

Diagnostic applications of molecular and serological assays for bluetongue and African horse sickness

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Summary

The availability of rapid, highly sensitive and specific molecular and serological diagnostic assays, such as competitive enzyme-linked immunosorbent assay (cELISA), has expedited the diagnosis of emerging transboundary animal diseases, including bluetongue (BT) and African horse sickness (AHS), and facilitated more thorough characterisation of their epidemiology. The development of assays based on real-time, reverse-transcription polymerase chain reaction (RT-PCR) to detect and identify the numerous serotypes of BT virus (BTV) and AHS virus (AHSV) has aided in-depth studies of the epidemiology of BTV infection in California and AHSV infection in South Africa. The subsequent evaluation of pan-serotype, real-time, RT-PCR-positive samples through the use of serotype-specific RT-PCR assays allows the rapid identification of virus serotypes, reducing the need for expensive and time-consuming conventional methods, such as virus isolation and serotype-specific virus neutralisation assays. These molecular assays and cELISA platforms provide tools that have enhanced epidemiological surveillance strategies and improved our understanding of potentially altered *Culicoides* midge behaviour when infected with BTV. They have also supported the detection of subclinical AHSV infection of vaccinated horses in South Africa. Moreover, in conjunction with whole genome sequence analysis, these tests have clarified that the mechanism behind recent outbreaks of AHS in the AHS-controlled area of South Africa was the result of the reversion to virulence and/or genome reassortment of live attenuated vaccine viruses. This review focuses on the use of contemporary molecular diagnostic assays in the context of recent epidemiological studies and explores their advantages over historic virus isolation and serological techniques.

Keywords

African horse sickness – Bluetongue – Diagnostic – Epidemiology – Molecular assay – Real-time reverse-transcription polymerase chain reaction – Serology.

Introduction and history

African horse sickness virus (AHSV) and bluetongue virus (BTV) are segmented, double-stranded RNA viruses that are included in the genus *Orbivirus*, of the family *Reoviridae* (1).

Both BTV and AHSV are transmitted by haematophagous *Culicoides* midges, and thus the epidemiology of these two viruses is similar (2, 3, 4). Furthermore, both BTV and AHSV can cause lethal diseases in their respective animal hosts. Specifically, BTV is the cause of bluetongue (BT)

in domestic and wild ruminants, and AHSV is the cause of African horse sickness (AHS) in horses (genus *Equus*). Fulminant BT and AHS are both characterised by vascular leakage and hypovolaemic shock, analogous to that which occurs in human viral haemorrhagic fevers, such as Ebola (2, 5).

Early detailed descriptions of BT and AHS and initial characterisation of BTV and AHSV were made in South Africa in the late 19th and early 20th centuries (6, 7, 8, 9, 10, 11). Whereas AHS was usually confined to sub-Saharan Africa until recent incursions into Asia, BTV infection occurs throughout temperate and tropical regions of the world (3, 4). Both BT and AHS were previously included in List A of the World Organisation for Animal Health (OIE), a list of diseases notifiable to the OIE that may have serious socio-economic or public health consequences. Both diseases therefore constitute considerable barriers to the international movement and trade of ruminant livestock and horses (3, 4, 12, 13, 14, 15). Indeed, AHS is one of only seven diseases for which the OIE recognises official disease freedom status for individual countries, and is the only disease of horses to be given this designation. The availability of accurate and reliable diagnostic tests for both BT and AHS is central to the regulation of international trade and movement of susceptible animals (16, 17, 18). The purpose of this review article is to focus on the use of contemporary serological and molecular diagnostic assays, exploring their strengths over historic techniques such as virus serum neutralisation assays. Additionally, the authors will highlight the importance of using validated tests for application in diagnostic and epidemiological studies to ensure accurate interpretation of results.

Evolution of laboratory diagnostic assays for bluetongue and African horse sickness

Virus detection

In experimental terms, intravenous inoculation of embryonating chicken eggs (ECE) was confirmed to be a more sensitive method of virus isolation than yolk-sac inoculation (19, 20). Propagation of BTV in cell cultures was first described by Haig (cited by Henning in 1956) and clearly provides a more convenient laboratory method for isolating and propagating BTV than inoculation of ECE or susceptible animals (7). However, the passage of suspect material in ECE before inoculation into cell culture increases the sensitivity of BTV isolation in *in vitro* assays (21, 22, 23). Once adapted, BTV is readily propagated in

cell culture (24). The process, isolation and characterisation of BTV using the methods described above is inherently time-consuming (typically taking several weeks), labour intensive, costly and often technically challenging.

As with BT, AHS diagnosis was historically based on the characteristic clinical signs and lesions exhibited by affected horses and by the epidemiological features of disease outbreaks (7). Identifying AHSV required expensive and laborious inoculation and monitoring of susceptible equids (horses and mules) until it was shown that AHSV could be cultivated in either ECE or suckling mouse brain (7, 25, 26, 27, 28, 29). The adoption of cell culture isolation for AHSV is attributed to Erasmus (30).

Molecular diagnostic tests

The advent of molecular techniques, notably real-time, reverse transcription polymerase chain reaction (RT-PCR), has facilitated the rapid, sensitive and specific detection of both BTV and AHSV, which, in turn, has expedited and transformed the accurate diagnosis of BT and AHS. While conventional PCR-based assays for detecting BTV nucleic acid were first developed over 25 years ago, the advancement of molecular tools has been central to understanding the epidemiology of both diseases in recent field studies (31, 32, 33). Although a number of different BTV gene segments have been targeted in individual assays, those targeting gene segment 10 (which encodes the non-structural protein NS3) consistently identify all serotypes of BTV, regardless of their geographic origin (16, 32, 34, 35, 36, 37). Additionally, these assays are analytically sensitive and specific, with sufficient experimental detail (Table I).

Similarly, real-time RT-PCR assays have been developed and, notably, those based on detection of the gene-encoding core viral protein 7 (VP7) of AHSV have been characterised and validated, according to OIE standards (16, 41, 42, 43). A highly sensitive and specific diagnostic assay, developed in A.J. Guthrie's laboratory, has proven useful in investigating suspected outbreaks and furthering epidemiological studies of AHS in South Africa (16, 41, 42, 44, 45, 46, 47, 48, 49). The diagnostic sensitivity and specificity of this AHSV pan-serotype, real-time RT-PCR assay was estimated using blood samples from 503 horses with clinical signs consistent with AHS. Five hundred and three of these were uninfected and unvaccinated from the AHS-free zone in South Africa, and 98 were adult horses from an international race meeting in Dubai (42). The application of standard statistical approaches with virus isolation as the reference standard (i.e. use of 2×2 tables for the counts of the joint test results) would have led to a conclusion of poor specificity for the real-time RT-PCR, even though an analytical validation of the assay had not found any cross-reacting viruses (41). To prevent bias because of an imperfect reference standard, Bayesian latent class models were used to assess assay performance

Table I
Commercially available bluetongue virus diagnostic assays

Method	Test kit	DSe/DSP*	Target	Comments	Company references/links
Agent identification					
Real-time RT-PCR	ID Gene™ BlueTongue Duplex	Not listed	Serotypes 1–27	Validated by the French National Reference Laboratory (38) and according to the French standard NFU47-600-2 Registered at the Federal Food Safety and Veterinary Office (OSAV/BLV/USAV)	www.id-vet.com/produit/id-gene-bluetongue-duplex/ www.labgene.ch/veterinar-diagnostics/504-id-gene-real-time-pcr-kits.html
	VetMAX™ BTVNS3 All Genotypes Kit	Not listed	Serotypes 1–27 By segment 10	Validated by the French National Reference Laboratory (38)	www.thermofisher.com/order/catalog/product/BTVNS3?us&en#/BTVNS3?us&en
	LSI VetMAX™ Bluetongue Virus Real-Time PCR Kit, BTV 1 typing – IAH	Not listed	Serotype 1		www.thermofisher.com/order/catalog/product/BTV1GIAH50#/BTV1GIAH50
	VetMAX™ BTV8 IAH Typing kit	Not listed	Serotype 8	Validated by the French National Reference Laboratory (38)	www.thermofisher.com/order/catalog/product/BTV8GIAH50?us&en#/BTV8GIAH50?us&en
	Bio-T® kit BTV all genotypes	Not listed	Serotypes 1–27	Validated by the French National Reference Laboratory (38)	biosellal.com/famille-bio-t-ruminants/173-bio-t-kit-btv-all-genotypes.html
	ADIAVET™ BTV REAL TIME (ADI352)	100%/100%	Serotypes 1–27 By segment 10	Validated by the French National Reference Laboratory (38)	www.biox.com/en/adi352-100-adiavet-btv-real-time-100r-p-395/ ADIAVET™ BTV Real Time Validation Data by ADIAGENE bio X diagnostics Instruction manual: ADIAVET™ BTV Real Time Test for the Detection of the Blue Tongue Virus by Real-Time Enzymatic Amplification (RT-PCR test)
	ADIAVET BTV TYPE 8 REAL TIME (ADI381)	Not listed	Serotype 8	Validated by the French National Reference Laboratory (38)	www.biox.com/en/adi381-50-adiavet-btv-type-8-real-time-50r-p-397/
	ADIAVET BTV ALL + TYPE 8 REAL TIME (ADI401)	Not listed	Serotypes 1–27 and serotype 8 specifically	Validated by the French National Reference Laboratory (38)	www.biox.com/fr/adi401-100-adiavet-btv-all-type-8-real-time-100r-p-399/
	ViPrimePLUS Bluetongue Virus RT-qPCR Kit	Not listed	Segment 10 target	Ct value 28 ± 3 with probability of 95% Detection limit is tested to 100 copies per reaction Primers and probes are 100% specific	www.vivanttechnologies.com/index.php?option=com_content&view=article&id=826:vi-primeplus-bluetongue-virus-rt-qpcr-kit&catid=78:ruminants&Itemid=107
	Primerdesign™ Ltd Bluetongue Virus Nonstructural protein 3 (NS3) gene genesig® Standard Kit	Not listed	Segment 10 target	Primers represent 100% homology with > 95% of the NCBI database reference sequences	www.genesig.com/assets/files/btv_std.pdf
	Creative Biogene Bluetongue Virus (BTV) Real Time RT-PCR Kit	Not listed	Not listed		www.creative-biogene.com/bluetongue-virus-btv-real-time-rt-pcr-item-pdps-ar074-440372.html
	QIAGEN virotype® BTV RT-PCR Kit	Not listed	Not listed	Approved by the Friedrich-Loeffler-Institut and registered in accordance with Section 17c of the German Law on Animal Diseases (FLI-B 482) for use in Germany for veterinary diagnostic procedures	Virotype BTV RT-PCR Kit Handbook 09/2014

Method	Test kit	DSe/DSp*	Target	Comments	Company references/links
Immune response detection					
cELISA	ID Screen® Bluetongue Competition	87.8%/98.2% (39)	Anti-VP7 antibodies		www.id-vet.com/produit/id-screen-bluetongue-competition/
	Bluetongue Virus Antibody Test Kit, cELISA v2	100%/99%	Not listed	Meets USDA requirements for serial release	vmrd.com/test-kits/detail/bluetongue-virus-antibody-test-kit-celisa-v2/ intermed.ua/up/catalog/VMRD.pdf
	IDEXX Bluetongue Competition Ab Test	100%/100%	Anti-VP7 antibodies		www.idexx.com/en/livestock/livestock-tests/ruminant-tests/idexx-bluetongue-competition-ab-test/ IDEXX Bluetongue Competition Ab test Validation Data Report
Indirect ELISA	ID Screen® Bluetongue Milk Indirect	98.9%/96.5% (40)	Anti-VP7 antibodies	Milk is the sample type	www.id-vet.com/produit/id-screen-bluetongue-milk-indirect/
AGID	Bluetongue Virus Antibody Test Kit, AGID	100%/99%	Not listed	Meets USDA requirements for serial release	vmrd.com/test-kits/detail/bluetongue-virus-antibody-test-kit-agid/ http://intermed.ua/up/catalog/VMRD.pdf

*DSe/DSp: diagnostic sensitivity/specificity

Ab: antibody

AGID: agar gel immunodiffusion

BTV: bluetongue virus

cELISA: competitive enzyme-linked immunosorbent assay

Ct: cycle threshold

ELISA: enzyme-linked immunosorbent assay

IAH: Institute for Animal Health, Pirbright (United Kingdom)

NCBI: National Center for Biotechnology Information (United States of America)

OSAV/BLV/USAV: Federal Food Safety and Veterinary Office (Switzerland)

RT-PCR: reverse-transcription polymerase chain reaction

USDA: United States Department of Agriculture

VP: viral protein

(42). In all models, the sensitivity of real-time RT-PCR was at least twofold higher than that of virus isolation in cell culture. For subclinical AHSV infection scenarios, there were no sensitivity data for real-time RT-PCR at that stage, and hence a more conservative sensitivity value of 91.4% (95% probability interval, 65.5-99.4%) for real-time RT-PCR was used in the quantitative risk assessment for AHS in live horses exported from South Africa (15).

Serotype-specific, real-time RT-PCR assays based on unique sequences of gene segment 2 (which encodes the serotype-specific, outer-capsid protein VP2) have been developed for the rapid serotyping of both BTV and AHSV, which is essential to select the most appropriate vaccine type during disease outbreaks and for epidemiological investigations (50, 51, 52, 53). Within the context of diagnostic or epidemiologic studies, it is important to recognise that situations may arise where a pan-serotype, real-time RT-PCR is positive while a serotype-specific, real-time RT-PCR assay remains negative. In these cases, the recent advent of whole genome sequence analysis has facilitated in-depth evaluation of circulating and outbreak viruses (47).

Some caution is needed in the interpretation of real-time RT-PCR results in the diagnosis of both BT and AHS. For example, BTV nucleic acid can be detected in blood from infected animals long after viable virus is no longer present (54, 55). Live attenuated vaccines can also be detected

by real-time RT-PCR and differentiation from field virus infection can be difficult (48). Therefore, a positive test result indicates that an animal has been infected or vaccinated at some point prior to the collection of the specimen, but the animal may not necessarily still be infectious to *Culicoides* insects (34). There are numerous molecular tests used to detect BTV and AHSV, and viral RNA detection is often used as a presumptive test, due to the labour and time involved in virus isolation. These molecular assays are an important part of the diagnostician's and researcher's toolbox. Evaluating test results in the context of the clinical, immune and exposure history of an animal or population of animals typically results in a correct diagnosis.

Serological assays

Serological detection of BTV exposure relies on serogroup-specific antibody detection targeting the VP7 protein, and this is important for identifying animals that have previously been infected, regardless of BTV serotype. Complement fixation, indirect immunofluorescent antibody, agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) have all been used for the detection of serogroup-specific antibodies against BTV (56, 57, 58, 59, 60, 61, 62, 63, 64). However, competitive ELISA (cELISA) assays using monoclonal antibodies against viral core protein VP7 are now routinely used as they are accepted by the OIE as a perfect reference

standard to provide a highly sensitive and specific method for the serological diagnosis of BTV infection of animals, regardless of the infecting virus serotype (16). Antibodies persist for long periods after natural infection, although it is not currently possible to distinguish animals that were naturally infected from those that were immunised with live attenuated vaccine. It is important to note that the presence of serum antibody only indicates prior exposure to BTV and implies nothing about disease causality. In addition, the sensitivity and specificity of all cELISA tests vary with the cut-off inhibition value used to declare a result positive or negative, and the cut-off value can change depending on the purpose of the test (e.g. clinical disease confirmation versus surveillance). For transparency in peer-reviewed publications, it is recommended that the sensitivity and specificity of cELISA be reported across a range of cut-off values to facilitate intra-study comparison of estimates.

Commercial cELISA test kits for BT are widely available, including in both North America and Europe (Table I). Although one of the advantages of the cELISA assay is that it can be used to test serum samples from a wide range of ruminant species, a more significant benefit is improved inter-laboratory reproducibility. For example, Singer *et al.* (65) compared AGID, cELISA and serum neutralisation (SN) assays in free-ranging desert bighorn sheep (*Ovis canadensis*) in New Mexico, the United States of America (USA), and concluded that all three assays gave comparable results for determining serogroup exposure (65).

One of the first validation studies of an in-house cELISA was performed in MacLachlan's laboratory and compared the sensitivity and specificity of cELISA, AGID and SN tests using experimentally inoculated ruminants and rabbits (*Oryctolagus cuniculus*) (66). After commercialisation of the Reddington *et al.* (66) assay, validation studies continued, comparing the agreement of a commercial cELISA assay with an in-house, non-standardised cELISA (66). In related studies, Afshar *et al.* (67) concluded that the commercial kit would detect animal antibody responses against South African, USA and Australian strains of BTV (67). Subsequently, Ward *et al.* (68) evaluated a commercial cELISA in ten cattle herds in northern Australia and found that, on average, cattle were detected positive by cELISA at least one month before they could be detected positive by SN (68). Although the SN is considered to be the gold standard, the sensitivity of the cELISA is superior (65).

Additional validation studies followed after the incursion of BTV into Corsica in 2000 and 2001, and the BTV-8 incursion that occurred in Belgium in 2006 and spread through much of Europe (39, 69). The latter study used Bayesian analysis and included comparison of the cELISA and real-time RT-PCR. The real-time RT-PCR had higher sensitivity (99.5%) than the cELISA (87.8%) during the epidemic, and both assays had specificities > 98% (39).

Although serum is the typical specimen for assessing prior BTV infection in animals, milk has also been validated as a suitable diagnostic sample and yields similar results. For example, only 10/470 coupled serum-milk samples gave discordant results in Dutch dairy cows (40). More recent studies in the European Union (EU) have involved expanding the specimen type used for the cELISA to include pooled samples (milk and serum) as part of ring trials (70, 71). Despite the international importance of BT, none of the cELISA test kits is currently certified by the OIE for inclusion on the OIE register of diagnostic kits (OIE Register).

The serum-virus neutralisation (VN) assay (or variants such as plaque neutralisation) is used to serotype virus isolates and for quantitation of antibodies against specific BTV serotypes (16, 63, 72, 73). Interpreting antibody responses against specific serotypes can be problematic, particularly where animals have been infected with more than one serotype. Similar serological methods and challenges are present when evaluating antibody responses against AHSV. Serological diagnosis of prior AHSV infection is similar to that of BTV; however, serogroup-specific ELISAs (either competitive or indirect ELISAs) are not widely used diagnostically (74, 75). Serotype-specific antibodies against each of the nine serotypes of AHSV are quantitated by VN assays (16).

Applying molecular diagnostics to epidemiological studies

Bluetongue in California

Initial epidemiological studies of BTV in California (c. 1980s) were reliant on virus isolation in ECE, with subsequent characterisation of viruses through propagation in cell culture and serotyping by VN assay, using serotype-specific antisera (22, 76). The advent of real-time RT-PCR assays has been crucial to both rapid diagnosis during disease outbreak investigations and comprehensive epidemiological investigations of BTV infection in California, using methods prescribed in the relevant chapters of the OIE *Terrestrial Animal Health Code (Terrestrial Code)* and *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual)* (16, 77, 78, 79, 80, 81). Specifically, sentinel cattle at dairy farms throughout the state were bled regularly and screened for seroconversion to BTV by serogroup-specific cELISA. Sequential blood samples from individual sentinel cattle that seroconverted to BTV were then evaluated for the presence of viral nucleic acid, first by pan-serotype, real-time RT-PCR and second, from any animals testing positive, using virus serotype-specific, real-time RT-PCR assays (81). Data from these studies were used to develop predictive models of BTV infection in California,

and evaluate the various effects of climate, region and anthropogenic influences (Fig. 1) (82). Identifying key drivers of BTV infection of ruminant livestock in the region is a prerequisite to the logical design and implementation of potential control strategies (83).

Vector midges were collected at many of the same study sites using different trapping methods, and pools of these midges were evaluated by pan-serotype, real-time RT-PCR. Samples that tested positive were further analysed by BTV-serotype-specific, real-time RT-PCR assays (81). Using these molecular tools to further understand BTV infection rates in midges circumvents the inherent limitations and complexities of conventional cell-culture-based detection methods. A key finding from these field studies was the presence of BTV-infected adult midges during the putative 'overwintering' period of the virus, which probably explains how this virus maintains itself between seasons within temperate regions (84). Furthermore, these field studies confirmed potentially altered behaviour of BTV-infected midges, notably their host-seeking behaviour.

The remarkable sensitivity of these molecular assays has also facilitated pathogenesis studies in the invertebrate host of BTV. These studies, which also included immunohistochemical staining analyses, confirmed viral deposition within the eyes of BTV-infected *Culicoides* midges that was associated with their altered attraction to light and host-seeking behaviour (85).

African horse sickness in South Africa

African horse sickness was first described in South Africa after horses were introduced to the region by European settlers during the 17th century (2, 7, 9, 10, 11). Dramatic outbreaks of AHS occurred during the 19th and 20th centuries, which in turn catalysed research efforts by Sir Arnold Theiler and others to find a solution to the problem. The epidemiological features of AHSV infection were characterised long before the advent of molecular diagnostics (7). However, the development of real-time RT-PCR assays (both group and serotype specific) has greatly expedited diagnostic investigation of disease outbreaks and provided further insight as to how the virus is maintained

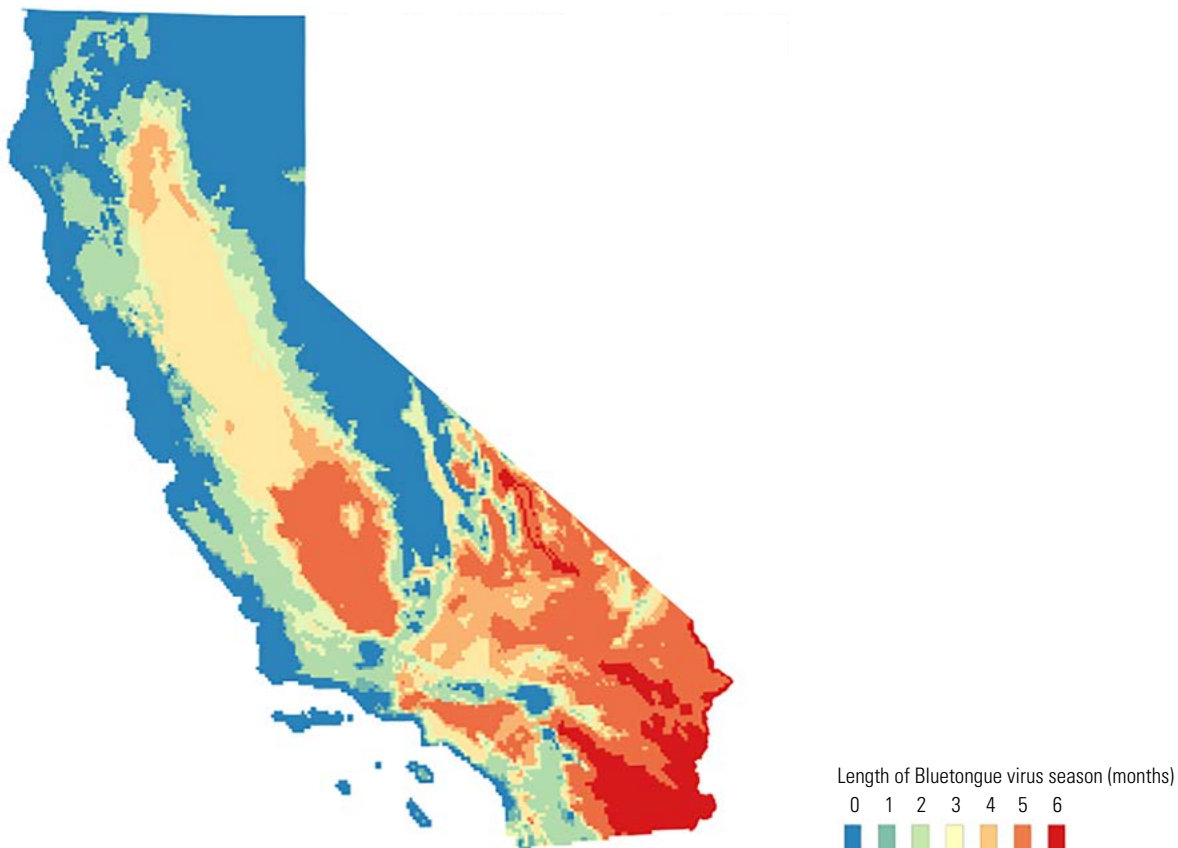


Fig. 1
Map of California depicting monthly basic reproductive rate (R_0) estimates defining the annual bluetongue virus season as the period (calendar months) when $R_0 > 1$ in different regions of the state (82)

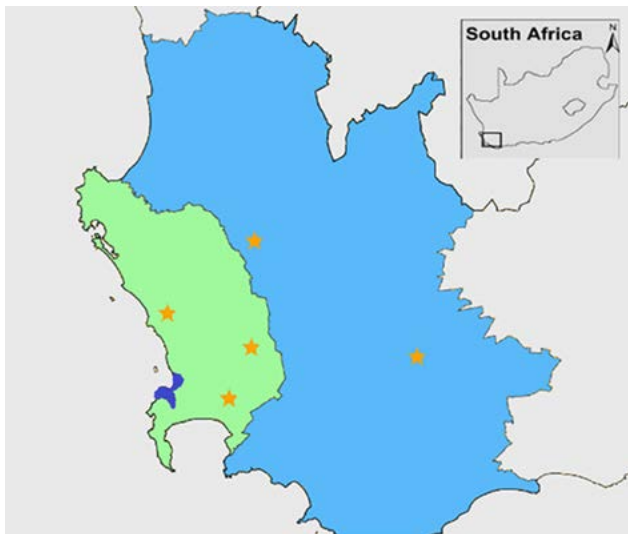


Fig. 2
The African horse sickness-controlled area of South Africa
 The location of the area within South Africa is shown in the inset. The African horse sickness (AHS) protection zone is light blue, the AHS surveillance zone green and the AHS-free zone is dark blue. Locations in the area of the outbreaks (2004–2014) attributed to reversion to virulence or reassortment of live attenuated vaccine viruses are shown as gold stars (47)

and spread (3, 15, 42, 44, 45, 46, 47). Specifically, these recent studies have confirmed that asymptomatic AHSV infection occurs in previously vaccinated horses. Clearly, such animals would not be recognised in surveillance programmes based solely on the detection of clinical disease (46, 49). Furthermore, use of the AHSV pan-serotype and serotype-specific, real-time RT-PCR assays, in conjunction with whole genome sequence analyses, has confirmed that several recent outbreaks of AHS in the legislatively defined AHS-controlled area of the Western Cape Province (Fig. 2) were caused by reversion to virulence and/or reassortment of live attenuated AHS vaccine viruses (47). Given the high diagnostic sensitivity and specificity of validated, real-time RT-PCR assays, coupled with appropriate quarantine, the likelihood of exporting horses with an undetected AHSV infection from endemic countries can be minimised (15). Furthermore, these diagnostic assays, alongside whole genome sequencing, provide tools for very rapid and confirmed diagnosis of AHS in previously infection-free regions, as recently occurred most unexpectedly in Thailand (86).

Conclusions

Serological assays for BT have an important role in sentinel surveillance and in monitoring exposure among a wide range of ruminant species. Additionally, molecular, real-time RT-PCR assays have facilitated the rapid and accurate

diagnosis of BT and AHS, an undertaking that was previously protracted, difficult, expensive and often inconclusive using earlier methods (e.g. VI, SN and AGID). The application of whole genome sequencing enriches molecular assays by completely characterising the complexity of orbivirus genomes beyond conventional serotyping nomenclature.

Although molecular analysis and typing of orbiviruses are essential tools for epidemiological surveillance, outbreak detection and control of BTV and AHSV, conclusions made based on these assays depend upon the epidemiological study design. Nucleic acid detection within the vector population has become one of the most popular methods of virus detection and has potentially displaced virus isolation as the new reference standard. Additionally, although a cold chain is still recommended, the use of real-time RT-PCR has demonstrated that it is possible to use real-time RT-PCR to detect viral RNA extracted from dead infected vectors kept for several weeks in hot and humid conditions (87, 88, 89).

The detection of viral RNA by PCR necessitates the further characterisation of the agent, either by serotype-specific, real-time RT-PCR or full genome sequencing. Advances in sequencing technologies have facilitated efficient characterisation of the full BTV genome. Full-length sequencing approaches have helped to identify novel serotypes as well as assisting characterisation of the genetic diversity of the virus. The continuing development of a growing database of fully sequenced BTV genomes has facilitated the design of conventional RT-PCR and real-time RT-PCR assays (90).

Thus, these modern diagnostic technologies improve characterisation and tracking of the circulating viruses identified in surveillance studies and, concomitantly, the information derived from these studies informs the future advancement of diagnostic assays.

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Applications diagnostiques des essais moléculaires et sérologiques de détection de la fièvre catarrhale ovine et de la peste équine

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Résumé

La disponibilité d'essais diagnostiques moléculaires et sérologiques rapides, hautement sensibles et spécifiques tels que l'épreuve immuno-enzymatique de compétition (ELISAc), a accéléré le diagnostic des maladies animales transfrontalières émergentes, dont la fièvre catarrhale ovine et la peste équine, et contribué à dresser un tableau épidémiologique plus complet de ces maladies. Grâce à la mise au point d'essais basés sur l'amplification en chaîne par polymérase en temps réel couplée à une transcription inverse (RT-PCR) qui permettent de détecter et d'identifier les nombreux sérotypes du virus de la fièvre catarrhale ovine et du virus de la peste équine, des études approfondies ont pu être conduites sur l'épidémiologie de l'infection par le virus de la fièvre catarrhale ovine en Californie et de l'infection par le virus de la peste équine en Afrique du Sud. L'évaluation postérieure des échantillons positifs à une RT-PCR en temps réel de groupe (détectant le virus quel que soit le sérotype) au moyen de RT-PCR spécifiques de chaque sérotype permet d'identifier rapidement le sérotype causal et de limiter le recours à des méthodes classiques onéreuses et chronophages comme l'isolement viral ou les essais de neutralisation virale spécifiques de chaque sérotype. Les outils fournis par ces essais moléculaires et par les plateformes ELISAc ont renforcé les stratégies de surveillance épidémiologique et permis de mieux connaître les altérations potentielles de comportement chez les moucheron *Culicoides* infectés par le virus de la fièvre catarrhale ovine. Ils ont également contribué à détecter des cas d'infection asymptomatique par le virus de la peste équine chez des chevaux vaccinés en Afrique du Sud. En outre, combinés à une analyse de séquences du génome entier, ces tests ont révélé que le mécanisme sous-jacent aux récents foyers de peste équine dans la zone de contrôle en Afrique du Sud correspondait à une réversion vers la virulence et/ou à un réassortiment du génome des souches de vaccin à virus vivant atténué. Les auteurs passent en revue l'utilisation des essais de diagnostic moléculaire de nouvelle génération dans le contexte de récentes études épidémiologiques et cherchent à établir leurs avantages par rapport aux techniques classiques d'isolement viral et de recherche sérologique.

Mots-clés

Amplification en chaîne par polymérase en temps réel couplée à une transcription inverse
– Diagnostic – Épidémiologie – Essai moléculaire – Fièvre catarrhale ovine – Peste équine
– Sérologie.



Aplicaciones de los ensayos moleculares y serológicos para el diagnóstico de la lengua azul y la peste equina

C.E. Mayo, C.T. Weyer, M.J. Carpenter, K.J. Reed, C.P. Rodgers, K.M. Lovett, A.J. Guthrie, B.A. Mullens, C.M. Barker, W.K. Reisen & N.J. MacLachlan

Resumen

La existencia de ensayos moleculares y serológicos de diagnóstico rápidos y de gran sensibilidad y especificidad, como el ensayo inmunoenzimático de competición (ELISAc), ha acelerado el diagnóstico de enfermedades animales transfronterizas emergentes, como la lengua azul o la peste equina, y facilitado una caracterización más exhaustiva de su epidemiología. La creación de ensayos basados en la reacción en cadena de la polimerasa acoplada a transcripción inversa (RT-PCR) en tiempo real para detectar y caracterizar los numerosos serotipos de los virus de la lengua azul y la peste equina ha ayudado a estudiar a fondo la epidemiología de sendos episodios infecciosos causados por el virus de la lengua azul en California y por el virus de la peste equina en Sudáfrica. El subsiguiente análisis de las muestras positivas a la prueba de RT-PCR en tiempo real de cualquier serotipo con empleo de ensayos RT-PCR dirigidos específicamente contra uno u otro serotipo permite identificar rápidamente los serotipos víricos, lo que hace menos necesario el uso de métodos convencionales más caros y largos, como el aislamiento del virus o técnicas de neutralización vírica adaptadas específicamente a un serotipo. Estos dispositivos de ensayo molecular o de ELISAc ponen a nuestra disposición herramientas que potencian las estrategias de vigilancia epidemiológica y ayudan a conocer mejor las eventuales alteraciones del comportamiento de los jevenes *Culicoides* al ser infectados por el virus de la lengua azul. Estas técnicas han ayudado también a detectar en Sudáfrica casos de infección asintomática por el virus de la peste equina en caballos vacunados. Estas pruebas, además, empleadas en combinación con el análisis de secuencias genómicas completas, han servido para aclarar que el mecanismo subyacente a los recientes brotes de peste equina surgidos en la zona de control en Sudáfrica fue fruto de la reversión a la virulencia y/o el reordenamiento genómico de virus vacunales atenuados. Los autores, centrándose en el uso de modernos ensayos moleculares de diagnóstico como parte de recientes estudios epidemiológicos, examinan las ventajas que ofrecen en comparación con las tradicionales técnicas serológicas y de aislamiento vírico.

Palabras clave

Diagnóstico – Ensayo molecular – Epidemiología – Lengua azul – Peste equina – Reacción en cadena de la polimerasa acoplada a transcripción inversa en tiempo real – Serología.



References

1. MacLachlan N.J. & Dubovi E.J. (eds) (2017). – Reoviridae, Orbivirus. Chapter 15. In Fenner's veterinary virology, 5th Ed. Academic Press, Oxford, United Kingdom.
2. Coetzer J.A.W. & Guthrie A.J. (2004). – African horse sickness. In Infectious diseases of livestock, 2nd Ed. Oxford University Press, Cape Town, Africa, 1231–1246.
3. Zientara S., Weyer C.T. & Lecollinet S. (2015). – African horse sickness. In New developments in major vector-borne diseases. Part 2: Important diseases for veterinarians (S. Zientara, D. Verwoerd & P.-P. Pastoret, eds). *Rev. Sci. Tech. Off. Int. Epiz.*, **34** (2), 315–327. doi:10.20506/rst.34.2.2359.
4. MacLachlan N.J., Mayo C.E., Daniels P.W., Savini G., Zientara S. & Gibbs E.P.J. (2015). – Bluetongue. In New developments in major vector-borne diseases. Part 2: Important diseases for veterinarians (S. Zientara, D. Verwoerd & P.-P. Pastoret, eds). *Rev. Sci. Tech. Off. Int. Epiz.*, **34** (2), 329–340. doi:10.20506/rst.34.2.2360.
5. MacLachlan N.J., Drew C.P., Darpel K.E. & Worwa G. (2009). – The pathology and pathogenesis of bluetongue. *J. Comp. Pathol.*, **141** (1), 1–16. doi:10.1016/j.jcpa.2009.04.003.
6. Spreull J. (1905). – Malarial catarrhal fever (bluetongue) of sheep in South Africa. *J. Comp. Path. Ther.*, **18**, 321–337. doi:10.1016/S0368-1742(05)80073-6.
7. Henning M.W. (1956). – Animal diseases in South Africa, 3rd Ed. Central News Agency Ltd, Johannesburg, South Africa.
8. MacLachlan N.J. (2011). – Bluetongue: history, global epidemiology, and pathogenesis. *Prev. Vet. Med.*, **102** (2), 107–111. doi:10.1016/j.prevetmed.2011.04.005.
9. Bayley T.B. (1856). – Notes on the Horse-sickness at the Cape of Good Hope, in 1854–'55. S. Solomon & Company, Cape Town, South Africa, 161 pp.
10. Fadyean J.M. (1900). – African horse sickness. *J. Comp. Path. Therap.*, **13**, 383 pp. Available at: krishikosh.egranth.ac.in/handle/1/2045358 (accessed on 4 February 2021).
11. Theiler A. (1921). – African horse sickness (*Pestis equorum*). *Union S. Afr. Dept Agric., Pretoria, Sci. Bull.*, **19**, 32 pp. Abstract available at: www.cabdirect.org/cabdirect/abstract/19211000431 (accessed on 4 February 2021).
12. MacLachlan N.J. & Osburn B.I. (2006). – Impact of bluetongue virus infection on the international movement and trade of ruminants. *JAVMA*, **228** (9), 1346–1349. doi:10.2460/javma.228.9.1346.
13. Dominguez M., Münstermann S., Murray G. & Timoney P. (2015). – High health, high-performance horses: risk mitigation strategies for OIE-listed diseases. *Rev. Sci. Tech. Off. Int. Epiz.*, **34** (3), 837–848. doi:10.20506/rst.34.3.2399.
14. Grewar J.D. (2016). – The economic impact of bluetongue and other orbiviruses in sub-Saharan Africa, with special reference to southern Africa. *Vet. Ital.*, **52** (3–4), 375–381. doi:10.12834/VetIt.503.2427.3.
15. Sergeant E.S., Grewar J.D., Weyer C.T. & Guthrie A.J. (2016). – Quantitative risk assessment for African horse sickness in live horses exported from South Africa. *PLoS One*, **11** (3), e0151757. doi:10.1371/journal.pone.0151757.
16. World Organisation for Animal Health (OIE) (2018). – Manual of Diagnostic Tests and Vaccines for Terrestrial Animals., 8th Ed OIE, Paris, France. Available at: www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/ (accessed on 7 January 2021).
17. World Organisation for Animal Health (OIE) (2019). – Infection with bluetongue virus. Chapter 8.3. In Terrestrial Animal Health Code. OIE, Paris, France. Available at: www.oie.int/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_bluetongue.pdf (accessed on 7 January 2021).
18. World Organisation for Animal Health (OIE) (2019). – Infection with African horse sickness virus. Chapter 12.1. In Terrestrial Animal Health Code. OIE, Paris, France. Available at: www.oie.int/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_ahs.pdf (accessed on 7 January 2021).
19. Goldsmit L. & Barzilai E. (1968). – An improved method for the isolation and identification of bluetongue virus by intravenous inoculation of embryonating chicken eggs. *J. Comp. Pathol.*, **78** (4), 477–487. doi:10.1016/0021-9975(68)90047-9.
20. Foster N.M. & Leudke A.J. (1968). – Direct assay for bluetongue virus by intravascular inoculation of embryonating chicken eggs. *Am. J. Vet. Res.*, **29** (3), 749–753.

21. Howell P.G., Kümme N.A. & Botha M.J. (1970). – The application of improved techniques to the identification of strains of bluetongue virus. *Onderstepoort J. Vet. Res.*, **37** (1), 59–66. Abstract available at: www.cabdirect.org/cabdirect/abstract/19712205249 (accessed on 26 January 2021).
22. Osburn B.I., McGowan B., Heron B., Loomis E., Bushnell R., Stott J. & Utterback W. (1981). – Epizootiologic study of bluetongue: virologic and serologic results. *Am. J. Vet. Res.*, **42** (5), 884–887. Abstract available at: www.ncbi.nlm.nih.gov/pubmed/6266296 (accessed on 12 January 2021).
23. Gard G.P., Shorthose J.E., Weir R.P., Walsh S.J. & Melville L.F. (1988). – Arboviruses recovered from sentinel livestock in northern Australia. *Vet. Microbiol.*, **18** (2), 109–118. doi:10.1016/0378-1135(88)90056-9.
24. Sawyer M.M. & Osburn B.L. (1977). – Isolation of bluetongue vaccine virus from infected sheep by direct inoculation onto tissue culture. *Cornell Vet.*, **67** (1), 65–71. Abstract available at: www.ncbi.nlm.nih.gov/pubmed/188594 (accessed on 12 January 2021).
25. Alexander R.A. (1933). – Preliminary note on the infection of white mice and guinea-pigs with the virus of horse sickness. *J. S. Afr. Vet. Med. Assoc.*, **4** (933), 1–9. Available at: journals.co.za/doi/pdf/10.10520/AJA00382809_3305 (accessed on 27 January 2021).
26. Alexander R.A. (1934). – Studies on the neurotropic virus of horsesickness. DVSc thesis, University of South Africa. Available at: repository.up.ac.za/handle/2263/41409 (accessed on 7 January 2021).
27. Alexander R.A. (1935). – Studies on the neurotropic virus of horsesickness II: some physical and chemical properties. *Onderstepoort J. Vet. Res.*, **4** (2), 291–322. Available at: repository.up.ac.za/handle/2263/53702 (accessed on 7 January 2021).
28. Alexander R.A. (1935). – Studies on the neurotropic virus of horsesickness III: the intracerebral protection test and its application to the study of immunity. *Onderstepoort J. Vet. Res.*, **4** (2), 349–377. Available at: repository.up.ac.za/handle/2263/53787 (accessed on 7 January 2021).
29. Alexander R.A. (1935). – Studies on the neurotropic virus of horsesickness IV: the pathogenesis in horses. *Onderstepoort J. Vet. Res.*, **4** (2), 379–388. Available at: repository.up.ac.za/handle/2263/53818 (accessed on 7 January 2021).
30. Erasmus B.J. (1963). – Cultivation of horsesickness virus in tissue culture. *Nature*, **200**, 716. doi:10.1038/200716a0.
31. Wilson W.C. (1994). – Development of a nested-PCR test based on sequence analysis of epizootic hemorrhagic disease viruses non-structural protein 1 (NS1). *Virus Res.*, **31** (3), 357–365. doi:10.1016/0168-1702(94)90028-0.
32. Bonneau K.R., Mullens B.A. & MacLachlan N.J. (2001). – Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and *Culicoides sonorensis*. *J. Virol.*, **75** (17), 8298–8305. doi:10.1128/jvi.75.17.8298-8305.2001.
33. Katz J., Alstad D., Gustafson G. & Evermann J. (1994). – Diagnostic analysis of the prolonged bluetongue virus RNA presence found in the blood of naturally infected cattle and experimentally infected sheep. *J. Vet. Diagn. Invest.*, **6** (2), 139–142. doi:10.1177/104063879400600201.
34. Bonneau K.R., DeMaula C.D., Mullens B.A. & MacLachlan N.J. (2002). – Duration of viraemia infectious to *Culicoides sonorensis* in bluetongue virus-infected cattle and sheep. *Vet. Microbiol.*, **88** (2), 115–125. doi:10.1016/s0378-1135(02)00106-2.
35. Orrù G., Ferrando M.L., Meloni M., Liciardi M., Savini G. & De Santis P. (2006). – Rapid detection and quantitation of bluetongue virus (BTV) using a molecular beacon fluorescent probe assay. *J. Virol. Meth.*, **137** (1), 34–42. doi:10.1016/j.jviromet.2006.05.028.
36. Balasuriya U.B.R., Nadler S.A., Wilson W.C., Pritchard L.I., Smythe A.B., Savini G., Monaco F., De Santis P., Zhang N., Tabachnick W.J. & MacLachlan N.J. (2008). – The NS3 proteins of global strains of bluetongue virus evolve into regional topotypes through negative (purifying) selection. *Vet. Microbiol.*, **126** (1–3), 91–100. doi:10.1016/j.vetmic.2007.07.006.
37. Hofmann M., Griot C., Chaignat V., Perler L. & Thür B. (2008). – Bluetongue disease reaches Switzerland. *Schweiz. Arch. Tierheilkd.*, **150** (2), 49–56. doi:10.1024/0036-7281.150.2.49.
38. Sailleau C., Viarouge C., Breard E., Vitour D. & Zientara S. (2017). – Ring trial 2016 for bluetongue virus detection by real-time RT–PCR in France. *Vet. Med. Sci.*, **3** (2), 107–114. doi:10.1002/vms3.63.
39. Vandebussche F., Vanbinst T., Verheyden B., Van Dessel W., Demeestere L., Houdart P., Bertels G., Praet N., Berkvens D., Mintiens K., Goris N. & De Clercq K. (2008). – Evaluation of antibody-ELISA and real-time RT–PCR for the diagnosis and profiling of bluetongue virus serotype 8 during the epidemic in Belgium in 2006. *Vet. Microbiol.*, **129** (1–2), 15–27. doi:10.1016/j.vetmic.2007.10.029.
40. Kramps J.A., van Maanen K., Mars M.H., Popma J.K. & van Rijn P.A. (2008). – Validation of a commercial ELISA for the detection of bluetongue virus (BTV)-specific antibodies in individual milk samples of Dutch dairy cows. *Vet. Microbiol.*, **130** (1–2), 80–87. doi:10.1016/j.vetmic.2008.01.004.
41. Quan M., Lourens C.W., MacLachlan N.J., Gardner I.A. & Guthrie A.J. (2010). – Development and optimisation of a duplex real-time reverse transcription quantitative PCR assay targeting the VP7 and NS2 genes of African horse sickness virus. *J. Virol. Meth.*, **167** (1), 45–52. doi:10.1016/j.jviromet.2010.03.009.

42. Guthrie A.J., MacLachlan N.J., Joone C., Lourens C.W., Weyer C.T., Quan M., Monyai M.S. & Gardner I.A. (2013). – Diagnostic accuracy of a duplex real-time reverse transcription quantitative PCR assay for detection of African horse sickness virus. *J. Virol. Meth.*, **189** (1), 30–35. doi:10.1016/j.jviromet.2012.12.01F4.
43. Agüero-García M., Castillo-Olivares J. & Münstermann S. (2016). – Validation of a real-time RT–PCR for the detection of African horse sickness virus genome. *OIE Bull.*, **2016** (1), 87–91. doi:10.20506/bull.2016.1.2504.
44. Scheffer E.G., Venter G.J., Joone C., Osterrieder N. & Guthrie A.J. (2011). – Use of real-time quantitative reverse transcription polymerase chain reaction for the detection of African horse sickness virus replication in *Culicoides imicola*. *Onderstepoort J. Vet. Res.*, **78** (1), 344. doi:10.4102/ojvr.v78i1.344.
45. Scheffer E.G., Venter G.J., Labuschagne K., Page P.C., Mullens B.A., MacLachlan N.J., Osterrieder N. & Guthrie A.J. (2012). – Comparison of two trapping methods for *Culicoides* biting midges and determination of African horse sickness virus prevalence in midge populations at Onderstepoort. *S. Afr. Vet. Parasitol.*, **185** (2), 265–273. doi:10.1016/j.vetpar.2011.09.037.
46. Weyer C.T., Quan M., Joone C., Lourens C.W., MacLachlan N.J. & Guthrie A.J. (2013). – African horse sickness in naturally infected, immunised horses. *Eq. Vet. J.*, **45** (1), 117–119. doi:10.1111/j.2042-3306.2012.00590.x.
47. Weyer C.T., Grewar J.D., Burger P., Rossouw E., Lourens C., Joone C., le Grange M., Coetzee P., Venter E., Martin D.P., MacLachlan N.J. & Guthrie A.J. (2016). – African horse sickness caused by genome reassortment and reversion to virulence of live, attenuated vaccine viruses, South Africa, 2004–2014. *Emerg. Infect. Dis.*, **22** (12), 2087–2096. doi:10.3201/eid2212.160718.
48. Weyer C.T., Grewar J.D., Burger P., Joone C., Lourens C., MacLachlan N.J. & Guthrie A.J. (2017). – Dynamics of African horse sickness virus nucleic acid and antibody in horses following immunization with a commercial polyvalent live attenuated vaccine. *Vaccine*, **35** (18), 2504–2510. doi:10.1016/j.vaccine.2017.03.005.
49. Grewar J.D., Weyer C.T., Guthrie A.J., Koen P., Davey S., Quan M., Visser D., Russouw E. & Bührmann G. (2013). – The 2011 outbreak of African horse sickness in the African horse sickness controlled area in South Africa. *J. S. Afr. Vet. Assoc.*, **84** (1), 1–7. Available at: www.scielo.org/za/scielo.php?script=sci_arttext&pid=S1019-91282013000100026&lng=en&nrm=iso (accessed on 4 February 2021).
50. Maan N.S., Maan S., Belaganahalli M.N., Ostlund E.N., Johnson D.J., Nomikou K. & Mertens P.P.C. (2012). – Identification and differentiation of the twenty-six bluetongue virus serotypes by RT–PCR amplification of the serotype-specific genome segment 2. *PLoS One*, **7** (2), e32601. doi:10.1371/journal.pone.0032601.
51. Maan S., Maan N.S., Belaganahalli M.N., Potgieter A.C., Kumar V., Batra K., Wright I.M., Kirkland P.D. & Mertens P.P.C. (2016). – Development and evaluation of real time RT–PCR assays for detection and typing of bluetongue virus. *PLoS One*, **11** (9), e0163014. doi:10.1371/journal.pone.0163014.
52. Weyer C.T., Joone C., Lourens C.W., Monyai M.S., Koekemoer O., Grewar J.D., van Schalkwyk A., Majiwa P.O.A., MacLachlan N.J. & Guthrie A.J. (2015). – Development of three triplex real-time reverse transcription PCR assays for the qualitative molecular typing of the nine serotypes of African horse sickness virus. *J. Virol. Meth.*, **223**, 69–74. doi:10.1016/j.jviromet.2015.07.015.
53. Bachanek-Bankowska K., Maan S., Castillo-Olivares J., Manning N.M., Maan N.S., Potgieter A.C., Di Nardo A., Sutton G., Batten C. & Mertens P.P.C. (2014). – Real time RT–PCR assays for detection and typing of African horse sickness virus. *PLoS One*, **9** (4), e93758. doi:10.1371/journal.pone.0093758.
54. MacLachlan N.J. (1994). – The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp. Immunol. Microbiol. Infect. Dis.*, **17** (3–4), 197–206. doi:10.1016/0147-9571(94)90043-4.
55. MacLachlan N.J., Nunamaker R.A., Katz J.B., Sawyer M.M., Akita G.Y., Osburn B.I. & Tabachnick W.J. (1994). – Detection of bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and *in vitro* feeding of *Culicoides variipennis*. *Arch. Virol.*, **136** (1), 1–8. doi:10.1007/BF01538812.
56. van den Ende M., Linder A. & Kaschula V.R. (1954). – Experiments with the Cyprus strain of blue-tongue virus: multiplication in the central nervous system of mice and complement fixation. *J. Hyg. (Lond.)*, **52** (2), 155–164. doi:10.1017/s0022172400027364.
57. Klontz G.W., Svehag S.E. & Gorham J.R. (1962). – A study by the agar diffusion technique of precipitating antibody directed against blue tongue virus and its relation to homotypic neutralizing antibody. *Arch. Gesamte Virusforsch.*, **12** (2), 259–268. doi:10.1007/bf01258136.
58. Pini A., Ohder H., Whiteland A.P. & Lund L.J. (1968). – Studies on the fluorescent and neutralizing antibodies to bluetongue virus in sheep. *Arch. Gesamte Virusforsch.*, **25** (2), 129–136. doi:10.1007/bf01258159.
59. Metcalf H.E. & Jochim M.M. (1970). – Bluetongue in cattle: efficacy of the agar gel precipitin test. *Am. J. Vet. Res.*, **31**, 1743–1749. Abstract available at: www.cabdirect.org/cabdirect/abstract/19712201732 (accessed on 7 January 2021).

60. Pearson J.E., Carbrey E.A. & Gustafson G.A. (1973). – Bluetongue virus in cattle: complement fixing antibody response and viremia in experimentally infected animals. *Proc. Annu. Meet. U.S. Anim. Hlth Assoc.*, **77**, 524–531.
61. Boulanger P. & Frank J.F. (1975). – Serological methods in the diagnosis of bluetongue. *Aust. Vet. J.*, **51** (4), 185–189. doi:10.1111/j.1751-0813.1975.tb00052.x.
62. Hübschle O.J., Lorenz R.J. & Matheka H.D. (1981). – Enzyme-linked immunosorbent assay for detection of bluetongue virus antibodies. *Am. J. Vet. Res.*, **42** (1), 61–65. Abstract available at: www.ncbi.nlm.nih.gov/pubmed/6261621 (accessed on 12 January 2021).
63. Huismans H. & Erasmus B.J. (1981). – Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort J. Vet. Res.*, **48** (2), 51–58. Abstract available at: www.ncbi.nlm.nih.gov/pubmed/6273773 (accessed on 12 January 2021).
64. Jochim M.M. & Chow T.L. (1969). – Immunodiffusion of bluetongue virus. *Am. J. Vet. Res.*, **30** (1), 33–41.
65. Singer R.S., Boyce W.M., Gardner I.A., Johnson W.O. & Fisher A.S. (1998). – Evaluation of bluetongue virus diagnostic tests in free-ranging bighorn sheep. *Prev. Vet. Med.*, **35** (4), 265–282. doi:10.1016/s0167-5877(98)00067-1.
66. Reddington J.J., Reddington G.M. & MacLachlan N.J. (1991). – A competitive ELISA for detection of antibodies to the group antigen of bluetongue virus. *J. Vet. Diagn. Invest.*, **3** (2), 144–147. doi:10.1177/104063879100300207.
67. Afshar A., Trotter H.C., Dulac G.C. & Reddington J.J. (1993). – Evaluation of a commercial competitive ELISA test kit for the detection of group-specific antibodies to bluetongue virus. *J. Vet. Diagn. Invest.*, **5** (3), 336–340. doi:10.1177/104063879300500305.
68. Ward M.P., Forbes-Faulkner J.C. & Duffy V.L. (1996). – Evaluation of a competitive enzyme-linked immunosorbent assay to detect infection of cattle in sentinel herds in Queensland, Australia with bluetongue viruses. *Vet. Microbiol.*, **49** (1–2), 117–125. doi:10.1016/0378-1135(95)00178-6.
69. Biteau-Coroller F., Gerbier G., Stärk K.D.C., Grillet C., Albina E., Zientara S. & Roger F. (2006). – Performance evaluation of a competitive ELISA test used for bluetongue antibody detection in France, a recently infected area. *Vet. Microbiol.*, **118** (1–2), 57–66. doi:10.1016/j.vetmic.2006.07.012.
70. Batten C.A., Bachanek-Bankowska K., Bin-Tarif A., Kgosana L., Swain A.J., Corteyn M., Darpel K., Mellor P.S., Elliott H.G. & Oura C.A.L. (2008). – Bluetongue virus: European community inter-laboratory comparison tests to evaluate ELISA and RT-PCR detection methods. *Vet. Microbiol.*, **129** (1–2), 80–88. doi:10.1016/j.vetmic.2007.11.005.
71. Batten C.A., Sanders A.J., Bachanek-Bankowska K., Bin-Tarif A. & Oura C.A.L. (2009). – Bluetongue virus: European community proficiency test (2007) to evaluate ELISA and RT-PCR detection methods with special reference to pooling of samples. *Vet. Microbiol.*, **135** (3–4), 380–383. doi:10.1016/j.vetmic.2008.09.080.
72. Svehag S.E. (1963). – Effect of different 'contact conditions' on the blue tongue virus-antibody reaction and on the validity of the 'percentage law'. *Arch. Gesamte Virusforsch.*, **12** (5), 678–693. doi:10.1007/bf01246389.
73. Svehag S.E. (1966). – Quantal and graded dose-responses of bluetongue virus: a comparison of their sensitivity as assay methods for neutralizing antibody. *J. Hyg. (Lond.)*, **64** (2), 231–244. doi:10.1017/s0022172400040493.
74. Maree S. & Paweska J.T. (2005). – Preparation of recombinant African horse sickness virus VP7 antigen via a simple method and validation of a VP7-based indirect ELISA for the detection of group-specific IgG antibodies in horse sera. *J. Virol. Meth.*, **125** (1), 55–65. doi:10.1016/j.jviromet.2004.12.002.
75. Durán-Ferrer M., Agüero M., Zientara S. [...] & Castillo-Olivares J. (2019). – Assessment of reproducibility of a VP7 blocking ELISA diagnostic test for African horse sickness. *Transbound. Emerg. Dis.*, **66** (1), 83–90. doi:10.1111/tbed.12968.
76. Stott J.L., Osburn B.I., Bushnell R., Loomis E.C. & Squire K.R. (1985). – Epizootiological study of bluetongue virus infection in California livestock: an overview. *Prog. Clin. Biol. Res.*, **178**, 571–582. Abstract available at: www.ncbi.nlm.nih.gov/pubmed/2989906 (accessed on 12 January 2021).
77. World Organisation for Animal Health (OIE) (2019). – Terrestrial Animal Health Code. OIE, Paris, France. Available at: www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/ (accessed on 7 January 2021).
78. Mayo C.E., Crossley B.M., Hietala S.K., Gardner I.A., Breitmeyer R.E. & James MacLachlan N. (2010). – Colostral transmission of bluetongue virus nucleic acid among newborn dairy calves in California. *Trans. Emerg. Dis.*, **57** (4), 277–281. doi:10.1111/j.1865-1682.2010.01149.x.
79. Mayo C.E., Mullens B.A., Gerry A.C., Barker C.M., Mertens P.P.C., Maan S., Maan N., Gardner I.A., Guthrie A.J. & MacLachlan N.J. (2012). – The combination of abundance and infection rates of *Culicoides sonorensis* estimates risk of subsequent bluetongue virus infection of sentinel cattle on California dairy farms. *Vet. Parasitol.*, **187** (1–2), 295–301. doi:10.1016/j.vetpar.2012.01.004.

80. Mayo C.E., Gardner I.A., Mullens B.A., Barker C.M., Gerry A.C., Guthrie A.J. & MacLachlan N.J. (2012). – Anthropogenic and meteorological factors influence vector abundance and prevalence of bluetongue virus infection of dairy cattle in California. *Vet. Microbiol.*, **155** (2–4), 158–164. doi:10.1016/j.vetmic.2011.08.029.
81. Mayo C.E., Mullens B.A., Reisen W.K., Osborne C.J., Gibbs E.P.J., Gardner I.A. & MacLachlan N.J. (2014). – Seasonal and interseasonal dynamics of bluetongue virus infection of dairy cattle and *Culicoides sonorensis* midges in northern California – implications for virus overwintering in temperate zones. *PLoS One*, **9** (9), e106975. doi:10.1371/journal.pone.0106975.
82. Mayo C., Shelley C., MacLachlan N.J., Gardner I., Hartley D. & Barker C. (2016). – A deterministic model to quantify risk and guide mitigation strategies to reduce bluetongue virus transmission in California dairy cattle. *PLoS One*, **11** (11), e0165806. doi:10.1371/journal.pone.0165806.
83. MacLachlan N.J. & Mayo C.E. (2013). – Potential strategies for control of bluetongue, a globally emerging, *Culicoides*-transmitted viral disease of ruminant livestock and wildlife. *Antiviral Res.*, **99** (2), 79–90. doi:10.1016/j.antiviral.2013.04.021.
84. Mayo C., Mullens B., Gibbs E.P. & MacLachlan N.J. (2016). – Overwintering of bluetongue virus in temperate zones. *Vet. Ital.*, **52** (3–4), 243–246. doi:10.12834/VetIt.521.2473.3.
85. McDermott E.G., Mayo C.E., Gerry A.C., Laudier D., MacLachlan N.J. & Mullens B.A. (2015). – Bluetongue virus infection creates light averse *Culicoides* vectors and serious errors in transmission risk estimates. *Parasit. Vectors*, **8** (1), 460. doi:10.1186/s13071-015-1062-4.
86. Lu G., Pan J., Ou J., Shao R., Hu X., Wang C. & Li S. (2020). – African horse sickness: its emergence in Thailand and potential threat to other Asian countries. *Trans. Emerg. Dis.*, e-pub. ahead of print. doi:10.1111/tbed.13625.
87. Bangs M.J., Pudiantari R. & Gionar Y.R. (2007). – Persistence of dengue virus RNA in dried *Aedes aegypti* (Diptera: Culicidae) exposed to natural tropical conditions. *J. Med. Entomol.*, **44** (1), 163–167. doi:10.1093/jmedent/41.5.163.
88. Burkhalter K.L. & Savage H.M. (2017). – Detection of Zika virus in desiccated mosquitoes by real-time reverse transcription PCR and plaque assay. *Emerg. Infect. Dis.*, **23** (4), 680–681. doi:10.3201/eid2304.161772.
89. Drummond C., Gebhardt M.E., Robles M.T.S., Carpi G., Hoyer I., Pastusiak A., Reddy M.R., Norris D.E., Pipas J.M. & Jackson E.K. (2020). – Stability and detection of nucleic acid from viruses and hosts in controlled mosquito blood feeds. *PLoS One*, **15** (6), e0231061. doi:10.1371/journal.pone.0231061.
90. Nomikou K., Hughes J., Wash R., Kellam P., Breard E., Zientara S., Palmarini M., Biek R. & Mertens P. (2015). – Widespread reassortment shapes the evolution and epidemiology of bluetongue virus following European invasion. *PLoS Pathog.*, **11** (8), e1005056. doi:10.1371/journal.ppat.1005056.
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