samples such as blood including sera and buffy coat, nasal swabs and various tissue specimens (Wang *et al,* 2014). The mononuclear cells in the buffy coat of the infected animals represent the ideal target for virus isolation. Meanwhile, the best tissue specimen for the BVDV isolation is the payer's batches in the regional lymphoid tissues, the mesenteric lymph nodes, spleen and the thymus (Turin *et al,* 2012). Based on the cytopathological changes triggered by BVDV infection in cell culture, two types of the virus were identified; the cytopathic (CP) and the non-cytopathic strains of BVDV (NCP) (Gillespie *et al,* 1962). To distinguish NCP-BVDV that show exaltation of Newcastle disease virus (END)+ from NCP-BVDV-END-, END method and interference with VSV were used, however, these techniques require the use of high virulent strains of NDV and VSV, necessitating the use of high laboratory containment measures. Alternatively, a safer technique termed Competitive Virus Assay (CVA) was developed to titerate either BVDV-END+ or BVDV with another heterologous interference phenomenon (Muhsen *et al,* 2013). One of the main limitations of the BVDV isolation from sera of young calves below 3 months of age is the presence of high titres of neutralising antibodies which inhibit virus replication in cell culture (Zimmer *et al,* 2004).

Detection of BVDV antigens

Antigen Capture -ELISA (AC-ELISA) is one of the most common technique for the detection of BVDV antigens in clinical samples including whole blood, nasal swabs as well as some skin lesions especially skin notch (Grooms, 2004). The fluorescent antibody technique (FAT) can also be used for the detection of BVDV antigens in some BVDV infected tissues (lymph nodes, spleen, etc) (Grooms, 2004). Immunohistochemistry (IHC) is also used to detect the BVDV antigens in some of the infected fixed tissues for a long time (Seong *et al,* 2015). IHC can also be performed on frozen ear notch tissue samples (Bedeković *et al,* 2011). Both the IFT and IHC techniques were recently used in the detection of the BVDV antigens in some of the PI animals (Edmondson *et al,* 2007).

The performance of commercial diagnostic tests used for virus/antigen detection in the German BVDV eradication program was evaluated on four positive and one negative ear notch as well as four positive and one negative serum samples. Testing of these samples with Erns-based AC-ELISA showed a diagnostic accuracy of 100% while testing of these samples with 7 commercial and one in-house real-time RT-PCR (rRT-PCR) revealed high agreement (Wernike and Beer, 2019). A targeted antigen may affect the ability of the ELISA test to detect BVDV antigen. In this regards, Erns-based AC-ELISA rather than NS2/3-based AC-ELISA was able to detect BVDV antigen from all over the skin of PI calves, though both tests were equally able to detect the antigen in the recommended sample, from ear notch (Vander Ley *et al,* 2012). Using commercial Erns-based AC-ELISA, tail skin biopsy and nasal swabs were reported to provide similar detection rates to that obtained with ear notch, while it was 92% for the serum and moderate to very low with conjunctival, oral, rectal, vaginal /preputial swabs from PI animals (VanderLey *et al,* 2011). The presence of a colostrum-derived antibody may interfere with antigen detection in serum, nasal and saliva, but not in-ear notch, samples from PI calves for up to 3 weeks (Lanyon *et al,* 2014c). To avoid such interference, a test can be performed on supernatant obtained from treating serum with ethylenediamine tetra-acetic acid at pH 5 ± 0.5, boiling and centrifugation (Lanyon and Reichel, 2016).

The antigenic similarity of Erns and NS2/3 in BVDV-1, -2 and -HoBi were higher than antigenic similarity in E2 protein. Comparing the performance of commercial Erns-based AC-ELISA in detecting BVDV-1,-2 and -HoBi antigens showed its ability to detect all types at the same sensitivity (Bauermann *et al,* 2012). However, due to variation in BVDV and reliance on commercial diagnostic assays, like AC-ELISA, on one or two monoclonal antibodies, the possibility of false negativity should be kept in mind. Hence, BVDV tests may require regular evaluation (Yan *et al,* 2016b, Gripshover *et al,* 2007).

The AC-ELISA was used to detect BVDV-antigen in pneumonic lungs from an apparently healthy camel in Sudan. Out of 474 and 186 lungs, AC-ELISA detected 28 (9%) and 13 (7%) positive samples, respectively (Saeed *et al,* 2015, Intisar *et al,* 2010). To confirm the results of the AC-ELISA, the 13 BVDV-positive lungs were tested again with FAT and RT-PCR, which generated compatible results (Intisar *et al,* 2010). Similarly, among 214 samples

of abomasal contents collected from camel aborted fetuses in Iran, AC-ELISA detected 27 (12.61%) while RT-PCR detected 32 (14.95%) BVDV-positive samples (Dehkordi, 2011). Combining of two ELISA tests, one to detect antibody and the other to detect antigen, for testing of the same sera panel showed unexpectedly high BVDV-antigen prevalence of 22% in Egypt (El Bahgy *et al,* 2018) and 41.4% in Algeria (Saidi *et al,* 2018) with seroprevalence of an anti-BVDV antibody of 33% and 9% respectively (Table 1).

The development of a rapid, sensitive and specific assay to detect BVDV-antigen under field conditions would be advantageous for control and eradication programs. Immunochromatography with anti-NS3 monoclonal antibody was developed and used to detect BVDV in leukocyte extract with sensitivity and specificity of 100% and 97.2%, respectively, relative to virus isolation (Kameyama *et al,* 2006). Similarly, immunochromatography assay based on recombinant E2 protein and chicken anti-E2 IgY was developed to detect BVDV antigen and showed an agreement of 90.91% with RT-PCR results (Zhang *et al,* 2016). A commercial rapid test based on lateral flow technology was evaluated for detection of BVDV in whole blood, plasma and ear notch from PI calves. Results revealed a sensitivity and specificity of ≥96%, relative to AC-ELISA, in all tested sample types with the ability to detect both BVDV-1 and -2 (Rammelt *et al,* 2013).

Detection of BVDV antibodies in sera and milk

Serological techniques are useful in the diagnosis of viral infections in the case of many viruses. However, due to the ubiquitous nature of the spread of the BVDV among the various population of animals and the massive administration of BVDV vaccines, thus, we have to use various serological techniques with extreme caution to diagnose BVDV infection in animals. Typically, seroconversion is the most feasible approach to use various serological techniques in the diagnosis of BVDV infections in animals. This can be achieved by the collection of paired serum samples, one sample should be collected at the acute stage of the disease while the other sample should be collected during 4 weeks apart from the acute sample and during the convalescent stage of the viral infection (Edmondson *et al,* 2007b). It is highly recommended to screen young animals under 6 months of age for possible BVDV exposure. This is to identify if the virus is circulating in some young animals and before using the potentially infected animals for breeding purposes. There are some serological techniques that can be applied to distinguish between the BVDV-1 and the BVDV-2 (Edmondson *et al,* 2007b), especially those targeting E2 protein (Bauermann *et al,* 2012). Several serological tests have been used to detect anti-BVDV antibodies in serum and to a lesser extent in milk, including Virus neutralisation test (VNT), Enzyme-Linked Immunosorbent assay (ELISA), Agar Gel Immunodiffusion test (AGID), Complement fixation test (CFT), Indirect Immunofluorescence Assay (IFA), Western Blotting and peroxidase linked assay. The two former tests are the most popular assays in diagnostic laboratories (Sandvik, 2005).

The virus neutralisation assay is one of the most common serological techniques used to diagnose

Table 1. Summary of the BVDV prevalence studies in camel in Arabian Peninsula and surrounding countries (with results of seroprevalence).

Country	# and type of samples	Test	Prevalence	Reference
Sudan	260 sera	i-ELISA*	84.60%	(Intisar *et al,* 2010)
	186 pneumonic lungs	AC-ELISA /FAT/RT-PCR	7%	
Iran	214 abomasal contents from aborted fetuses	AC-ELISA	12.61%	(Dehkordi, 2011)
		RT-PCR	14.95%	
Sudan	474 peumonic lungs	AC-ELISA	9%	(Saeed *et al,* 2015)
Egypt	200 sera	c-ELISA	33% (47.5% smuggled, 11.2% local	(El Bahgy *et al,* 2018)
		AC-ELISA	22% (31.6% smuggled, 7.5% local)	
KSA	182 sera	ELISA	29.10%	(Khalafalla *et al,* 2017)
	97 uterine swabs from camel with reproductive failure	RT-PCR	0%	
Algeria	111 sera	c-ELISA	9%	(Saidi *et al,* 2018)
		AC-ELISA	41.40%	

* with anti-camel conjugate

BVDV infection in animals. It is highly sensitive and specific (Sandvik, 2005), hence it remains the gold standard for the detection of antibodies against BVDV (Wernike and Beer, 2019). VNT can be used to detect and distinguish antibodies against different types and subtypes of BVDV (Minami *et al,* 2011). It is highly recommended to assess both the acute and convalescent samples using the same reference strain to avoid any misinterpretation of the obtained results (Edmondson *et al,* 2007b, Grooms, 2004). However, the use of different virus strains and cells lines made it difficult to compare results from various laboratories (Dubovi, 2013). Additionally, VNT is laborious, costly, time-consuming, needs well-trained workers and limited to virology laboratories (Sandvik, 2005). To overcome many of these disadvantages, a recombinant BVDV that expresses EGFP was constructed and used in flow cytometry-based-VNT (FC-VNT). The FC-VNT showed the ability to be automated, high-throughput, reproducible and can be performed in 48 hours (Gebauer *et al,* 2014). Colostrum has a cytotoxic effect on cells, impairing the use of VNT for the detection of anti-BVDV antibodies. A modified VNT (mVNT) was developed to overcome this challenge. In mVNT, a high density of MDBK cells was incubated for 3 hours to prevent detachment of cells from the well surface. The cytotoxic effect of colostrum complement on cells was minimised by heat inactivation of the colostrum and reducing the incubation time of colostrum-virus mixture on cells for 1 hour followed by five times washing of MDBK cells with Dulbecco modified Eagle medium (DMEM). The mVNT showed sensitivity and specificity of 100% compared with standard VNT and showed a high correlation between antibody titers in serum and colostrum (Bedeković *et al,* 2013).

ELISA is a widely used serological technique to diagnose BVDV in several clinical samples including blood and milk samples. The Ig-based ELISA is commonly used to detect the antibodies in sera of infected animals rather than for the detection of antigens (Edmondson *et al,* 2007b, Grooms, 2004). It is mandatory to know the vaccination history of the tested animals to avoid any misinterpretations in case of the vaccinated animals (Lanyon *et al,* 2014b). Positive results for serum samples collected from pregnant cattle indicated previous infection or vaccination history with the BVDV (Lanyon *et al,* 2014b). Negative results of screening a statistically representative sample per each herd using the Ig-based ELISA for BVDV may suggest the PI animals. Further confirmation should be conducted to identify the PI animals.

The performance of several commercial ELISA kits for the detection of anti-BVDV antibodies in sera and milk were evaluated in several laboratories participating in German BVDV eradication program. Results showed that some commercial ELISA kits repeatedly generate false-negative results, especially for milk, suggesting its need for optimisation (Wernike and Beer, 2019). Similar studies in the context of the Belgium BVDV eradication programme showed that using of indirect ELISA (i-ELISA) for testing individual serum samples from unvaccinated animals generate the best results for sero-monitoring (Hanon *et al,* 2017, Hanon *et al,* 2018). Pooling of serum samples from viremic seronegative cattle with seropositive sera did not generate false-negative results in i-ELISA and c-ELISA (Graham *et al,* 2019). Similarly, ELISA-detection of anti-BVDV antibody in serum and individual or bulk milk samples showed good agreement (Lanyon *et al,* 2014a). However, using of commercial ELISAs to detect antibodies in bulk milk showed sensitivity and specificity of 70 to 92% and 77 to 88%, respectively, compared to ELISA detection of antibodies in sera at herd level (Eiras *et al,* 2012). It has been reported that there was a diagnostic gap for up to 12 days after cattle parturition for ELISA-detection of anti-BVDV antibody in serum, but not by VNT. In contrast, colostrum was strongly seropositive in ELISA after parturition, though positivity decreases in the next few days (Bachofen *et al,* 2013).

Unlike VNT, the ELISA test is mostly developed and validated for use in certain species, mostly bovine and linked to species-specific reagents, limiting their use in heterologous host species. Competitive or blocking ELISA (c-ELISA) may not link to species-specific reagents but need to be validated for the targeted animals' species (Dubovi, 2013). A study on the diagnostic performance of two bovine commercial ELISAs and AGID test to detect anti-BDV and anti-BVDV in sheep showed that all tests have a specificity of 100% but varies widely in their sensitivity. The authors recommend optimising the threshold value of the ELISA test to obtain >95% diagnostic sensitivity and specificity (Evans *et al,* 2017). Similar optimisation of bovine diagnostic tests would be necessary before using in camels and other species. A study that involved both ELISA and VNT (table 2) showed that out of 812 samples tested with c-ELISA, three positives and 2 suspected samples turned to be negative when tested with VNT. The author concludes that the used ELISA test provides reliable results in the detection of anti-BVDV in camel sera (Taha, 2007).

Several investigations were performed to determine the seroprevalence of BVDV in camels in Arabian Peninsula and surrounding countries. Different serological tests were used, with most studies before 2010 using the VNT while most studies performed thereafter were based on the ELISA test (table 2). Seroprevalence of BVDV in camel varies widely with the majority of the investigations reported it between 5% and 30%. However, a comprehensive study based on a random representative sample that involves the whole camel population seems scarce. Seropositivity of 9% and 29.1% were reported respectively from Algeria and Saudi Arabia randomly collected serum samples, (Khalafalla *et al,* 2017; Saidi *et al,* 2018). Seroprevalence as high as 52.5% and 58.7% were also reported at subnational administrative divisions in Egypt (Zaghawa, 1998) and Turkey (Erol *et al,* 2020), respectively. The highest seroprevalence of 84.6% was reported from a Sudanese national study based on randomly collected samples (Intisar *et al,* 2010). Noteworthy, this seems to be the only study that performed with species-specific anti-camel conjugate in i-ELISA. Most of the studies in this regard were performed with VNT or c-ELISA format; while a couple of studies used i-ELISA format but without specifying the used conjugate or with other than anti-camel conjugate. As stated earlier, evaluation of several commercial ELISA assays showed that, for serum, i-ELISA targeting the whole virus was more sensitive than i-ELISA targeting NS3 or other competitive ELISA (c-ELISA) (Hanon *et al,* 2017).

Detection of BVDV-Nucleic acids

Nowadays, detection of the viral nucleic acids in clinical specimens is the gold standard technique for BVDV diagnosis. Some molecular diagnostic assays, especially the RT-PCR have been used successfully used for the detection of various BVDV strains (Laamanen *et al,* 1997). Some studies reported that the stability of the BVDV-1-RNA in the foetal brain was much higher than that in foetal skin, muscle, ear, or pooled tissues (Ridpath *et al,* 2014). The copy number of viral RNA detected in peripheral blood mononuclear cells (PBMCs), ear notches and the hair bulbs were much higher than that detected in plasma or dry blood (Zoccola *et al,* 2017). The detectability of BVDV in buffy coat samples was significantly higher than that in serum or nasal swab samples from transiently infected calves (Peddireddi *et al,* 2018). Faecal and aerosol samples from persistently infected cattle are suitable samples for the detection of

Table 2. Seroprevalence of anti-BVDV antibody in camel in Arabian Peninsula and surrounding countries.

Country	# of serum sample	Test	Result	Refer
Oman	30	SNT	6.7%	(Hedger *et al*, 1980)
Sudan	102	SNT	15.70%	(Bornstein and Musa, 1987)
UAE	1000		9.2% for breeding females 3.6% for racing females	(Wernery and Wernery, 1990)
Egypt	59	SNT	52.5%	(Zaghawa, 1998)
Egypt	365	SNT	1% for abattoir group, 1.7% for camel in contact with cattle	(Eisa, 1998)
KSA	2472		18%	(Al-Afaleq *et al*, 2007)
UAE	812	c-ELISA, SNT	0%	(Taha, 2007)
UAE	1119		1.6%	(Wernery *et al*, 2008)
Iran	137	SNT	19.70%	(Raoofi *et al,* 2010)
Sudan	260	i-ELISA*	84.60%	(Intisar *et al,* 2010)
Egypt	165	SNT	14.54%	(Elbayoumy *et al,* 2013)
Iraq	88	i-ELISA	13.63%	(Al-Rubayie, 2016)
Egypt	200	c-ELISA	33% (47.5% for smuggled from sudan, 11.2% local camel)	(El Bahgy *et al,* 2018)
KSA	182	ELISA	29.10%	(Khalafalla *et al,* 2017)
Algeria	111	c-ELISA	9%	(Saidi *et al,* 2018)
Egypt	92	i-ELISA	27.20%	(Malek and Madkour, 2017)
KSA	316	c-ELISA	10.8%	(Intisar, 2019)
Turkey	92	SNT	58.70%	(Erol *et al,* 2020)

* anti-camel conjugate was used.

BVDV-1 by most common molecular techniques such as the TaqMan real-time RT-PCR (rRT-PCR) or SYBR Green rRT-PCR, respectively (Liang *et al*, 2019; Hou *et al*, 2020). The use of tissue swabs for rRT-PCR detection of BVDV was reported to yield comparable results to that obtained from tissue pieces with the possibility of reducing lysis time (Errington *et al,* 2014). Like blood samples, hair samples (30-100 pooled hairs from each animal) were successfully used to detect PI calves using rRT-PCR (Singh *et al,* 2011). With pooled samples of ear notch or sera, the Ct of the rRT-PCR significantly correlated with pool size (number of animals). The cost-effective pool size to screen with rRT-PCR was reported to be 25 or 50 samples at BVDV prevalence of 0.75-2% and 0.25 to 0.5%, respectively (Yan *et al,* 2011). Both the proper sampling, the presence of the pre-existing anti-BVDV antibodies in sera of tested animals affects the performance of serological techniques. For example, the presence of a higher level of anti-BVDV neutralising antibody reduces the number of BVDV positive RT-PCR days and increases the average Ct value over-tested days for buffy coat samples (Peddireddi *et al*, 2018). Colostrum-derived IgG, on the other hand, showed no inhibitory effect on the performance of the RT-PCR used to detect BVDV in calves blood at the age of 48 hours (Chigerwe and Crossley, 2013). Direct amplification of the BVDV-1- 5'UTR from as low as 0.5 µl of the heat-treated serum or plasma samples with no RNA extraction was successful in producing sufficient PCR product for sequencing purposes (Bachofen *et al*, 2013b). Similarly, direct detection of BVDV in hair bulbs-supernatant was capable of detecting all PI animals using TaqMan rRT-PCR (Zoccola *et al*, 2017). Magnetic particle technology was also used successfully to shorten the time of extraction of BVDV from blood or serum samples (Aebischer *et al*, 2014a). A chemically treated paper that upon adding biological samples serve to lyse cells and stabilise nucleic acids was developed and termed Fast Technology Analysis (FAT) card. Using this card for collection and transport of nucleic acid of BVDV and other bovine respiratory viruses was reported to maintain nucleic acid in a quality suitable for rRT-PCR testing over a wide range of temperature (-14°C to 47°C) for 14 days (Liang *et al,* 2014).

There are two new types of amplification assays (1) the thermal cycling techniques with the ligase chain reaction (LCR) and (2) the isothermal amplification techniques like loop-mediated isothermal amplification (LAMP), Rolling circle amplification (RCA) and hybridisation chain reaction (HCR) (Monjezi *et al*, 2016)

The development of an RT-PCR capable of detecting all types the BVDV was a fundamental issue for virus monitoring and surveillance programs. The genetic diversity of Hobi-like viruses was reported to represent a challenge for the developing diagnostic RT-PCR and rRT-PCR assays for this virus (Moorthy *et al*, 2019). Additionally, frequent mutations within the 5'UTR region reduce the performance of the regular rRT-PCR (Yan *et al,* 2016a). The conventional RT-PCR (cRT-PCR) (Monteiro *et al*, 2019), TaqMan-rRT-PCR (Losurdo *et al*, 2015) and the Multiplex-TaqMan-rRT- PCR (Mari *et al*, 2016) capable of detecting all three BVDV types have been reported with a detection limit of as low as 100 to 101 viral RNA copies in case of the TaqMa- rRT-PCR. However, failure in the detection of BVDV or HoBi-like viruses is not uncommon. For example, cRT-PCR and rRT-PCR designed for detection of either BVDV or HoBi-like viruses were used in the detection of HoBi-like virus in serum, buffy coat and ear notch from PI calves. According to the used test and sample type, the percentage of false-negative was 17-75% using BVDV cRT-PCR, 0-17% using BVDV-rRT-PCR, 13-25% using HoBi-like cRT-PCR and 4-38% using HoBi-like rRT-PCR. (Bauermann *et al*, 2014). Expanding the diagnostic capacity to include other viruses related to bovine Pestiviruses was also attempted. The cRT-PCR followed by ELISA was used to detect the PCR product successfully used to simultaneously detect BVDV-1, BVDV-2, as well as the Border disease virus in cattle, sheep and goats with high sensitivity, specificity and detection limit of 10 TCID50/ml (Dubey *et al*, 2015). Similarly, triplex TaqMan-rRT-PCR was successfully used to detect and differentiate wild-type classical swine fever virus, hog cholera lapinised vaccine (HCLV) and BVDV-1 with a detection limit of 3.2 TCID50 for BVDV-1 (Zhang *et al*, 2012). Some studies developed a five-plex cRT-PCR was developed to detect five viruses related to bovine respiratory disease (BRD) or bovine enteric disease (BED), followed by differentiation on an electronic microarray cartridge (Thanthrige-Don *et al*, 2018).

Another multiplex cRT-PCR assay was developed to do simultaneous detection of three BED viruses, including BVDV, with dual-priming oligonucleotide (DPO) primers combined with nanoparticles assisted PCR (DPO-nano-PCR). Results showed that the DPO-nano-PCR was able to detect the targeted pathogens with higher specificity and sensitivity compared to the cRT-PCR (4.09X101 versus 4.09X104 copies/µl regarding BVDV) (Wang *et al*, 2019). Similarly, another multiplex rRT-PCR was

developed to simultaneously screen 16 pathogens involved in the BRD syndrome, including the BVDV (Kishimoto *et al*, 2017). Similarly, it was used to detect and differentiate between several notifiable diseases of cattle, including BVDV (Wernike *et al*, 2015), with a detection limit of ≤100 copies/reaction.

Several isothermal amplification techniques were developed for the detection of BVDVs. The reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) was adapted to detect BVDV-RNA with high specificity and sensitivity. This technique was used successfully to detect as low as 4.67X100 RNA copies of the viral genome (Fan *et al*, 2012) and 70 PFU/ml of the virus (Tajbakhsh *et al*, 2017). A single-step RT-LAMP test for detection and differentiation of BVDV-1-2 was described with a detection limit of 103 copies of synthesised DNAs (Mungthong *et al,* 2021). For the rapid detection of BVDV, an assay based on recombinase polymerase amplification (RAP) using primers and probes targeting the 5'UTR was developed. After 15 minutes of amplification at 38°C, the RAP product can be detected on the lateral flow dipstick (LFD). RAP-LFD assay was specific with a detection limit comparable to rRT-PCR (20 copies/reaction) (Hou *et al*, 2018).

Three promising techniques, including LAMP, recombinase polymerase amplification (RPA) and high-speed rRT-PCR (hrRT-PCR), were tested for suitability for field use and performance compared with standard rRT-PCR for detection of BVDV. All the three assays may provide results within 30 minutes, hrRT-PCR was superior regarding sensitivity and speed of assay design, the LAMP was the best assay regarding specificity, simplicity (portable equipment) and cost-effectiveness, while RPA was as simple as LAMP (Aebischer *et al*, 2014b). The cross-priming amplification (CPA) assay targeting the 5'-UTR region of the BVDV was successfully used in detecting BVDV-1 and -2 with a detection limit of 3500 and 80000 copies/reaction, respectively, in samples from PI animals (Kuta *et al*, 2015). The other isothermal technique is the hybridisation chain reaction (HCR) assay with colorimetric detection of gold nanoparticles. HCR assay based on a salt-induced aggregation of unmodified gold nanoparticles was developed to detect BVDV-RNA with a detection limit of 0.1 and 0.008 TCID50/reaction for visual and spectrophotometer measurement, respectively (Monjezi *et al*, 2016). Analogous detection of unamplified BVDV-RNA was successful with a detection limit of 200 TCID50/ml by naked eye (Heidari *et al*, 2016). Similar HCR assay based on peptide nucleic acid (PNA) induced aggregation of unmodified gold nanoparticles showed high sensitivity with a detection limit of 10.48 and 1.05 ng/reaction of BVDV-RNA for visual and spectrophotometer measurement, respectively (Askaravi *et al*, 2017).

An assay with dual ssDNA aptamers capable of binding to BVDV-1, one for immobilisation of BVDV-1 (capturing probe) and the other conjugated with gold nanoparticles (reporting probe), was developed and successfully used to detect as low as 800 copies per ml, a sensitivity equivalent to that of rRT-PCR (Park *et al*, 2014). Another rapid screening test is a dot blot assay that developed based on the conjugation of BVDV specific affinity peptides with unmodified gold nanoparticles. Conjugate is added to bind to immobilised BVDV on nitrocellulose strips; then copper nano-polyhedral shells are added to amplify a signal. The detection limit of this test was reported to be 4.4 copies/ml (Kim *et al*, 2020). For *in situ* detection and quantification of BVDV in single-cell, RNA probe was used to specifically bind Npro-Erns coding region in BVDV-2a followed by flow cytometry and fluorescent microscopy (Falkenberg *et al*, 2017). Next-generation sequencing was successfully used to sequence a panel of 21 BVDV isolates and produce near-full complete genome sequences (Neill *et al,* 2014).

Future perspectives on the diagnosis of BVDV in Camelids

Despite the ubiquities nature of the spread of BVDV among cattle and other species of animals, still is still known about its prevalence, pathogenesis and diagnosis in dromedary camels. Currently, well-known diagnostic assays and approaches are adopted for the diagnosis of BVDV in other species of animals including dromedary camels. It is highly recommended to develop specific diagnostic assays for the detection of the BVDV in dromedary camels.

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