DEVELOPMENTS ON DIAGNOSIS, PATHOGENESIS, AND CONTROL OF BLUETONGUE DISEASE VIRUS

Von

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Contents

1. Introduction

Bluetongue virus (BTV) is a double stranded RNA virus of the family *Reoviridae*, genus *Orbivirus,* with 10 genome segments [1]. The virus protein 2 (VP2), encoded by segment 2, is the serotype defining protein of the outer capsid shell carrying the neutralising epitopes. Up to now, 24 notifiable so-called “classical” BTV serotypes (BTV 1-24) are differentiated by using the virus neutralisation test (VNT). However, in the last years, a rising number of additional serotypes and strains phylogenetically distinct from classical BTV, and therefore referred to as “atypical” BTV, were discovered. Most atypical BTV were detected in healthy small ruminants.

BTV is a non-contagious arbovirus transmitted by hematophagous *Culiocoides* midges as biological vector. Nearly all domestic and wild ruminants are susceptible to BTV infection, whereas BTV-related clinical disease is mostly reported in sheep. However, also cattle and other ruminants can develop clinical disease, but at a lower frequency, as reported during the Northern European BTV-8 epidemic during the years 2006 to 2009. BTV is a haemorrhagic fever, thus, the clinical signs are mostly related to vascular injuries. Affected animals can develop fever, ulcers and erosions, lameness and coronitis, weakness and depression, as well as oedema. The pulmonary oedema can lead to death of the infected animal. Bluetongue disease is mainly located in regions with a tropical and subtropical climate connected to the occurrence and spread of *Culicoides* midges. However, globalisation with global trade, global warming and climate change brought BTV and in some regions also its vector in more Northern regions of the world. Since 1998, several serotypes are endemic in Europe, and since the BTV-8 incursion in 2006 also in Northern Europe.

This thesis targets new developments in control, diagnosis and pathogenesis of BTV. One of the most important tools in disease control and eradication is the vaccination. Now, several years after the BTV vaccination campaign with inactivated BTV-8 vaccines in Europe, one could analyse the longevity of vaccine-derived antibodies towards BTV-8. Furthermore, with the invasion of several different serotypes in Europe, the need for a fast and reliable diagnostic tool for molecular ‘serotyping’, including the differentiation between classical and atypical BTV, led to the development of the optimized diagnosis tool “BlueTYPE array”. Moreover, monitoring of a BTV-25-positive goat flock in Bavaria allowed detailed research on the pathogenesis of this atypical strain, and the study is the first to succeed in cell culture propagation of BTV-25 for further analyses.

2. Literature overview

2.1. Bluetongue virus taxonomy, structure and proteins

Bluetongue virus (BTV) forms one of the 22 species of the genus *Orbivirus* within the family *Reoviridae* (respiratory enteric orphan virus), subfamily *Sedoreovirinae*. The currently 15 genera within the family *Reoviridae* are organised on the basis of 9 to 12 linear double stranded (ds) RNA segments (seg). Interestingly, mature virions don't have a lipid envelope, with the exception of *Orbiviruses* and *Rotaviruses*, which can temporally have an unstable lipid envelope after virus morphogenesis and release [2].

The ten ds RNA segments of BTV are ranging from 3954 to 822bp forming three size classes: large with 3.9 to 2.8 kbp (Seg 1-3), medium with 2.0-1.6 kbp (Seg4-6) and small with 1.2-0.8 kbp (Seg 7-10). Each of the ten BTV segments has identical conserved terminal sequences at the 5' –NTRs (5'-GUUAAA) and 3' NTRs ((A/G)CUUAC-3') with only a few exceptions [3]. The segments 1 to 8 have each one ORF coding for one viral protein, whereas the segments 9 and 10 have one large and one small ORF and therefore encode two proteins. In total, the 10 segments are coding for seven structural (VP1-VP7) and six non-structural proteins (NS1-NS5, NS3a) [1, 4-6]. The BTV non-structural viral proteins are mainly important for the virus life cycle and are not part of the virion structure [5].

The subcore capsid shell of BTV (T=2 symmetry) is made of 60 dimers of VP3 (901 aa) associated to 12 decamers and encloses the 10 viral genome segments as well as the transcriptase complex consisting of a VP1 monomer (1302 aa; polymerase), a VP4 dimer (644 aa; capping enzyme) and a VP6 hexamer (329 aa; helicase) [7, 8]. Altogether, the subcore capsid shell, the transcriptase complex and the RNA segments form the sub-core particle with less than 59 nm in diameter. The intermediate or core-surface layer consists of 260 trimers of VP7 and the diameter is less than 73 nm [9]. The name of the genus *Orbivirus* was given due to the hexameric rings (in Latin "orbi" for ring) of the VP7 (349 aa) capsomeres visible by electron microscopy, once the icosahedral symmetric outer capsid layer is removed. Subsequently, the core particle of BTV comprises the sub-core particle with the VP7 layer. Finally, the outer capsid layer of BTV is formed by 120 VP5 trimers (526 aa) serving as underlay for 60 sail-shaped VP2 surface trimers (961 aa) [7]. The diameter of the BTV particle is approximately 90 nm (see figure 1).

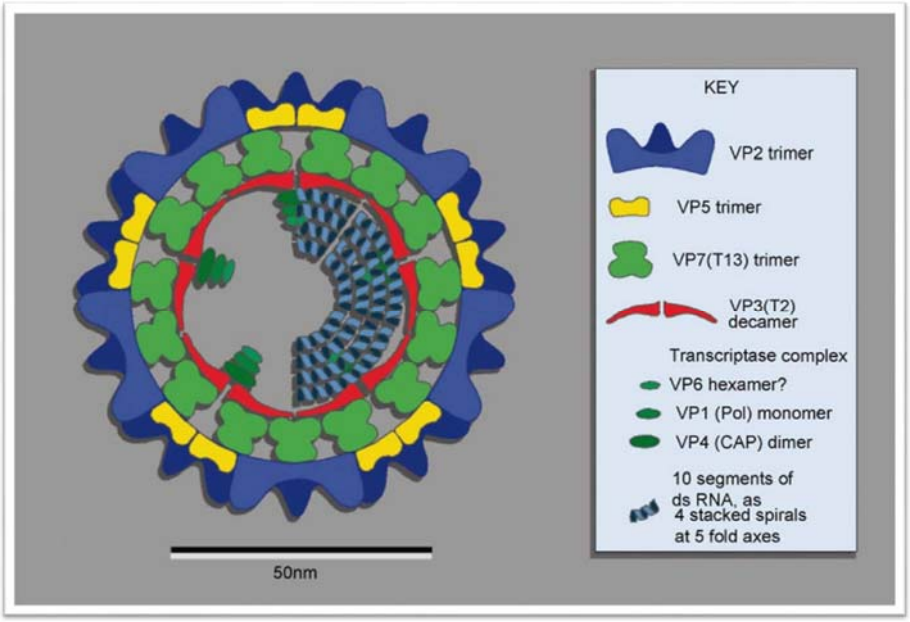

Figure 1: Overview of the BTV virus particle [1]. (Source: Mertens et al., 2004, page 30).

The outer capsid layer of viruses within the family *Reoviridae* is highly variable and relevant for transmission, cell attachment, mammalian host immune system interaction and penetration of different cell types. Consequently, for BTV, VP2 and VP5 are the most variable viral proteins as components of the outer capsid layer. VP2 is responsible for receptor binding using cell surface glycoproteins, for hemagglutinating properties, and the external tip part is carrying the neutralising epitopes essential for the production of neutralising antibodies [10]. Therefore, VP2 determines the BTV serotype analysed by serum neutralisation [11] assays, and currently 24 classical BTV serotypes are known [12]. Phylogenetic comparisons revealed that variations in the nucleotide sequences of segment 2 and 6 highly correlate with the respective viral serotype [13, 14]. VP5 is relevant for membrane penetration and after pH-dependent conformational changes for fusion with cellular membranes [15].

NS1 (64 kDa; 553 aa) - encoded by segment 5 - strongly enhances viral protein synthesis [16], and forms tubules in the cytoplasm of infected cells [17]. NS2 (355aa) is the major component of the viral inclusion bodies, in which viral replication and assembly takes place [18]. NS3 (230 aa) and its N-terminal truncated form NS3a (216 aa) are the main proteins for BTV morphogenesis and release in the non-lytic egress in insect cells, but they play also an important role in both the non-lytic and the cytopathogenic release in mammalian cells [19, 20]. The complex of NS3/NS3a is necessary for intracellular trafficking, budding and viral release from infected cells [19, 21]. NS3 plays furthermore a role for the induction of the host innate immune response by downregulating its type 1 interferon synthesis [22] and increasing

the viral replication by using the MAPK/ERK cellular pathway [23]. NS4 (78aa) is encoded by a second small ORF of segment 9 [5] and is an interferon antagonist downregulating several promotors such as the IFN-ß promotor and therefore is a factor of virulence [4]. The NS5 protein (59aa) is encoded by the ORF 2 of segment 10 and might play a synergistic role to NS4 [6].

2.2. Bluetongue virus replication

The replication cycle of BTV consists of virus entry, replication, assembly and egress. Generally, BTV replicates rapidly and avoids cellular apoptosis (see figure 2) [24].

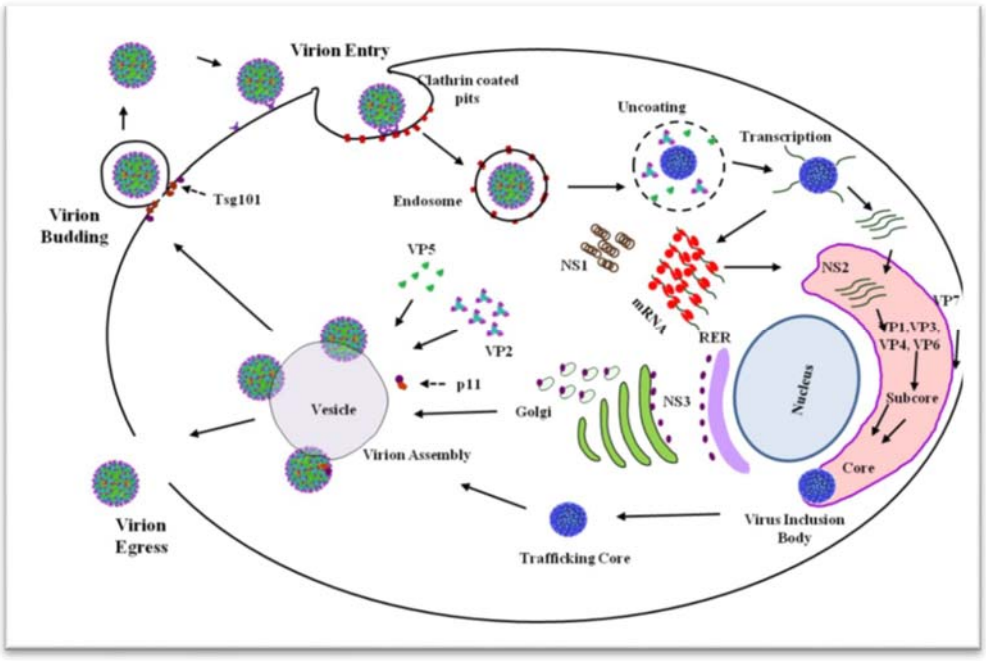

Figure 2: Overview of the BTV replication cycle [25]. (Source: Mohl, B.P. and P. Roy, 2014; page 3253).

For virus entry, VP2 binds to the cell membrane via surface glycoproteins, but possibly also other receptors. However, the BTV core particle is infectious as well and can bind to the cell surface via interaction of VP7 and host cells glycosaminoglycans [1]. Hence, antibodies reactive to VP7 bind and neutralise core particles but not the fully intact virus [1]. Then, the BTV particle is internalised by the host cells clathrin-mediated endocytosis. From the clathrin vesicles the BTV particles are delivered to the early endosomes. The early endosomal low pH leads to the rapid degradation of VP2, which triggers structural modifications of VP5 and might lead to the exposure of its amphipathic helices. Hence, pores in the endosomal membrane can be formed, through which the transcriptionally active core particle can egress in the

cytoplasm, whereas VP5 is retained in the endosomes due to its membrane binding ability [1, 15, 24].

BTV replicates in the cytoplasm of infected cells, but necessarily within the BTV core to avoid the host cell defence activated by direct contact with dsRNA [26]. For initial mRNA synthesis, BTV carries the transcriptome complex formed by VP1, VP4 and VP6 into the host cell [1]. The removal of VP2 and VP5 activates the transcriptase complex and the ten genomic segments are transcribed into single stranded mRNAs [12]. VP4 is capping the mRNA molecules by its guanylyl-transferase and transmethylase activity [12]. These capped, viral mRNAs then leave the core particle and are released into the cytoplasm. Within two hours post infection, the translation of BTV proteins begins by the hosts ribosomes [12], whereas other mRNAs are further processed and packaged as part of novel progeny virus particles [1]. BTV NS1 enhances the translation of ssRNA and forms tubules, whereas the phosphorylated NS2 is forming VIBs (Virus inclusion bodies) within the cytoplasm containing the core proteins and ssRNA [27, 28]. The polymerase VP1 synthesizes dsRNA from the viral plus-strand RNA in both insect and mammalian cells [29]. The viral assembly is located in the VIBs starting with the correct encapsidation of the RNA within the VP3 shell by the help of VP1, VP4, VP6 and NS2 [30]. As the VP3 subcores are rather unstable, they act as a frame for the addition of VP7 trimers. Assembled core particles are trafficked on exocytotic vesicles involving VP2/vimentin interactions for cell egress [12, 27]. During the process of egress into the host cell cytoplasm, the outer capsid proteins VP2 and VP5 are added as the outer capsid shell. Both, the cell lytic egress with extensive cytopathic effects particularly in mammalian cells, but also the non-lytic cell egress mainly in insect cells occur [31]. On the one hand, NS3 can function as a viroporin protein, mediating virus release by membrane permeabilization. Thus, the assembled particles can leave the host cell by lysis. On the other hand, NS3 can bind to the Tsg101 cellular protein (human tumour-susceptibility gene 101), which facilitates virus release by vesicle formation at the cytoplasmic membrane. Thus, the BTV particle can leave the host cell via budding, which might be important for insect cells [31].

2.3. Bluetongue virus vectors and ways of transmission

Arboviruses naturally infect hematophagous arthropods through ingestion of infected vertebrate blood (see figure 3). After replication in the arthropod vector, arboviruses are transmitted via bite to susceptible vertebrates [32]. The role of *Culicoides* (Diptera: family Ceratopogonidae) in Bluetongue virus transmission was firstly discovered in South Africa in

1944 [33]. Today, it is well established that *Culicoides* midges are the main biological vector of BTV [34, 35].

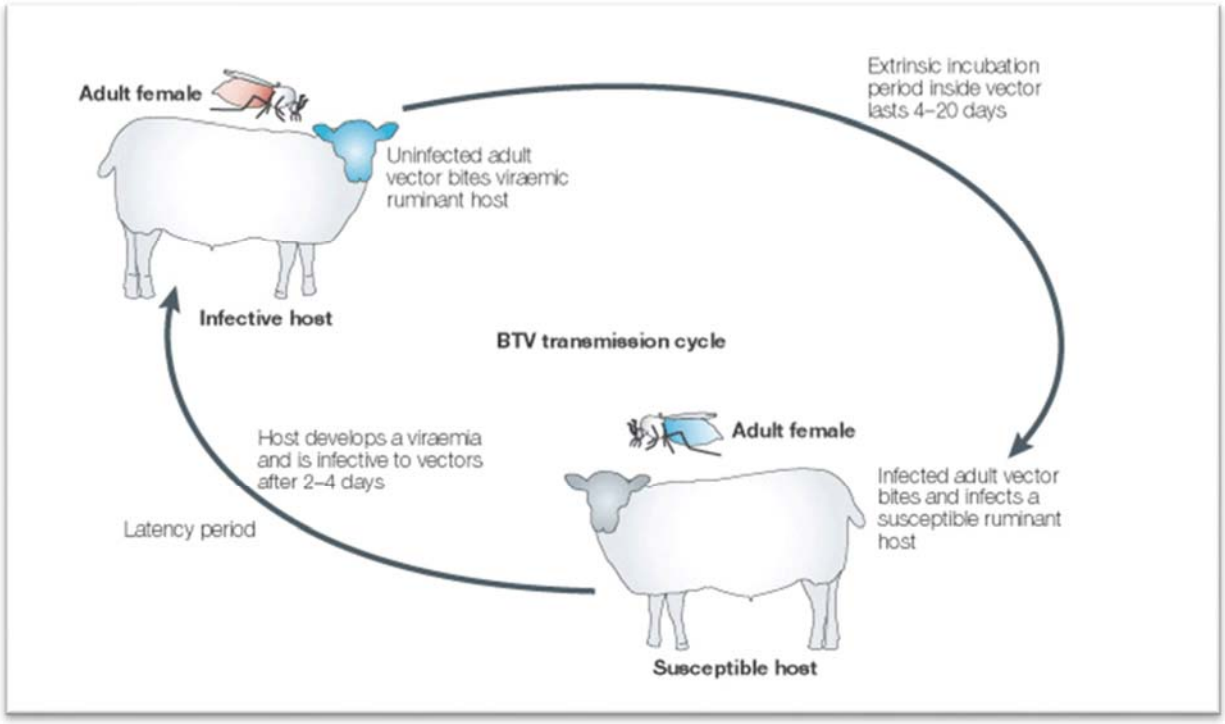

Figure 3: BTV transmission cycle [36]. (Source: Purse, et al 2005; page 174).

The transmission cycle of BTV starts with the blood meal of the female *Culicoides* midges on the viraemic ruminant host [37]. *C. variipennis* females are able to ingest approximately 0.1 to 1 µl blood [38]. When the BTV positive blood is incorporated, the blood meal enters the mid-gut diverticulum and for 1-2 days, nearly no virus is detectable in the midge (eclipse or partial eclipse). After BTV successfully passes the mesenterion infection barrier (MIB), it replicates in the mid-gut cells until a stable virus concentration is reached, typically after 5-9 dpi. After passing the mesenterion infection barrier (MEB), BTV is released into the haemocel, where it needs to pass the fat body as part of the midges' immune system, representing the dissemination barrier (DB). The haemocel subsequently transports BTV in the insect body to the salivary glands. After passing the salivary gland infection barrier (SGIB), BTV replicates in the salivary gland cells as secondary target cells. BTV reaches the saliva by passing the salivary gland escape barrier and can be transmitted by the next blood meal to a new vertebrate host [39, 40].

The time span between infectious blood meal until virus release in the salivary glands can vary from few days up to several weeks and is related e.g. to temperature and BTV

serotype [41]. With a single bite of a BTV positive *Culicoides* midge about 0.32 to 7.79 $TCID_{50}$ can be transmitted [39]. Despite the low titers, already one single bite of a *Culicoides* midge can ascertain bluetongue infection in sheep [42]. The probability of virus transmission from an infectious vector to the susceptible ruminant host is close to 100 % [41]. Low titres of $<10^{0.5}$ to $10^{2.4}$ $TCID_{50}$ per ml blood of viraemic sheep and cattle are enough to infect *Culicoides* midges [39]. However, the transmission efficacy from the vertebrate host to a blood feeding midge is low. After BTV infection, the *Culicoides* midges remain life-long BTV-positive [39].

More than 1000 *Culicoides* species are known, but only about 30 species are reported to be capable of transmitting BTV [43]. In Africa and the Middle East, the major vector of BTV is *Culicoides imicola*, whereas in other parts of the world other *Culicoides* are present like *C. sonorensis* and C. *variipennis* in the Inited States of America (USA), *C. breviataris* and *C. wadai* in Australia and Asia, as well as *C. insignis* in both North and South America. In Europe, *C. imicola* (Southern Europe), *C. obsoletus* (see figure 4)and *C. pulicaris* but also *C. derwulfi* are the major vectors of BTV [44].

Figure 4: A female C. obsoletus [45]. (Source: van der Meide, 2012 with permission; Appendix A).

Environmental conditions such as seasonality and meteorology influence the occurrence of *Culicoides,* and consequently determine Bluetongue virus spread [46]. A warmer climate increases the vector competence of the poikilothermic *Culicoides* in many ways e.g with higher biting and virus replication rates, shorter extrinsic incubation times, but also modified mortality rates of midges [41, 47]. The minimum temperature required for *Culicoides* to replicate BTV is between 10 to 15°C [48]. Therefore, it's not surprising, that historically BTV has existed in tropical and subtropical regions of the world between latitudes

of approximately 40° North and 35° South [49, 50]. In most endemic regions, vectors peak in late summer or autumn [46]. In Germany during the years 2006 to 2008 the *Culicoides* appearance peaked in autumn [51], and in 2019, the appearance of *Culicoides* in Germany peaked twice a year, in May/June and August/September. Most *Culicoides* were found near to dung sites of farms and renaturation areas/floodplains, less often in meadows or swampy forests, but highly depending on the species [52].

Several overwintering strategies were suggested for BTV in Northern regions. [53]. Currently there is no evidence for transovarial transmission in the vectors [39, 54]. More likely, the virus persists undetectable in the ruminant host for three to four months and bridges the time of the vector absence during winter until re-infection of the next vector population [53, 55]. Furthermore, the prolonged survival of BTV-infected *Culicoides* midges contributes to the inter-seasonal overwintering [53, 54]. Furthermore, the transplacental transmission might contribute to overwintering in epidemic areas [56]. Transplacental BTV infection is a non-vector transmission way, where BTV crosses the placenta and invades the foetus [56]. Already in the 1950's an increase of stillbirth, weak, spastic or blind lambs in vaccinated flocks with MLV vaccines was reported [57]. Transplacental transmission has been mainly associated with cell-culture adapted live vaccine virus strains and has been reported for multiple serotypes [56]. The high reassortance rates of BTV and the yearlong usage of live-attenuated vaccines worldwide made it difficult to find field strains free from vaccine virus derived genome sequences [56]. Interestingly, the European BTV-8 strain showed a higher vertical transmission rate than other serotypes, further supporting a possible connection to cell culture adaptation [56].

Vector-free horizontal transmission of BTV is an infrequent event requiring very close animal contacts or contact to highly infectious materials. Calves possibly became BTV-infected by intake of infectious colostrum, and other cattle due to ingestion of BTV-contaminated placentas [58, 59]. In addition, saliva- or blood-contaminated feed or water was proposed to infect cattle with BTV-8 and BTV-1 during experiments [56, 60].

Alternative biological vectors like ticks are in discussion [61] as well as alternative mechanical vectors like *Melophagus ovinus* [62]. BTV can be also transmitted iatrogenically e.g. by the use of shared needles [63]. Besides, infected rams and bulls occasionally shed the virus in the semen together with blood cells. Consequently, during viremia venereal transmission can be possible. Hence, BTV testing is required for breeding bulls and rams

before semen exportation. Nevertheless, the BTV-8 re-emergence in 2015 might be linked to contaminated bull semen [53].

2.4. Epidemiology and History

Bluetongue is an arthropod-borne disease (Arbovirus) and its appearance is connected to the distribution of its biological vector, the *Culidoides* midges. In the 19th and 20th century, Bluetongue disease was located in regions with a tropical and subtropical climate between 40°N and 35°S of the world. First mentioned in 1876 in sheep in South Africa as 'Malarial catarrhal fever' or 'Bekziekte' (Africaans for moth-sickness), the first outbreaks of Bluetongue were related to the introduction of highly susceptible merino sheep in the former South African colony [64]. The name Bluetongue derived from the English translation from the Dutch word '*Blaauwtong',* which described the observed cyanotic blue coloured tongue in severely diseased sheep. Spruell and Hutcheon described the disease, epidemiology and first results of animal experiments in the early 19's. Theiler and Robertson identified as causative agent a non-filterable virus [65, 66]. Furthermore, Theiler developed the first live attenuated BTV vaccine by serial passaging and in South Africa between 1907 and 1943 more than 50 million vaccine doses were used [66-68]. In 1943, the usage of this first monovalent vaccine was stopped, due to insufficiencies in immunity [69, 70]. In the 1940's, evidence for the existence of different serotypes was provided linked to the optimised growth of BTV in embryonated chicken eggs [64]. By 1942, already six serotypes were identified and multivalent live-attenuated vaccines were produced [64]. In 1948, immunisation studies proved the serotype specific immunity with only little or no cross immunity to other strains, which forms a major milestone in efficient disease control up to now [64, 69].

Today, 24 classical and 3 atypical BTV serotypes are classified [1] by virus and serum neutralisation assays with more or less observed cross reactions between serotypes. The virus/serum neutralization test (V/SNT) has become the reference method for serotype identification. With the rapid progress in genomics in recent decades, more and more BTV sequence data have become available. The segment-2/VP2 sequence identities correlate perfectly with the respective BTV serotype [71, 72], and in 2011, an international working group suggested levels of maximum and minimum nucleotide (nt) and amino acid (aa) identities within segment-2 of the BTV genome as an alternative to the traditional serotyping methods [72]. Within the same serotype, the minimum levels of Seg2/VP2 sequence identities were defined as 68.4% on the nucleotide level, (nt) and 72.6% amino acids (aa) in the year

2010, accordingly, the maximum levels of variation were 31.6% on nt level / or 27.4% on the aa level, respectively. Viruses belonging to different serotypes can show up to 71.5% nt and 77.8% aa identity, and consequently a minimum variation of 28.5% nt / 22.2% aa, respectively [71-74].

However, it has to be considered that a high genetic diversity has been described among BTV strains even within the same serotype. One reason of the high diversity rate is most likely genetic shift occurring by reassortment of one or more segments in the insect vector or mammalian host cells (see figure 5) but also the general properties of RNA viruses with their error-prone polymerase, with short generation times and high progeny. Genetic drift is the other factor contributing to genetic diversity [70]. For BTV the existence of quasispecies was shown in both, the mammalian and the insect host [75]. The founder effect, which can occur every time during altering virus transition from the mammalian host to the insect vector, can lead to the fixation of random genetic variations. Furthermore, the founder effect can be responsible for the development of geographic topotype variation within the same serotype (see figure 6), e.g when a new strain enters a previously free area. [70, 75].

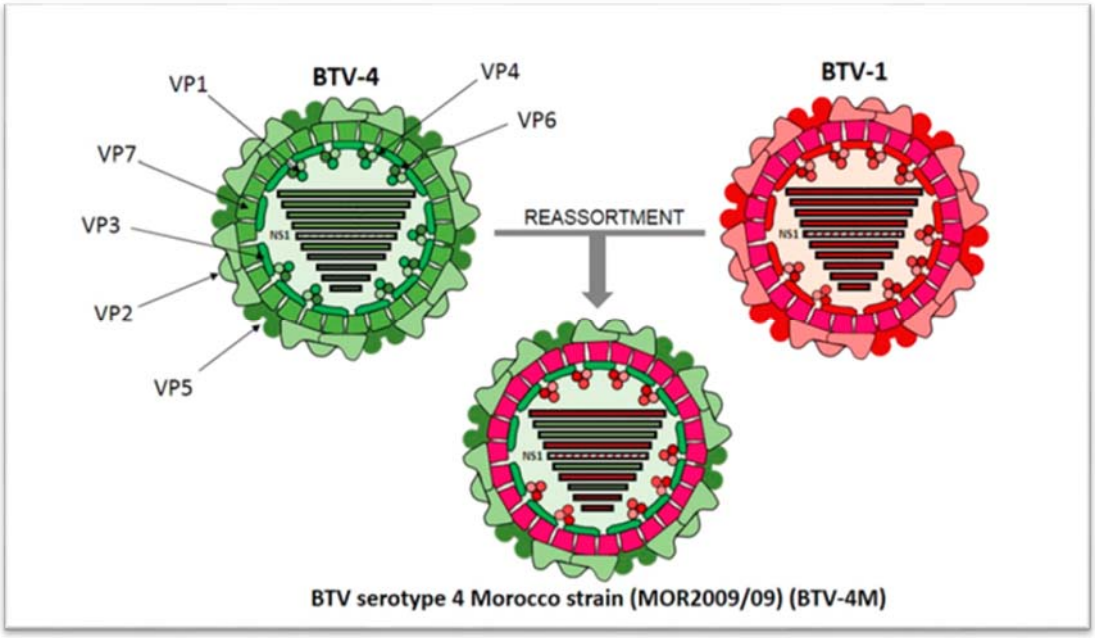

Figure 5: Schematic representation of the reassortant virus BTV-4 from Morocco (MOR2009/09), a BTV strain generated as a consequence of a reassortment event between BTV-4 and BTV-1 serotypes. Segments 1, 4, 5, 7, 9 and 10, deriving from BTV-1, are represented in red. Segments 2, 3, 6 and 8, deriving from BTV-4, are colored green. Segment 5 is striped [76]. (Source: Utrilla-Trigo, S., et al, 2020; page 346).

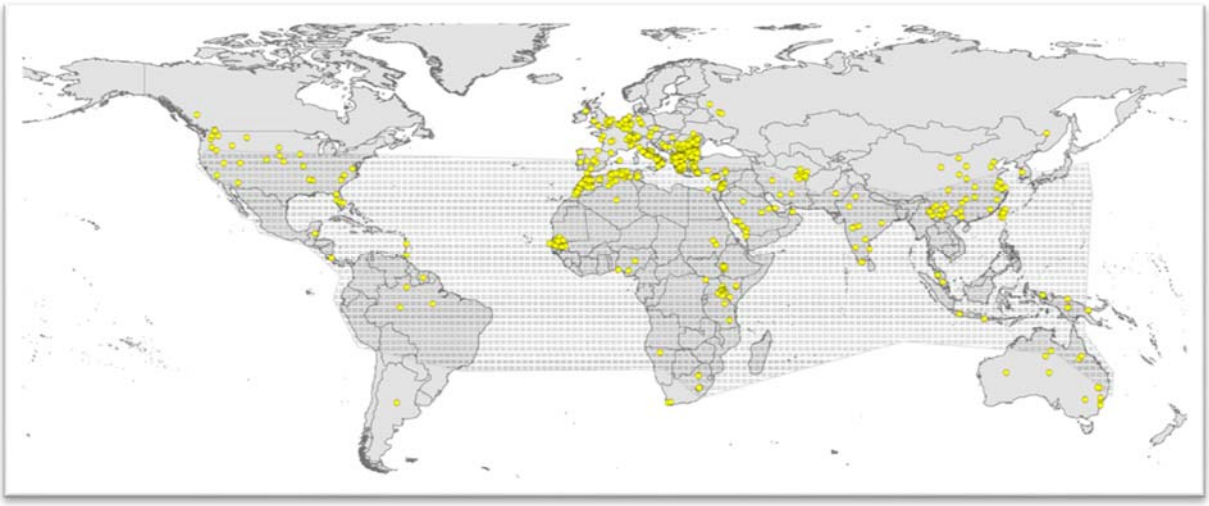

Figure 6: Summary of bluetongue virus occurrences (yellow points) worldwide (2016). Dotted black shading represents the early belt of BTV occurrence [44]. (Source: Samy et al., 2016; page 5).

First outbreaks in Europe were reported in Cyprus in 1943, in Israel in 1949, in northern Australia in 1977, in parts of the Indian Subcontinent with Pakistan in 1959 and India in 1964 and north America in 1948 [67, 77, 78]. Interestingly, two ancestral lineages were identified worldwide, the western topotype isolates from Africa, Europe and the Americas, whereas the Eastern topotype isolates occur in Australia, Japan, China, India and Southeast Asia (see figure 7) [13, 71, 74, 79].

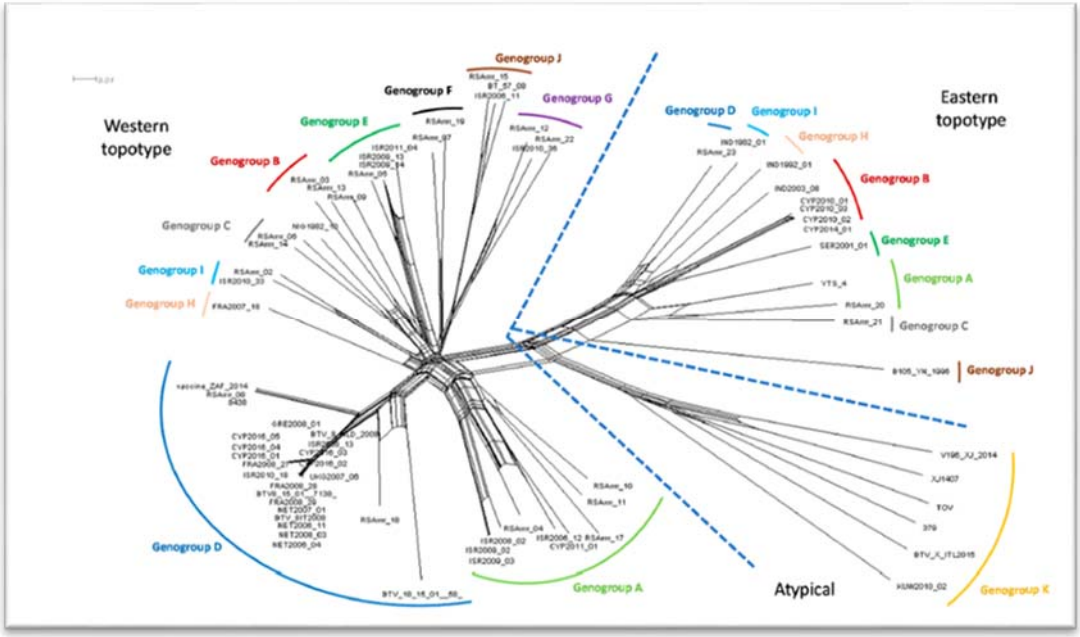

Figure 7: The Neighbor-Net network was estimated from an alignment of 18,621 characters for each of 73 taxa, which sequence composed of the concatenated coding regions for the BTV Segment 2 [80]. (Source: Rajkow-Nenow, et al. 2020; page 15).

Culicoides midges, the vectors of Bluetongue disease are distributed worldwide and determine the distribution of BTV [68]. In particular *Culicoides imicola* plays the major role in Bluetongue transmission as the 'Vector of the Old World' in Africa and Asia [81]. Wind can transport infected *Culicoides* midges naturally over distances greater than 170 km [82], which might explain the northward spread of BTV through routes from Turkey to Greece-Bulgaria, from Algeria-Tunisia to Italy and from Morocco-Algeria to Spain [41, 83]. *Culicoides imicola* was introduced firstly in southern Europe in 1982 causing several Bluetongue outbreaks in the following years [36, 81]. Thereby, the global warming facilitated the northwards spread of the tropical and subtropical *Culicoides* midges, and *C. imicola* was even found in Ticino in Switzerland [36]. However, Bluetongue outbreaks occurred in regions, where *C. imicola* was reported absent like in mainland Greece and Bulgaria in the late 90's [36]. Here, since long-time local *Culicoides* species as *C. obsoletus* and *C. pulicaris* were causing the outbreaks and environmental changes may have been responsible for their capability to serve as efficient Bluetongue vectors [36]. It was concluded that in Europe, two episystems of Culicoides exist, the episystem in Southern parts of Europe (Mediterranean Basin), where *C. Imicola* predominates and the episystem in Southern, Central, and Northern Europe, where the *Obsoletus* complex act as main vectors [84].Thereby, the global spread of BTV is a complex, multifactorial development driven by environmental and anthropogenic factors. Modulations revealed that BT might have the potential to spread even to Iceland or the southern part of Alaska in the coming years [44, 70]

A recently developed computer modulation about the air-borne spread of *Culicoides* analysed the route from Morocco-Algeria to Spain and showed a high correspondence between the model results and the real bluetongue introduction and spread in southern Spain [85]. The anthropogenic is an important factor for the worldwide spread of BTV. With the international trade between e.g. Europe and Africa, infected animals were moved to new regions. Moreover, the use of live attenuated vaccines in endemic countries like South Africa for nearly a century contributed to the spread of bluetongue, because Live attenuated vaccines can cause sufficient levels of viraemia in vaccinated animals for infecting *Culicoides* midges and enable their transmission in the field [36]. The circulation of various vaccine strains

in Culicoides was reported and the unauthorised international movement of vaccine viruses was responsible for outbreaks in the Mediterranean basin in the past [86]. Usage of contaminated biological products as fetal bovine serum or contaminated canine vaccines and the usage of shared needles could be also factors for the introduction and spread of BTV [49, 58, 63, 70, 87].

In Europe, before 1998 BTV appeared only sporadically. However, in Cyprus BTV occurred regularly before the 1990s and is historically considered as BTV endemic zone [41]. Since 1998 the BTV situation in Europe changed radically with the presence of *Culicoides* midges in more northern regions and the endemic circulation of several bluetongue virus serotypes in southern parts of Europe up to now [41, 88] (see figure 8 and 9). Several BTV incursions including five different BTV serotypes (BTV-1, -2, -4, -9 and -16) led to high losses in the ruminant population in the countries of the Mediterranean Basin. BTV-1 entered Europe by using the 'Eurasian ruminant street' coming from southern Asia. In the year 2000, BTV-2 was most likely imported from the sub-Saharan West Africa to northern Africa from where infected vectors spread hundreds of kilometres most likely via wind to Italy. Also BTV-4 was probably introduced to Gibraltar through wind-transported infected *Culicoides* from Morocco and animal movements led to the further spread of BTV-4 in Spain and Portugal during the years 2004-2005 [46].

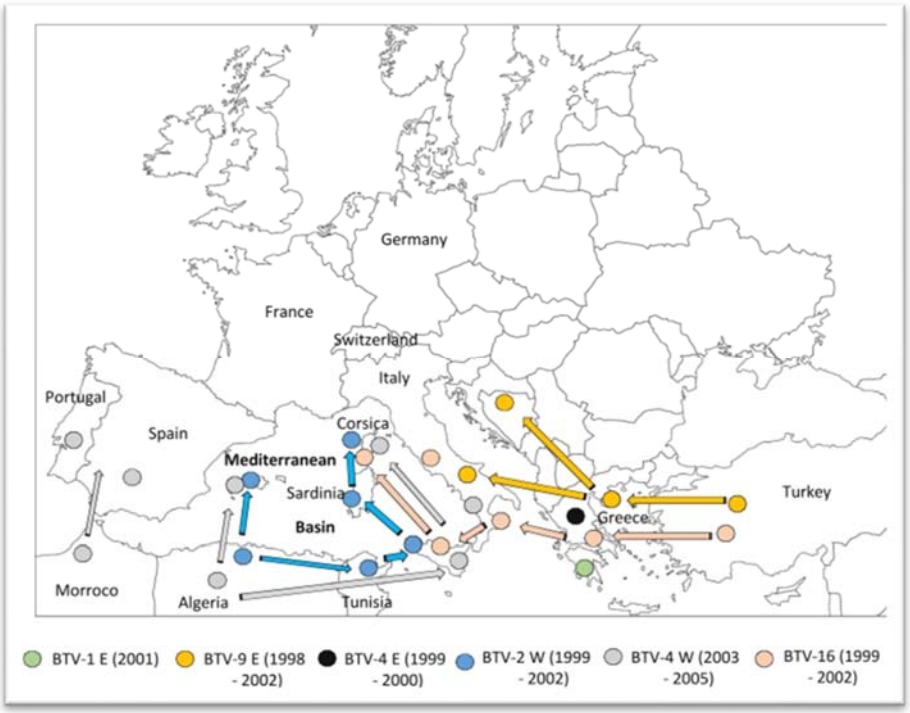

Figure 8: Bluetongue virus context from 19989 to 2005. Spread of BTV serotypes and strains (BTV-x) during the period indicated in brackets. E: Eastern strain; W: Western strain [89]. (Source: Kundlacz, et al. 2019; page 3).

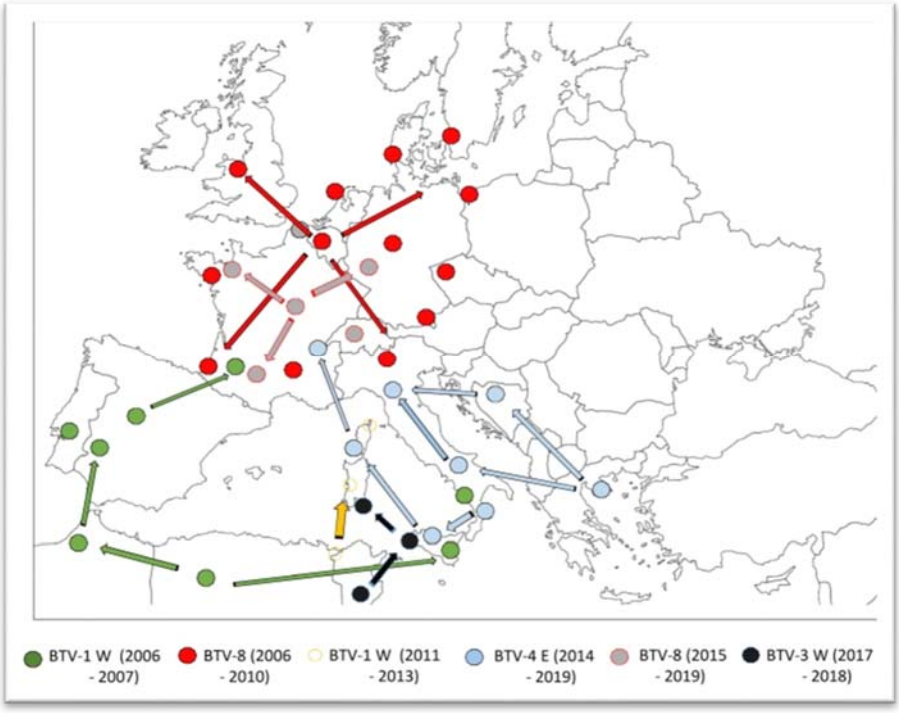

Figure 9: BTV spread from 2006 to 2019. Spread of BTV serotypes and strains (BTV-x) during the period indicated in brackets. E: Eastern strain; W: Western strain [89]. (Source: Kundlacz, et al. 2019; page 5).

In August 2006, the next level of the northern movement of BTV begun. Bluetongue virus serotype 8 reached northern Europe occurring in the Netherlands, Belgium, Luxembourg, France and Germany. The BTV-8-epidemic had huge economic impacts ongoing from 2006 to 2009 [90, 91]. The European BTV-8 strain was virulent for sheep, but severe clinical signs were also reported in cattle [92-94]. Therefore, the BTV-8 epidemic had a huge economic impact in form of direct losses from mortality and reduced production, as well as indirect losses caused by trade restrictions and costs of control measures including mass vaccination, one of the biggest cost factors [95]. The origin of the European BTV-8 strain might have been a modified life vaccine from the sub-Saharan Africa, as the transplacental transmission ability of the European BTV-8 strongly suggests the origin in a modified life vaccine [96, 97]. In addition, the climate conditions in 2006 most likely contributed to the wide spread of BTV-8 [47]. Finally, the BTV-8 epidemic from 2006 to 2009 was successfully controlled and eradicated with the help of obligatory mass vaccination programs with inactivated BTV-8 vaccines [95]. In

Germany, the epidemic resulted in more than 24 000 reported BTV-8 cases until 2008 with a peak in 2007 [98] (see figure 10). The mandatory mass vaccination program in Germany started in early summer 2008 based on a monovalent inactivated BTV-8 vaccine. A vaccine coverage in cattle with up to 83 % led to a considerable case decrease and the last reported incident in Germany in November 2009. The obligatory vaccination program was switched to voluntary vaccination in January 2010 [90, 98], and on the 15th of February 2012, Germany declared itself official free from BTV [98].

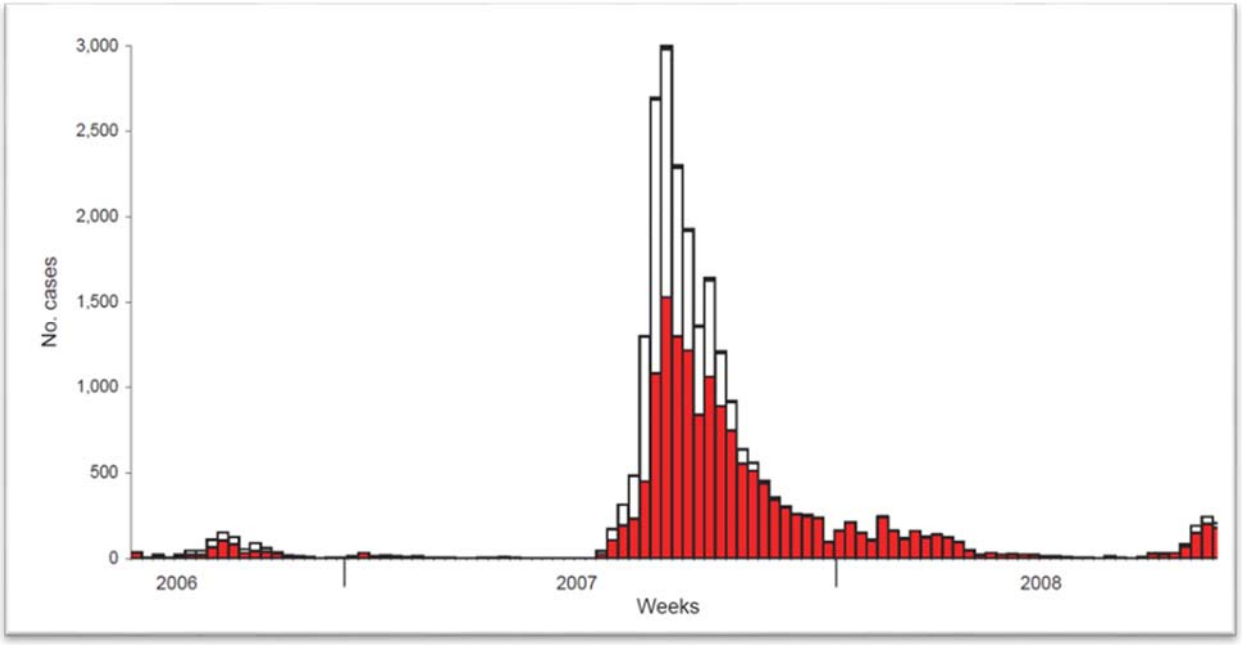

Figure 10: A) Number of new cases/outbreaks of bluetongue disease per calendar week in cattle (red), sheep (white), and goats (black) in Germany during 2007 and 2008 [90]. (Source: Conraths, et al. 2009; page 434).

Next to BTV-8, BTV-1 spread from Southern Spain to Portugal and up to North East France in 2007 [99]. During the BTV-1 epidemic, vaccination significantly slowed the speed of virus spread [100], and both BTV-1 and BTV-8 could be eradicated in France 2010 [89]. In 2008, illegal use of live-attenuated vaccines might have introduced BTV-6 into Netherlands and Germany. Interestingly, BTV-6 disappeared from Europe without any further control measures [101, 102]. In Belgium, BTV-11 was isolated from cattle [103], but only limited spread was reported without clinical signs [89]. In Poland, a BTV-14 vaccine strain was circulating from2012 to 2014 [104].

In August 2015, a French clinical diseased ram was tested positive for BTV-8 and marked the start of the first BTV-8 re-emergence in Europe after 2006 [105]. At the time point of re-emergence, the French cattle population was estimated to be ≥ 80% naive with only 18% BTV-8 pre-immune cattle [106]. BTV-8 could have continued to spread subclinically in domestic or wild ruminants before the re-emergence in 2015 [106]. However, comprehensive phylogenetic analyses revealed too high sequence identities between the BTV-8 isolates from 2015 and older BTV-8 isolates from 2007. With only seven mutations in difference, the expected evolutionary changes of the BTV-8 genome during a 5-year subclinical spreading period were absent. Most likely, the second BTV-8 endemic was caused by an accidental release e.g. through BTV-8 contaminated frozen material like bull semen. An unlikely and implausible alternative for the second outbreak could have been an undetected ongoing persistent infection without high level viral replication for several years [107]. Nevertheless, the few observed mutations in the new BTV-8-genome led to a less virulent re-emerged BTV-8 strain with reduced pathogenicity [108]. Unfortunately, only a limited amount of BTV-8 vaccines was available in 2015/16 from vaccine producers, and together with high vaccine costs, it was problematic to eradicate BTV-8. Since 2018, due to the increased case numbers in France, the whole country was declared enzootic for both BTV-4 and BTV-8 [89]. Furthermore, BTV-8 was firstly reported again in Germany (2018), in Switzerland (2019), in Luxembourg (2019), and in Belgium (2019) [107, 109, 110]. In Germany, the first case of BTV-8 since 2009 was detected in two clinically healthy cattle in context with an export investigation on the 12th of December 2018, and consequently, a restriction zone of 150 km was implemented. For 2018, only one outbreak was reported in Germany, followed by 59 in 2019 and BTV-8 still keeps ongoing in 2020 with 2 cases [111]. The current BTV restrictions zones in the EU from January 2021 are shown in figure 11.

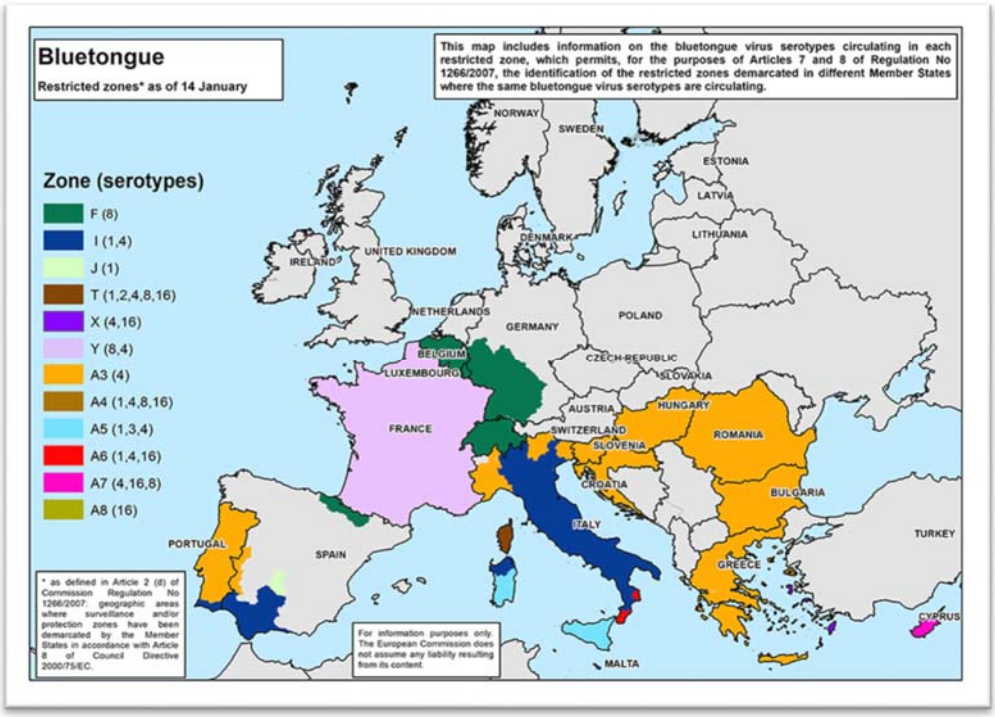

Figure 11: Map of BTV restricted zones in the EU (Source: European Commission, 2021).

2.5. Host range of Bluetongue virus infection

Bluetongue is a major disease of sheep, but there are breed-specific differences and especially European fine wool and mutton sheep breeds become severely disease. Possibly, Bluetongue virus can infect all ruminant species, but BTV-related clinical disease occurs only sporadically in other animal species like BTV-infected cattle or South American camelids [49]. Goats are also susceptible to BTV and might play a role as host reservoir, but in contrast to sheep, they rarely show clinical symptoms [112]. Viremia in infected cattle, sheep and goats is prolonged but transient [113]. Detectable viremia in cattle is reported up to 63 dpi [114], whereas RNA is much longer detectable for up to 222 dpi [113]. Duration of viremia after experimental infection in small ruminants lasted about 34 to 37 days without significant difference between sheep and goats [115]. Interestingly, eastern Mediterranean small ruminant breeds appear to have a longer viremia than other breeds [115]

South American camelids are susceptible to Bluetongue virus infection, but play only a negligible role in BTV epidemiology. Seroprevalences were found to be higher in regions near BTV outbreaks and therefore BTV exposure and infection pressure might play a significant role for the risk of BTV infection in South American camelids [116]. Viremia observed in alpacas and llamas was shorter compared to cattle, which is in line with the reduced BTV binding efficiency to red blood cells of South American camelids [117]. Clinical disease in South

American camelids was not or only rarely observed in the field and after experimental infection [116, 117]. However, sporadically, fatal cases were reported in connection to BTV-1, BTV-8 or BTV-11 infections [116, 118, 119].

Most wild ruminant species are susceptible for BTV infection, but BTV infection leads to a very variable clinical outcome from subclinical infection to mild or severe cases and in single cases also death [120]. Most wild ruminants remain asymptomatically, particularly indigenous animals in regions where BTV is endemic [120]. BTV pathogenesis and transmission routes seem very similar in wild ruminants compared to sheep and cattle [120-122]. Also wild sheep such as bighorn (*Ovis canadensis*) and mouflon (*Ovis aries musimon*) can show clinical disease, even fatal [120, 123]. Likewise, pronghorn antelope (*Antilocapra americana*), American bison (*Bison bison*), and African buffalo (*Syncerus caffer*) show sometimes clinical disease [120, 124]. On the other hand, no clinical signs were reported for the susceptible blesbock (*Damaliscus pygargus*) [125] and the mountain gazelle (*Gazella gazelle*) [120], as well as the North American elk as close relative to the European red deer [120, 126]. Bluetongue outbreaks in domestic animals appeared to be more or less correlating with the prevalence of BTV in wild ruminants, suggesting that wild ruminants might play a role in bluetongue epidemiology [127]. In Europe, the red deer population is the most relevant wild ruminant species for BTV transmission, due to its high population and density [128] and unsurprisingly, partly high seroprevalences for BTV were reported in red and fallow deer [120]. Experimental and natural BTV infection in red deer lead to asymptomatic or mild disease with RNA detection up to 112 dpi [129]. BTV infection in red deer populations did not lead to a higher mortality rate [130] and lately, a French long term monitoring study suggested, that the red deer might not play an important role as maintenance host for spreading BTV [131]. Overall, in Europe the wild ruminant populations seem to be infected as long as the virus is circulating in sheep and cattle. After eradication of BTV-8 due to vaccination the seroprevalence in wild ruminants faded out and there was no indication of a wild ruminant reservoir [131], which was e.g. confirmed by the German BTV monitoring program [132].

Carnivores are susceptible for BTV infection and clinical disease was reported in dogs including abortions, pulmonary oedema and even death [133, 134]. Canine BTV cases might be linked to the vaccination with BTV contaminated canine vaccines or the consumption of BTV contaminated raw meat. However, vector-borne transmission in single cases is discussed

as well [133-136]. Two Eurasian lynx were fed with ruminant foetus and stillborn animals and died due to BTV-8 infection [137].

2.6. Bluetongue virus pathogenesis and clinical disease

Bluetongue disease can cause a highly variable disease outcome depending on a variety of factors as breed, virus strain, environment and immunological status whereas serotype does not seem to determine virulence. In the majority of ruminants, BTV infection leads to no clinical disease or mild symptoms typically in BTV endemic areas. Disease occurs mostly when susceptible animals are introduced in an endemic region or when virus spreads in areas with immunological naive animals [51]. Most BTV strains do not cause a clinical severe picture in cattle, but the BTV-8 strain circulating in Europe during 2006-2009 did lead to clinical disease in a part of the infected population [92]. Sheep are developing more severe clinical signs than cattle, which can be explained by the different susceptibly of bovine and ovine endothelial cells [138, 139]. Furthermore, the ratio of thromboxane to prostacyclin is higher in BTV-infected sheep and thromboxane is a great inducer of microvascular injury and thrombosis [12, 138, 140]. Indeed, the virulence of different strains in sheep is correlating with the severity of vascular lesions they induce and with the serum concentrations of acute phase proteins, but not directly with viral loads [141]. BTV circulation is linked to the life span of blood cells, because BTV is highly-cell associated. Platelets are short-lived, but the longer life span of erythrocytes leads to a prolonged viremia in the blood and enables even the co-circulation of neutralizing antibodies and virus for several weeks [12, 140]. Infectious BTV persists in invaginations of the cell-membrane of erythrocytes, and is a main factor for cattle as natural hosts [12].

Bluetongue virus enters the host via the bite of an infected *Culicoides* midge through the skin. After inoculation, BTV reaches the draining regional lymph node, where the first virus replication takes place and the immunological response of the host starts [12]. Interestingly, BTV can also replicate in the skin itself [142]. Peripheral mononuclear blood cells are the secondary sites of replication, but also the endothelium, peri-endothelial cells, pericytes of capillaries, small arterioles and venules particularly of the lung and the spleen [70, 140]. With ongoing replication, BTV spreads with the blood and lymph stream through the body and consequently most organs and tissues contain a certain amount of virus [12, 140]. BTV infection is followed by a pan-leukopenia with its peak at 7-8 dpi [12]. The relatively transient BTV replication in the target endothelium cells lasts around 10 days and causes cell injuries

and necrosis. Furthermore, BTV and the host induce the production of vasoactive and pro inflammatory mediators as cytokines and prostanoides contributing to the increased vascular permeability and the clinical picture of a haemorrhagic fever [12]. This vascular permeability can lead to extensive oedemas, and the pulmonary oedema can be fatal typically after two or more weeks post infection [140]. A disseminated intravascular coagulation was reported in highly susceptible white-tailed deer [70, 140].

The first clinical signs develop mostly within the first week post infection. Infarction of the small blood vessels in the oral cavity leads to erosions and ulcers of the oral mucosa, whereas affected blood vessels in the coronet lead to coronitis and lameness. Serous to bloody nasal discharge with crusts around the nares and muzzle can be seen. Vascular thrombosis and infarcts occur also in the upper gastrointestinal tract, subcutis, heart and skeletal muscle as well. Oedema of the head, neck, lungs and thoracic cavity, as well as abdominal, pleural and pericardial effusions are followed by difficulties in breathing and respiratory problems [121, 140]. Less common is the cyanosis of the tongue and oral mucous membranes leading to a purple/blue discoloration which gave the disease the characteristic name (see figure 12). Furthermore, cardiac necrosis may result in sudden death at any time even in an animal that appears to be recovering. Severely affected cattle occasionally develop severe pulmonary oedema, whereas pleural, pericardial and abdominal effusions are apparently not as characteristic of severe BT in cattle as in sheep [94, 140, 143]. For several animals surviving acute BTV infection, chronic dermatitis as well as vesicular and erosive lesions at interdigital and mucosal surfaces were reported [12, 140].

Figure 12: Moderate (left) and severe (right) clinical signs of Bluetongue in sheep [53]. (Source: Wilson, et al. 2008; page 1613).

Interestingly, congenital BTV infection can lead to teratogenic defects in ruminants. Particularly teratogenic are strains modified by growth in embryonated chicken eggs or cell culture as performed for modified live vaccine strains. For a numerous number of serotypes transplacental transmission and teratogenic defects were reported [144]. BTV is destructing the neuronal and glial cell precursors, which are in young foetuses in the subependymal region of the developing cerebrum. After migration to the cerebral cortex and subcortical white matter, they are less susceptible for BTV and mature neurons and glial cells are resistant to productive, lytic BTV infection [144]. Already in the 1950's, the vaccination of pregnant ewes with a BTV-10 modified life vaccine strain in California led to congenital deformities, cerebral abnormalities such as hydranencephaly, and the birth of viraemic calves [145-147]. Some BTV-1 and BTV-8 infected calves showed severe necrotizing encephalopathy, meningitis as well as hydranencephaly or porencephaly [148]. Particularly during the BTV-8 epidemic in Northern Europe, the number of congenital malformations and abortions increased [60, 147-149]. The genetically very closely related re-emerged BTV-8 strain in 2015, showed a reduced pathogenicity compared to the BTV-8 strain during 2006-2009 [108]. Nevertheless, in a high number of calves, clinical signs connected to the central nervous system were reported indicating the remaining potential of the re-emerged BTV-8 strains to cross the placenta and cause congenital malformation and damage of the nervous system [150].

2.7. Bluetongue virus specific immune responses

Both, the innate and the adaptive immune system are involved during BTV infection. The innate immune system is important to enhance the adaptive immune response. The adaptive immune system can protect the animal from BTV disease, either cellular mediated by T cells and also mediated by antibodies [12].

The innate immune response is the hosts' first line defence after BTV enters the animals' skin through the bite of a *Culicoides* midge. Dendritic cells, recruited in a high number, quickly transport the virus into the regional lymph node. The initial replication occurs in dendritic cells, macrophages, endothelium and lymphocytes [141, 151]. Then, the virus disseminates further in the body to secondary replication sites [112, 121, 141]. The different pattern recognition receptors especially recognize the viral dsRNA and trigger on different

signalling pathways the production of Type I, Type III interferons, and other proinflammatory and vasoactive mediators like interleukins, prostanoids and tumor necrosis factor α (TNF-α). The virus-induced cytokine and chemokine mediators limit and control the infection and promote the development of a strong immune response. As a consequence, the cytokine storm contributes to the BTV clinical disease of a haemorrhagic fever, with the capillary leakage syndrome and coagulopathy. On the other hand, the virus tries to escape the immune system of the host and particularly the NS3 protein of BTV interferes with the IFN production [22].

Cell-mediated immunity limits viral spread of acute viral infections by the destruction of virus-infected cells. The cell-mediated immunity in ruminants is poorly characterized, although BTV infection clearly results in alterations in lymphocyte populations locally and systemically [151, 152]. Nevertheless, BTV infection first leads to a transient pan-leukopenia and decrease of CD8+ cytotoxic T lymphocytes followed by an increase of CD8+ cytotoxic T lymphocytes later on and thus a decrease of the CD4/CD8 ratio during the convalescent period [152, 153]. Interestingly, when the CD8+ cytotoxic T lymphocytes from a previously BTV infected sheep were transferred to a BTV naive sheep, the CD8+ T lymphocytes did only partially protect the sheep against BTV challenge infection [153, 154]. The major targets of the ovine cell-mediated immunity might be the viral NS1 and VP2 proteins [141, 155]. The NS1 protein is conserved among most BTV serotypes and thus different approaches for polyvalent BTV vaccines were made, however with only little progress [141, 156, 157].

BTV infection induces the production of antibodies directed towards both structural and non-structural viral proteins [141, 158]. Antibodies formed towards the VP7 are serogroup specific, as VP7 is a more conserved protein amongst BTV serotypes and strains [153, 159]. However, antibodies formed towards the outer core protein VP7 might bind and neutralise core particles, but not the fully intact virions [1]. Commonly used ELISAs in BTV diagnostics and serological surveys are usually targeting the VP7 protein due to the cross-serotype reactivity [153, 160, 161]. The neutralizing epitopes of BTV are located on the VP2, which is a protein located on the virus outer capsid and furthermore the most variable viral protein defining BTV serotypes [121, 153, 159]. VP2 is therefore essential for protection and induces also the production of neutralizing antibodies, which can be analysed by neutralization assays [155, 162]. BTV serotype specific neutralizing antibodies enable a long-lasting resistance to reinfection with the homologous serotype, which is the basis of BTV vaccination strategies

[70, 95, 163, 164]. However, BTV is highly cell associated and connected to erythrocytes, so that BTV can co-circulate for several weeks together with neutralizing antibodies and escape neutralization [141, 165, 166]. Important for efficient neutralization is the conformation of the VP2, but also the conformational interaction with VP5, the second outer capsid protein. Several BTV serotypes carry multiple common neutralization epitopes allowing a serological cross reactivity. This can lead to the development of neutralizing antibodies without actual exposure of the animal to that certain serotype [141, 167].

2.8. Bluetongue virus vaccination

Vaccination is the major disease control measure and prevention strategy for blocking Bluetongue virus disease and spread next to implementation of restriction zones and export bans [168]. The aims of BTV vaccination strategies are preventing the disease, reducing the BTV spread for eradication and safe animal movements [95, 163]. Other control strategies directed against the vector population like repellents can be very challenging and insufficient particularly for extensively kept livestock [160, 168]. Nevertheless, protection afforded by BTV-vaccines is serotype specific [169]. Two different vaccine types are commercially available: (i) modified-life virus (MLV) vaccines, which consist of attenuated virus strains and (ii) inactivated vaccines including killed/inactivated virus strains combined with an adjuvant [168].

The first MLV vaccine used in South Africa from 1907 to 1943 was the monovalent Theiler's strain [64, 66, 69]. Nowadays, the used MLV vaccines in South Africa are polyvalent and each of the three formulations contains five different BTV serotypes [168, 170-172]. Methods for attenuation of life BTV are alternate passaging in cell culture and embryonated chicken eggs [168]. The MLV vaccines are effective in local animal populations or in early stages of recent outbreaks to minimise BTV circulation [173]. Consequently, MLV vaccines have been applied in endemic countries like South Africa, Italy, Bulgaria, Israel, France, India, Turkey and Spain [168, 174]. The great advantages of MLV are the cheap and easy production and the long-lasting protective immunity achieved with a single dose [168]. However, the usage of MLV vaccines is responsible for several vaccine-induced BTV outbreaks in the past [168]. Some of the live attenuated strains are e.g. virulent for European sheep breeds after prolonged circulation in the field, and MLV strains did lead to sufficient high vaccine virus titers during viremia for infecting vectors [173, 175, 176]. Overall, the safety of BTV-MLV can be problematic and vaccine and wild-type strains can exchange genome segments when

simultaneous infection of the ruminant host or *Culicoides* midge happens [163]. Reassortment is not limited to phylogenetically related viruses or vaccine serotypes [177]. The circulation of different vaccine serotypes in *Culicoides* and susceptible hosts might even provide the ideal environment for reassortment [172]. Moreover, live vaccines have been linked to higher abortion rates and decreased milk production [178].

The first approach of an inactivated BT vaccine was described in 1975 for use in sheep [179], but however, inactivated vaccines haven't become commercially available until 2005 [164, 168]. The first developed BTV inactivated vaccine was using an Italian BTV-2 field isolate [180]. Virus inactivation is achieved by treatment with heat, ultraviolet radiation or chemical methods using hydroxylamine or binary ethylenimine [168, 179, 181-183]. Inactivated vaccines contain the killed virus mixed with an adjuvant, which stimulates non-specifically the immune response. In the last years, mono- and multivalent vaccines were developed for BTV-1, -2, -4, -8, and -9 [168]. The big advantage of inactivated BTV-vaccines is their very good safety in comparison to MLV vaccines. Inactivated vaccines cannot reassort, reverse to virulence or lead to viremia, and consequently cannot infect insect vectors. The usage of more than 100 million vaccine doses during the European BTV-8 epidemic during 2006-2009 did contribute to the successful eradication of BTV in northern Europe [95]. The target of national vaccination campaigns with inactivated vaccine was to reach at least 80 % coverage of domestic ruminants [164, 178]. Disadvantages of the inactivated vaccines are the higher production costs and the requirement of booster immunizations [95]. Nevertheless, inactivated vaccines can be also highly efficient [95], and some studies even revealed a six-year antibody longevity of BTV-8 group- and serotype-specific neutralising antibodies, as well as the transmission of neutralizing BTV-8 antibodies through colostrum to calves [184]. Recently, presence of antibodies in sheep was shown for 7.5 years after BTV-8 vaccination [185].

Furthermore, numerous other novel BTV vaccine types were developed during the last years by using e.g. genetically modified BTV viruses, recombinant vectors for the expression of immunogenic BTV proteins, or immunogenic proteins themselves as subunits [168, 186, 187]. Recombinant vector vaccines induce a strong neutralizing immunity and have the potential for a "single dose immunity". Several different non-pathogenic viruses were used as expression vectors so far [168, 187]. With reverse genetics disabled infectious single cycle (DISC) and disabled infectious single animal (DISA) vaccines have been also developed, lacking

one or more essential BTV genes. Recently, a DISA vaccine with live-attenuated BTV lacking the dispensable non-structural NS3/NS3a protein allowed in addition the differentiation of vaccinated from naturally infected animals (DIVA) [168, 186, 188]. Virus-like particle vaccines (VLP) are produced by infecting insect cells with genetically modified baculovirus vectors expressing several BTV virus proteins [168]. These proteins assemble to so-called “empty viral particles” and mimic the immunogenic structure of native BTV [168]. Multiple doses of the VLP mixed with adjuvants might be necessary, but the vaccine is stable, highly safe and efficient [168, 189]. Unfortunately, the biggest disadvantage of all these novel vaccine types are the high production costs compared with MLV or inactivated vaccines, because they are more difficult to design and produce. Consequently, none of these novel vaccines is commercially available up to now [168].

2.9. Diagnostics of bluetongue virus

BTV infection is followed by viremia in the blood and can be detected by virus isolation through embryonated chicken eggs (ECE), by cell culture or less commonly by inoculation of sheep. BTV can also be detected by antigen ELISAs and the viral RNA by diverse RT-PCR methods from an extracted blood or tissue sample. The time window of BTV RNA detection is with 111-222 dpi quite prolonged comparing to the actual viremia lasting less than ≤9 weeks which is further advantage of BTV PCR-diagnostics [113]. An Overview of all diagnostic methods recommended by the OIE is shown in Table 1.

Method	**Purpose**					
	Population freedom from infection	**Individual animal freedom from infection prior to movement**	**Contribute to eradication policies**	**Conformation of clinical cases**	**Prevalence of infection & surveillance**	**Immune status in Individual animal or populations post-vaccination**
Agent identification						
Real-time RT-PCR	no	recommended	no	recommended	suitable	no
RT-PCR	no	recommended	no	recommended	suitable	no
Classical virus isolation	no	recommended	no	recommended	no	no
Detection of immune response						
cELISA (Serogroup specific)	suitable	recommended	suitable	no	suitable	suitable
Virus Neutralization	suitable	recommended	suitable	no	suitable	suitable

(serotype specific)						
Agar gel immunodiffusion	maybe	no	maybe	no	maybe	maybe
Complement fixation test	maybe	no	maybe	no	maybe	maybe

Table 1: Adapted from the OIE terrestrial Manual 2019 [190], page 340.

The ideal sample for virus isolation represents the fresh blood sample collected into an anticoagulant like EDTA (ethylamine diamine tetra-acetic acid), but also heparin or sodium citrate can be used. Prior to the virus isolation attempt, the blood sample need to be washed in phosphate buffered saline (PBS) to remove neutralising antibodies and stored at 4°C when not immediately used. Mosquito or tissue samples can be used as inoculum as well [190]. One of the most sensitive isolation methods is the inoculation of 9-12 days old embryonated chicken eggs (ECE) by using the intravascular (IV) inoculation route [191]. Highest recovery rates are reported by isolation in ECE followed by passages in cell culture for further replication. For virus isolation in cell culture several mammalian and insect derived cell lines are in usage. Interestingly, the insect-derived cell lines do not show necessarily CpE (Cytopathic Effect), but in mammalian cells CpE is normally prominent. Commonly used cell lines are the insect derived *Culicoides sonorensis* cell line (KC), the *Aedes albopictus* clone C6/36 cell line and for mammalian cell lines the baby hamster kidney cells (BHK-21), a clone of BHK cells (BSR5), and African green monkey kidney (Vero) cells. For confirmation of a successful virus isolation, BTV genome can be detected with molecular methods such as RT-PCR. Alternatively, viral antigen can be directly detected by using the immunofluorescence test or antigen capture enzyme-linked immunosorbent assays. By using virus neutralisation procedures as the microtiter neutralisation test, plaque inhibition or plaque reduction assays, the BTV serotype can be defined [190]. However, also concepts of serotype-specific ELISAs were developed, like for BTV-4, but not commercialised [192].

For genome detection, the real-time RT-PCR (RT-qPCR) based on TaqMan technology has become the most popular RT-qPCR technique [193]. Numerous RT-qPCR assays have been developed over the time targeting different BTV genome segments. BTV group-specific RT-qPCR assays target the more conserved regions of the BTV genome segments like segment 1, 5 or 10. The Pan-BTV-S10-RT-qPCR from Hofmann, Griot, et al. 2008, detects all known BTV serotypes and strains including the atypical BTV currently circulating in the world and is therefore recommend for broad BTV-diagnostics by the OIE [190, 194]. Serotype-specific RT-

PCRs are targeting segment 2 of the BTV genome. Particularly, the emergence of bluetongue serotypes in Europe has led to the publication of numerous serotype-specific assays like for BTV-1, -6, and -8 [195], for BTV- 15 [196], BTV-1, -6, -8, and -11 [197], and for BTV-2 [198]. Primers for RT-qPCRs for typing the serotypes 1, 2, 4, and 9, as well as approaches for differentiation of field and vaccine strains in Europe were e.g. published by the Pirbright Institute (TPI) in 2004 [14],and in an expanded version for BTV-1, -2, -4, -8, -9, and -16 in 2007 [199]. RT-PCR assays for 26 serotypes were published by TPI in 2012 [200], and in 2016 TaqMan-RT-qPCRs for 27 BTV serotypes including one putative novel alpaca serotype were reported [74]. Outside Europe, RT-PCR assays for typing BTV were developed as well. Thus, a study in India published serotype-specific TaqMan assays for the circulating serotypes 1, 2, 9, 10, 12, 16, 21, and 23, specifically adapted to strains from India [201]. In the USA and Australia, a number of RT-PCR tests are in use for typing as well [202-205]. Far more serotype-specific RT-PCR and RT-qPCR assays have been published over the years, including assays for newly discovered serotypes. A different approach for serotyping is the nCounter® Analysis System Microarray platform[206].

Various serological methods can be used to detect group and serotype specific BTV-antibodies which are usually generated within the first week post infection. In common use for detecting BTV group-specific antibodies are commercially available competitive enzyme-linked immunosorbent assays (cELISA), which are using monoclonal antibodies (MAbs) reactive to the amino terminal region of the major core protein VP7 [190, 207], however, also indirect ELISAs for milk samples are in usage [190, 208]. For double antigen or sandwich ELISAs even a higher sensitivity was shown for serum samples of vaccinated sheep, but they are not commonly used [209]. VNTs and SNTs are reference methods for serotype identification by detecting serotype-specific antibodies and of particularly interest in endemic regions with more than one BTV serotype circulating. For an SNT the test serum is incubated together with the reference virus strains, whereas for a VNT the test virus strain is incubated with the BTV reference sera. The cell culture is then scored for CpE or replication of BTV is detected by (immunofluorescence) staining. Both methods are labour intensive and require the availability of reference sera and the respective BTV serotypes. Less commonly used serological methods nowadays are the complement fixation or the agar gel immunodiffusion tests [190].

2.10. Atypical Bluetongue viruses

Genetically clearly distinct from the 24 notifiable so-called “classical BTV serotypes” are the “atypical” or “small ruminant” BTV [210, 211]. The atypical BTV were numbered consecutively starting with BTV-25, the very first reported atypical BTV serotype, which occurred in Switzerland in the Toggenburg region. Up to now, atypical BTV are reported in goats and sheep, but in no other ruminant species like cattle. In general, atypical BTV do not cause clinical disease in goats and no or only a mild clinical disease in sheep with the exception of BTV-28 [210-214]. Thus, atypical strains do not cause economically losses and no restriction measures or control strategies are implemented. Consequently, atypical BTV strains are not notifiable in the European Union [211]. For the atypical BTV strains, serotyping can be difficult due to unsuccessful virus isolation in cell culture [210, 212, 215, 216] or the lack of antisera reactive against atypical BTV strains in most laboratories [210, 217]. Interestingly the NS3/NS3a of BTV-25 and BTV-26 are clearly distinct from the NS3/NS3a of classical BTV, which could be one of the reasons for the distinct characteristics of atypical BTV [218].

Atypical BTV-25

BTV-25 (Toggenburg Virus—TOV) was detected in two different asymptomatic goat flocks in the Toggenburg region in Switzerland [219]. Similarly to the naturally infected goats, experimentally TOV-infected goats did not develop any clinical signs typical for BTV, even though they exhibited a high virus replication rate [215, 220]. Experimentally TOV-infected sheep presented a very mild clinical disease [220]. Horizontal transmission of TOV seems unlikely, as direct contact animals did not get infected, and all swabs as well as milk and saliva samples revealed negative results [215]. The systemic spread of TOV in goats was described as rather slow [215]. Nevertheless, the high seroprevalence rates of goat flocks in combination with an extremely low vector activity in Switzerland provided some indication for the presence of an alternative transmission route [215, 220]. Furthermore, there are indicators for transplacental infection with TOV [220, 221]. TOV RNA could be detected for up to 25 months in infected small ruminants, and the infectivity of blood during that period was demonstrated [222]. In addition, the antibody response of experimentally infected animals was described as slow and weak [215]. All attempts of cell culture-based virus isolation remained unsuccessful [215]. Thus, for the use in VNTs, a chimeric classical BTV/TOV virus was generated by reverse genetics [221]. In 2018, another TOV-related BTV strain (BTV-Z ITL2017) was described in the Piedmont region in Italy and likewise could not be cultivated [212]. This TOV-related strain

was found in healthy goats and showed a high identity with TOV, both on the nucleotide (nt) and the amino acid (aa) level. Nevertheless, the serotype remained undefined due to the failure of ELISA-positive sera to neutralize the reference BTV and atypical BTV serotypes [212].

Atypical BTV-26

In Kuwait, a further atypical BTV, BTV-26, was discovered in two sheep and successfully isolated using mammalian cell lines [213]. The flock of origin was clinically diseased, but this was reported as not related to BTV infection [72]. However, mild clinical disease was described for experimentally infected Dorset Poll sheep including conjunctivitis, reddening of the mouth mucosal membranes, slight oedema of the face and nasal discharge [223, 224]. The levels of BTV-26 genome loads were low and short-lived in comparison to classical BTV [224]. Furthermore, as BTV-25, also BTV-26 did not replicate in experimentally infected *Culicoides* and *KC* cells in contrast to classical BTV strains. The inability of BTV-26 to infect/replicate in KC cells was shown to be associated with differences in Seg-1/VP1, Seg-2/VP2, Seg-3/VP3 and Seg-7/VP7 [225]. This difference in tropism may further indicate an alternative way of transmission [224]. BTV-26 did not cause clinical signs in naturally and experimentally infected goats, but the high replication levels suggest goats as natural reservoir [226]. Interestingly, the contact goat inclusively two nasal swabs became viral RNA positive. A following study in goats supported the suspicion of horizontal transmission via direct contact for BTV-26 in goats as all direct contact control goats became infected. However, the exact route of transmission needs to be further analysed [223]. Nasal or ocular secretion, as well as oral transmission or mechanically by infected blood via small injuries cannot be excluded [226]. [211]. Interestingly, BTV-26 antibodies were found to circulate in cattle and dromedaries in Mauretania [227]

Atypical BTV-27

During the BTV-1 vaccination programme in Corsica in 2014, BTV-27/FRA2014/v01 was isolated from asymptomatic goats, later on two more variants, BTV-27/FRA2014/v02 and BTV-27/FRA2014/v03 [228, 229]. BTV-27/FRA2014/v03 was detected in few goat flocks affected by BTV-27v01 as well, whereas BTV-27v02 was isolated from only one single goat and thus seems to be rare [229, 230]. The origin for the spatial dissemination of the three BTV-27 variants are unknown, but sera from BTV-27v01-infected goats did not neutralize BTV-27v02 and v03 strains or vice versa [229]. All three variants could be isolated on mammalian cell

lines, but interestingly not on KC cells [229, 230]. In experimental infections with the three variants of BTV-27, sheep were not susceptible for any variant, which is consistent with the field data. It is not assumed that cattle are susceptible either, however, it has been up to now only proven experimentally for BTV-27/FRA2014/v02. Most of the experimentally infected animals did not develop any clinical disease and only very few did show a slight body temperature rise and mild serous nasal discharge. Goats are most likely the natural host for BTV-27, especially as the antibody levels are slowly increasing and no clinical disease is seen. Furthermore, contact transmission for BTV-27/FRA2014/v02 was reported [230].

Atypical BTV-28

Three atypical BTV strains, BTV-28/1537/14, SPvvvv/02 and SPvvvv/03, were isolated from contaminated commercial batches of a sheep pox and lumpy skin disease vaccine [214, 231, 232]. SPvvvv/03 is genetically closely related to BTV-28/1537/14, whereas SPvvvv/02 is more distinct and phylogenetically related to BTV-26 [231]. BTV-28/1537/14 and BTV-26 are sharing high sequence similarities, which might indicate a common ancestor, whereas segment 9 resembles to BTV-Y TUN2017 (Tunisian atypical strain detected in 2017) suggesting reassortment [214]. The experimental infection in ewes with BTV-28/1537/14 showed moderate clinical disease including fever, conjunctivitis, inflammation of gums, coronitis and tongue cyanosis. BTV-28 is pathogenic and might be also transmitted directly as control goats became viral RNA positive and clinical diseased [214]. All experimentally infected ewes seroconverted and developed group specific and neutralising antibodies from 7 dpi onwards similar to BTV infection with classical strains [214].

Other putative atypical BTV

In Xinjiang in China, BTV-XJ1407 was isolated from clinical healthy sheep and goats [217]. VNTs were performed with the antisera of BTV-1-24, but reactive sera against BTV-25, 26 and 27 were missing for assignment to a novel BTV serotype. Nevertheless, the sequence analysis suggests, that BTV-XJ1407 is most likely a novel BTV serotype or a subgroup of BTV-25 or 27 [217].The V/196/XJ/2014 strain was collected from a Chinese goat and fully sequenced as well.

During a serological screening in Sardinia, clinical healthy goats were found positive for BTV-X ITL2015, another non-pathogenic atypical BTV strain related to BTV-27 and BTV-XJ1407 in segment 2 [210]. Both strains, BTV-X ITL2015 strain 34200 and BTV-X ITL2015 strain 33531,

could not isolated in cell culture. The high seroprevalences of up to 100 %, and high monthly rates of new infections in affected farms suggest an even more efficient transmission route than vector-borne. BTV-X-ITL2015 might have been introduced to Sardinia by import of affected goats [210].

Lately, another novel BTV strain related to BTV-26 and BTV-28 was described in asymptomatic sheep in Libya in 2017. Unfortunately, virus isolation attempts remained again unsuccessfully.

Further remarkable was the finding of a BTV-3 TUN2016/Zarzis and BTV-Y TUN2017 co-infected sheep, which is the first field report of a co-infection with an atypical and a classical BTV strain [216].

3. Objectives

This thesis describes recent developments in control, diagnosis and pathogenesis of BTV with a focus on the following three objectives.

Objective I: Duration of vaccine-induced BTV-8 immunity

One of the most important tools in BTV control and eradication is vaccination. In Europe during the first BTV-8 epidemic from 2006-2009 huge vaccination campaigns with inactivated BTV-8 vaccines led to a vaccine coverage in cattle of more than 80%. Serum samples from cattle, which received their last vaccination dose 5 to 8 years ago, were available for serological analysis. The presence of vaccine-derived antibodies of at least 8 years post vaccination could play an important role in future BTV control strategies.

Objective II: Modern diagnostics for molecular BTV serotyping

Several different serotypes invaded Europe and serotype specific vaccination is the major control measure. Our novel developed diagnostic tool “BlueTYPE” RT-qPCR array can be used for the swift identification of outbreak serotype(s), but also in daily laboratory work for excluding cross contaminations e.g. in cell culture propagated viruses. Therefore, the developed “BlueTYPE” array contains RT-qPCRs for all classical serotypes condensed in one array format and enables fast and reliable molecular ‘serotyping’. Furthermore, by including a newly developed RT-qPCR assay detecting only classical serotypes, the differentiation between classical and atypical strains can be done easily.

Objective III: Characterization of atypical BTV-25 including cell culture isolation

Recently, several novel, so-called atypical BTV were discovered in mostly clinically healthy goats and sheep. We reported the occurrence of a BTV-25 positive goat flock in Bavaria and surveyed the BTV-25 dynamics in the goat flock for several months regularly. We obtained the first BTV-25 isolate propagated in cell culture and the full-genome sequence, which enables further research on the pathogenesis and epidemiology of this atypical strain in comparison to classical BTV.

4. Results

The manuscripts are presented in the form they were accepted for publication.

Each manuscript has its own reference section formatted in the style of the respective journal; references and abbreviations from the manuscripts are not included in the relevant sections at the end of this document. Figures and tables are numbered individually within each of the manuscripts.

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Short communication

BTV antibody longevity in cattle five to eight years post BTV-8 vaccination

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ABSTRACT

The Bluetongue virus serotype –8 (BTV-8) epizootic in Germany (2006–2008) was successfully eradicated, essentially by the massive application of commercially available inactivated BTV-8 vaccines. While a six-year antibody longevity of BTV antibodies post BTV-8 vaccination in cattle has been described previously, our study investigated the BTV-8-vaccine antibodies in cattle for up to eight years. In total, 157 bovine serum samples were analysed for the presence of group-specific BTV antibodies in both a commercial cELISA, and a BTV-8- specific serum neutralization test. A robust number of cattle were seropositive for group- and serotype-specific neutralising antibodies for five or more years. In selected animals, born and vaccinated in 2009 or later, the presence of BTV antibodies for up to eight years post BTV-8 vaccination could be confirmed. Our data also show, that booster vaccination prolonged the antibody longevity of vaccine-induced antibodies and the number of serologically positive cattle.

1. Introduction

In 2006, BTV-8 occurred in northern Europe for the first time [1]. The BTV-8 outbreak in Germany between 2006 and 2008 was controlled by an obligatory vaccination program, and caused enormous economic damages [2]. The last BTV-8 case was reported in Germany in November 2009 [3]. Based on the successful eradication program, the vaccination strategy changed from obligatory to voluntary in January 2010 [2,4]. On February the 15th, 2012, Germany declared itself free from BTV [2].

Over the last years, several studies investigating the antibody longevity of BTV-specific vaccine antibodies were published. In a study performed in Bavaria, 110 cattle sampled 4 weeks post initial vaccination (basic immunisation) in 2008 were tested with a cELISA with a seroprevelance rate of 82%, whereas only one out of ten cattle pre-selected for the serum neutralization test (SNT) showed serotype-specific neutralizing antibodies. After revaccination in 2009, 28 out of 28 pre-selected cattle were also positive in the BTV-8-specific SNT with a median titer of 20 [5]. Furthermore, a British study with 40 cattle with a basic immunization status revealed that group-specific antibodies were present in 95%, and serotype-specific neutralizing antibodies in 97.5% of the cattle for at least three years post vaccination [6]. Subsequently, the follow up study with 29 animals of the cattle group demonstrated a 97% seropositivity rate of both group and neutralizing BTV-8 antibodies four years post vaccination [7]. A further study showed a six-year antibody longevity of BTV-8 group- and serotype-specific neutralising antibodies post natural BTV-8 infection in twelve cows [8] and another study demonstrated BTV-8 neutralizing antibodies even six years post vaccination in dams, and the transmission of colostral neutralizing BTV-8 antibodies to their calves [9]. In addition, a seroprevalence study in France in 2016 suggested the antibody longevity of BTV group-specific antibodies for at least 5 to 6 years after natural infection or vaccination [10].

Our study investigated the long-term humoral immune response following BTV-8 vaccination. Since "fresh" BTV infections can be excluded in Germany since 2010 until the end of 2018, we were able to investigate the presence of group and neutralising BTV-8 antibodies in cattle for up to 8 years. Furthermore, the immune response after initial basic immunization and several booster vaccinations were tested. The presented data about antibody longevity of BTV-8 vaccinated cattle provides important information especially for diagnostics and epidemiological analyses.

2. Material and methods

2.1. Animals

The study included serum samples from 157 cattle of different breeds, born and vaccinated in Germany between 2009 and

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2012. The registration of all BTV vaccinations in Germany in the national database for identification and registration of animals (HI-Tier) is mandatory [3]. Only cattle with a completed basic immunization (initial two shots) and cattle with up to three annually booster vaccinations were integrated in the study. All samples were taken 5 to 8 years after the last BTV-8 vaccination during routine controls. Hence, sampling time points were documented in quarterly periods, but not precisely per month. The used vaccines were BLUEVAC®8 (CZ Veterinaria S.A., Spanien), Zulvac®8 (Fort Dodge, The Netherlands), BTVPUR® AlSap8 (Merial, Frankreich), and Bovilis BTV8 (Intervet Deutschland GmbH). We presume that the local veterinarians performed all vaccinations according to the manufacturers' instructions. In detail, the animals were categorized into four groups (5, 6, 7 and 8) according to the time span in years between the last BTV-8 vaccination and the sampling. Cattle with 4 years and 5 months up to 5 years and 4 months between the last BTV-8 vaccination and sampling were combined in group 5. The assignment to the groups 6, 7 and 8 were handled in a similar way using the rounded time intervals between BTV-8 vaccination and sampling, respectively. Ninety-two of the 157 cattle received the initial BTV-8 basic immunisation (vaccination status = 1). Additionally, within one year after the basic immunisation 53 cattle received one boost (basic immunisation plus one boost; vaccination status = 2). Within the following second year past basic vaccination, 10 cattle received a second boost vaccination (basic immunisation plus two boosts; vaccination status = 3) and 2 cattle received the basic immunisation plus three boosts (vaccination status = 4).

2.2. Serology

The 157 serum samples were screened for group specific antibodies using a commercial cELISA (ID Screen® Bluetongue Competition, ID-Vet, France) according to the manufacturer's instructions.

A serum neutralization test was performed for detection of serotype 8-specific neutralizing antibodies according to the standard protocol of the EU Reference Laboratory for BT (The Pirbright Institute, UK) [11]. The used virus stock was based on a German BTV-8 isolate of 2008 with a titre of $10^{5.83}$ $TCID_{50}$/ml. A positive serotype-specific BTV-8 serum and a negative reference serum were used as controls. Briefly, the sera were diluted in log2-steps (1:10–1:1280), and titrated against 100 $TCID_{50}$ of the BTV-8 virus. Plates were incubated for 1.5 h at 37 °C before overnight incubation at 4 °C. The following day, 100 μl of a Vero cell suspension with 30 000 cells/ml were added per well. After incubation for 5–6 days at 37 °C, all wells were stained with crystal violet and scored for a cytopathic effect (CpE). The neutralization titer was determined as the dilution of serum giving a 50% neutralization and was calculated according to the method of Spearman and Kärber [20]. Samples with a neutralizing antibody titre of $\geq \log_{10}$ 1 were considered as positive.

3. Results

The ELISA and SNT results of all 157 cattle were analysed according to two variables: (i) the approximated time spans of 5–8 years (groups 5–8) between the latest BTV-8 vaccination and the time point of sampling, and (ii) according to the respective number of boost vaccinations (basic immunisation plus one, two or three booster vaccinations). The serological results are summarised in Table 1 and Fig. 1, statistics in supplementary Table S1. In total, 111 samples (70.7%) were positive in the cELISA, and 128 samples (81.5%) in the SNT. Group-specific antibodies were found in group 5 in 23 cattle (71.9%), in group 6 in 45 cattle (66.2%), in group 7 in 35 cattle (81.4%) and in group 8 in 8 cattle (57.1%). With the BTV-8 SNT, neutralizing titres ranged from $\log_{10}$ 1.15 – to 2.81. BTV-8 seropositive cattle were detected in group 5 in 30 cattle (93.8%), in group 6 in 54 cattle (79.4%), in group 7 in 36 cattle (83.7%) and in group 8 in 8 cattle (57.1%). Sorted by the number of vaccinations, antibodies were detected with the ELISA in 55 animals (59.8%) having received the basic immunization only, in 46 animals (86.8%) with one boost vaccination, in 8 animals (80%) with two boost vaccinations and in two

Table 1
Overview of all tested bovine serum samples in the cELISA and the BTV-8-SNT, sorted according to their group (group 5–8 represents the approximated time span of 5–8 years between the last BTV-8 vaccination and time of sampling), and the number of received vaccinations.

Group°	Number of cattle	Positive in ELISA (%)	Positive in SNT (%)	Number of V.*	Number of cattle	Positive in ELISA (%)	Positive in SNT (%)
5	32	23 (71.88%)	30 (93.75%)	1	13	7 (53.85%)	11 (84.62%)
				2	19	16 (84.21%)	19 (100%)
6	68	45 (66.18%)	54 (79.41%)	1	43	24 (55.81%)	32 (74.42%)
				2	16	14 (87.5%)	14 (87.5%)
				3	7	5 (71.43%)	6 (85.71%)
				4	2	2 (100%)	2 (100%)
7	43	35 (81.40%)	36 (83.72%)	1	24	18 (75%)	18 (75%)
				2	16	14 (87.5%)	15 (93.75%)
				3	3	3 (100%)	3 (100%)
8	14	8 (57.14%)	8 (57.14%)	1	12	6 (50%)	6 (50%)
				2	2	2 (100%)	2 (100%)
5–8	157	111 (70.70%)	128 (81.53%)	1	92	55 (59.78%)	67 (72.83%)
				2	53	46 (86.79%)	50 (94.34%)
				3	10	8 (80%)	9 (90%)
				4	2	2 (100%)	2 (100%)

° Groups 5–8: Approximated time (5–8 years) between the last received BTV-8 vaccination and sampling time. Group 5 (approx. 5 years) = 4 years 5 months to 5 years 4 months, in group 6 (approx. 6 years) = 5 years 5 months to 6 years 4 months, in group 7 (approx. 7 years) = 6 years 5 months to 7 years 4 months, in group 8 (approx. 8 years) = 7 years 5 months to 8 years 4 months.

* Number of vaccinations: 1 = basic immunised cattle; 2 = basic immunised cattle + one boost (boost vaccination one year past basic immunisation); 3 = basic immunization + two boosts (boost vaccination one year past basic immunisation + one boost within the second year past basic immunisation); 4 = basic immunization + three boosts (boost vaccination one year past basic immunisation + two boosts within the second year past basic immunisation).

animals (100%) with three boost vaccinations. Positive SNT results were found in 67 cattle (72.8%) with only the basic immunization, in 50 cattle (94.3%) with one boost, in 9 cattle (90%) with two boosts and in 2 cattle (100%) with three boosts. The Fisher's exact test was applied to examine any statistically significant difference between basic immunised (V = 1) and revaccinated cattle (V = 2–4) and between positive and negative serological result in SNT. The test revealed that the revaccination significantly ($P < 0.05$) improved the serological results in the SNT.

Of the BTV antibody-positive cattle, 17 animals had mismatches between the cELISA and the SNT results. In all 17 mismatch cases, the samples were negative in the cELISA but positive in the BTV-8-specific SNT with titres between $\log_{10}$ 1.15–2.05 (Fig. 2). 29 of all the 157 cattle were seronegative in both the cELISA and the SNT (Table 2). These samples were from 2 cattle (6.3%) of group 5, 14 cattle (20.6%) of group 6, 7 cattle (16.3%) of group 7 and 6 cattle (42.9%) of group 8. Among these seronegative cattle were 25 cattle (27.2%) having received basic immunization, three cattle (5.7%) with one boost, one cattle (10%) with two boosts and no cattle with three boosts.

Twenty-one of all 157 cattle showed negative ELISA results with S/N values higher than 90. Fifteen of these cattle were also negative in the BTV-8-specific SNT, among them 14 cattle with the basic immunization only, and 1 cattle with a single booster vaccination. The six cattle with ELISA S/N values higher than 90, but positive BTV-8-SNT results had low titres ranging between $\log_{10}$ 1.15–1.60 and all had received the basic immunization only.

4. Discussion

The mandatory mass vaccination campaign, which started in 2008 with inactivated BTV-8 vaccines in Germany, contributed significantly to the eradication of the virus [2]. In 2009, only cattle with very low BTV-genome loads in the blood could be detected in the first six months of the year. These weak PCR positive samples can be interpreted as the confirmation of old infections within the year 2008. No "fresh" BTV infection could be detected in 2009 in Germany and the following years. In 2010, the vaccination program switched to a voluntary campaign. On February 15th, 2012,

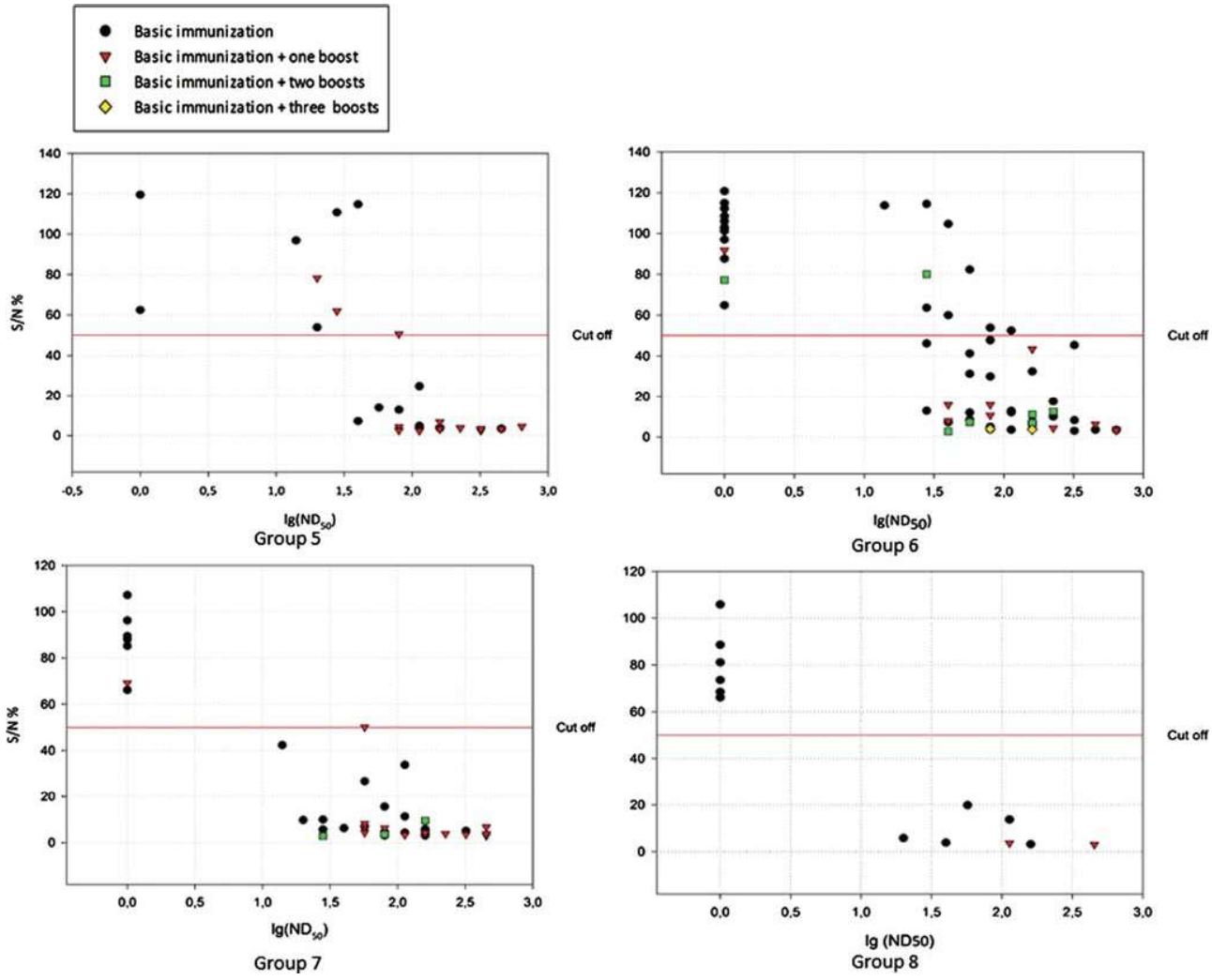

Fig. 1. Serum samples sorted by the group (5–8) and the number of vaccinations (1–4). The y-scale shows the ELISA results. The cut-off line divides the ELISA results in positive samples with less than 50% S/N and negative samples with more than 50% S/N value. The x-scale shows the SNT results. Samples with a $\log(ND_{50}) \geq 1$ are considered as positive, negative samples in SNT are shown with value 0. *Number of vaccinations: see legend of Table 1.

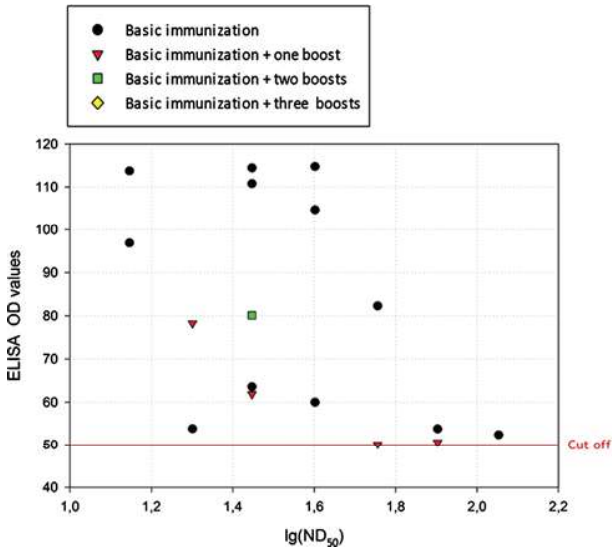

Fig. 2. Serum samples negative in the cELISA, but positive in the BTV-8-SNT sorted by the number of vaccinations. Six cattle with the basic immunization reacted positive in the BTV-8-SNT but strongly negative in cELISA. The y-scale shows the ELISA results. The cut-off line divides the ELISA results in positive samples with less than 50% S/N and negative samples with more than 50% S/N value. The x-scale shows the SNT results. Samples with a $\log(ND_{50}) \geq 1$ are considered as positive, negative samples in SNT are shown with value 0. *Number of vaccinations: see legend of Table 1.

Germany declared itself free from BTV [2] and BTV-8 vaccination was stopped completely for several years.

The mandatory and voluntary BTV-8 vaccination campaign over the years 2008 to 2012, the obligatory registration of BTV vaccinations in an official database, and the absence of "fresh" outbreaks of BTV since 2009 in Germany enabled us to the investigate the long-term humoral response after BTV-8 vaccination in older cattle. We investigated the antibody longevity in cattle for up to eight years post BTV-8 vaccination and the influence of booster vaccinations during this time. Two different serological methods (cELISA and SNT) were used for the investigations. Seven years post BTV-8 vaccination 83.7% of the analysed cattle were seropositive in both the cELISA and the SNT, and eight years post BTV-8 vaccination still more than 50% were seropositive. So far, antibody longevity of BTV-8 antibodies has been described for up to six years post vaccination [9,10]. Our results of BTV-8 seropositive cattle for five and six years post BTV-8 vaccination (93.8% and 79.4%) are comparable to the published seroprevalences for these time windows [9,10]. Less than 20% of the vaccinated cattle were seronegative for BTV even after several years. The existence of non- or poor responders after vaccination is a well-described phenomenon for several viral vaccines including BTV vaccines [12,13]. Differences in the immune response in cattle as shown for different booster vaccines [14], but also the training of veterinarians in vaccine application could influence the vaccination success [15]. Furthermore, the antibody responses to vaccination are modified by environmental and host genetic factors [16].

As there was not a single serum positive in the cELISA and negative in the BTV-8-SNT, the specificity of the used cELISA was 100%. The mismatches of the used serological methods were solely based on negative cELISA and positive SNT results, which was reported in previous serological studies as well [6]. The two serological methods target different BTV antibodies. VP7 as the major determinant of serogroup specificity is used in the cELISA, whereas the SNT detects with a high sensitivity antibodies against the serotype specifying VP2. A certain booster effect after re-vaccination could be observed, since the percentage of seropositive animals increased with the number of vaccinations. Cattle with only the basic immunization achieved a seroprevalence of 72.8%, whereas in cattle with one to three boost vaccinations, seropositivity increased to >90%. This statistically significant data support the efficiency of the booster vaccination as reported before [5].

However, the performed study does not provide information on the development of antibody titres over time in individual animals and is not able to show differences in the efficiencies of the commercially available BTV-8 vaccines. The selected bovine samples from older cattle do not reflect the current BTV-8 seroprevalence in Germany. As no challenge experiments were done, no statement with regard to the status of protection in cattle can be made, even though, a strong correlation between seropositivity and protection has been described previously [17–19]. This fact is supported by the latest findings from BTV-8 re-emergence in France in 2015, where seropositive cattle did not show a BTV-8 infection [10].

The results of our study show the huge power of inactivated BTV-8 vaccines and their long-term benefit, as antibodies were still detectable up to eight years after the last BTV-8 vaccination. All serology-based BTV screening programs are influenced by these long persisting vaccine-induced antibodies. This has to be taken into account by diagnosticians and epidemiologists.

Table 2
Overview of seronegative bovine serum samples in the cELISA and the BTV-8 SNT sorted by their group (group 5–8 represents the approximated time 5–8 years between the last BTV-8 vaccination and time of sampling), and the number of vaccinations.

	Seronegative cattle based on different numbers of vaccination*				Seronegative cattle depending of the groups	
Group°	1	2	3	4	Number	Percentage
5	2	0	0	0	2 of 32	6.25%
6	11	2	1	0	14 of 68	20.59%
7	6	1	0	0	7 of 43	16.28%
8	6	0	0	0	6 of 14	42.86%
5–8	25 of 92	3 of 53	1 of 10	0 of 2	29 of 157	18.47%
Percentage	27.17%	5.66%	10.00%	0%	18.47%	

° Groups 5–8: see legend of Table 1.
* Number of vaccinations: see legend of Table 2.

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Authors' contribution

CR participated in design of the study, collected, interpreted the data and wrote the manuscript. BH designed the study, interpreted the data, wrote the manuscript and participated in revision of the manuscript. MB contributed to the study design with his expert knowledge on bovine vaccinology, in data interpretation and participated in the revision of the manuscript.

Declaration of interest

The authors have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.03.082.

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BlueTYPE – A low density TaqMan-RT-qPCR array for the identification of all 24 classical Bluetongue virus serotypes

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ABSTRACT

Bluetongue virus is a double-stranded RNA virus with 10 genome segments. VP2 is the primary target for neutralising antibodies and defines the serotype. Today, more than 27 serotypes are known, 24 are defined as "classical", and new serotypes are under investigation. Beside group-specific BTV-genome detection, additional serotype characterisation is important for disease control and epidemiological investigations.

Therefore, a low-density RT-qPCR array representing a panel of group- and serotype-specific assays, was combined with an internal control system. For BTV serotype detection, both published and the newly developed in-house PCR systems were combined. The different primer-probe-mixes were placed in advance into a 96-well plate stored at −20 °C until use. At the time of analysis, the only template RNA was added to the prepared primer-probe-mixes and heat denatured at 95 °C for 3 min. After cooling, the master mix was added to each well and the PCR could run for around 90 min.

The presented low-density TaqMan-RT-qPCR array enables fast and precise characterisation of the BTV serotype in clinical cases. Furthermore, mixed infections can be easily identified. In addition, the newly developed low-density RT-qPCR-array can easily be adapted to novel BTV strain variants or extended for relevant differential diagnosis.

1. Introduction

Bluetongue virus (BTV; family *Reoviridae,* genus *Orbivirus*) is an arthropod-transmitted pathogen causing Bluetongue disease in ruminants (Schwartz-Cornil et al., 2008). Bluetongue disease is an OIE-listed notifiable disease with a large economic impact, involving trade restrictions (Zientara and Sanchez-Vizcaino, 2013). It causes a variable clinical outcome ranging from mild and apparent clinical signs to death of the infected host (Erasmus, 1975; Parsonson, 1990; Maclachlan et al., 2009). As a characteristic for the genus *Orbivirus*, family *Reoviridae*, the BTV consists of 10 genomic dsRNA segments (Mertens et al., 2004), coding for seven structural proteins (Mertens et al., 2004; Roy and Noad, 2006) and five non-structural proteins (Belhouchet et al., 2011; Stewart et al., 2015; Ratinier et al., 2016). The three layers of the bluetongue virus (BTV) particle include the sub-core shell (VP3), core-surface layer (VP7) and outer capsid layer (VP2 and VP5) (Mertens et al., 2004). The protective immune response of the host targets the outer surface protein VP2, which contains the majority of epitopes for inducing neutralising antibodies (Huismans and Erasmus, 1981). Consequently, the VP2 protein shows higher levels of sequence variations (Maan et al., 2004a). By using the serum neutralisation test (SNT), the interaction of the virus particle with the neutralising antibodies led to the well-known classification of the 24 classical BTV serotypes (Mertens et al., 2004). In the last years, several 'atypical' serotypes were discovered and defined serologically. Toggenburg virus (TOV) in Switzerland (Hofmann et al., 2008), BTV-26 in Kuwait (Maan et al., 2011), or BTV-27 in Corsica (Zientara et al., 2014). In addition, putative novel serotypes based on genetic data from isolates from China (Sun et al., 2016), Italy (Savini et al., 2017), Israel (Bumbarov et al., 2016), and Mongolia (publication in preparation) have been described. All the so-called 'atypical' BTV were up to know identified in small ruminants, mostly without any clinical sign.

The SNT continues to be a reference method for serotype identification and confirmation of novel BTV serotypes (Maan et al., 2016). However, the segment-2 sequences, encoding VP2, of the 24 classical serotypes correlate perfectly with the respective BTV serotype (Maan et al., 2007). Within the same serotype, the minimum levels of Seg2/VP2 sequence identities were defined as 68.4% nucleotide (nt) / 72.6% amino acid (aa) in the year 2010, accordingly the maximum levels of variation were 31.6% nt level / 27.4% aa level, respectively (Maan et al., 2010, 2016). Viruses belonging to different serotypes can show up to 71.5% nt and 77.8% aa identity, and consequently a minimum

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variation of 28.5% nt / 22.2% aa, respectively.

The real-time PCRs based on the TaqMan technology using a pair of primers and a dually labelled probe enable a simple assay design, a multiplexing strategy, and are easy to use. Therefore, the TaqMan qPCR technology has become the most popular qPCR technique representing one of the most powerful diagnostic methods (Nagy et al., 2016). For BTV diagnostics, several PCRs have been developed over the past years. The BTV group-specific PCRs target the more conserved regions of the BTV genome. Several PCR systems have been published, which target different BTV segments, e.g. for seg-1 / seg-5 (Toussaint et al., 2007), seg-1 (Shaw et al., 2007), seg-3 (McColl and Gould, 1991; Harding et al., 1995), seg-5 (Katz et al., 1993; Aradaib et al., 2003; Jimenez-Clavero et al., 2006; Yin et al., 2010), seg-6 (Bandyopadhyay et al., 1998), seg-7 (Anthony et al., 2007), and seg-10 (Akita et al., 1992; Billinis et al., 2001; Orru et al., 2006; Hofmann et al., 2008). The Pan-BTV-S10-RT-qPCR from Hofmann, et al. 2008, detects all currently known BTV serotypes and strains currently circulating in the world, and is recommend by the OIE (Hofmann et al., 2008). For BTV serotyping, RT-PCRs targeting seg-2 of the BTV genome are in use. The emergence of bluetongue serotypes in Europe has led to the publication of several serotype-specific assays, e.g. for BTV 1, 6, and 8 (Hoffmann et al., 2009), BTV 1, 6, 8, and 11 (Vandenbussche et al., 2009), and for BTV 2 (Orru et al., 2004). Primers for RT-PCRs for typing the serotypes 1, 2, 4, and 9, as well as approaches for differentiation of field and vaccine strains in Europe were published by the Pirbright Institute (TPI) in 2004 (Maan et al., 2004b), and in an expanded version for BTV 1, 2, 4, 8, 9, and 16 in 2007 (Mertens et al., 2007). The development of RT-PCRs for differentiation of 26 serotypes followed in 2012 (Maan et al., 2012). For serotypes 14 and 15, further RT-qPCR protocols were developed by the FLI (Eschbaumer et al., 2011). In 2016, TPI published a set of TaqMan-RT-qPCRs for 27 BTV serotypes and one putative novel alpaca serotype (Maan et al., 2016).

Outside Europe, PCR assays for typing BTV were developed as well. Thus, a study in India published serotype-specific TaqMan assays for the circulating serotypes 1, 2, 9, 10, 12, 16, 21, and 23, specifically adapted to strains from India (Reddy et al., 2016). In USA and Australia, several PCR tests were in use for typing as well (McColl and Gould, 1991; Wilson and Chase, 1993; Johnson et al., 2000; Krishnajyothi et al., 2016). Far more serotype-specific assays have been published over the years, including assays for newly discovered serotypes. The nCounter® Analysis System Microarray platform represented a different strategy for serotyping (Curini et al., 2019). Nevertheless, the TaqMan probe based real-time RT-PCRs developed by Maan et al., 2016 were mostly robust and reliable for serotyping BTV by covering the major eastern and western topotype variations (Maan et al., 2016). The specificity of each assay was evaluated using a wide range of BTV isolates from the Orbivirus Reference Collection (ORC) at TPI (Maan et al., 2016).

Rapid investigation of the broad spectrum of serotypes can only be achieved by performing several single serotype-specific RT-PCRs in parallel or by multiplexing. The disadvantages of the multiplexing strategy are extensive validation work, increased costs for the probes, expected reduction of the analytical sensitivity for some assays, and substantial re-validation procedure after adaption of single primers or probes. On the other hand, reduced costs for the master mix and increased number of simultaneous tests in one real-time PCR cycler are pros for the multiplexing strategy.

In our study, a low-density RT-qPCR array, called “BlueTYPE”, which can run 29 TaqMan-real-time RT-PCR assays in parallel for identification of all 24 classical BTV serotypes, was developed. A similar approach called “RITA” (Riems Influenza Typing Array) was successfully validated in our lab for subtyping of avian and mammalian influenza viruses (Hoffmann et al., 2016). For BlueTYPE, the published and the newly developed serotype- or group-specific RT-PCR assays were selected and combined with an extraction control system (Toussaint et al., 2007). Beside these FAM-labelled PCR assays, a second heterologous internal process control system was integrated and co-detected in the HEX channel (Hoffmann et al., 2006). The development and evaluation of this double-controlled BlueTYPE low-density RT-qPCR array for molecular serotyping of BTV positive samples in less than two hours, will be presented.

2. Material and methods

2.1. Primers and probes

For each of the 24 classical BTV serotypes, the respective serotype-specific RT-PCR assays published previously by TPI were selected (Maan et al., 2016). Furthermore, these assays were combined with published serotype-specific RT-PCR assays for serotypes 1, 6, and 8 (Hoffmann et al., 2009), 14 and 15 (Eschbaumer et al., 2011), as well as FLI-in-house assays for serotypes 4, 5, and the newly designed assays for serotypes 23 and 24. The Pan-BTV-S10 assay targeting seg-10 (Hofmann et al., 2008) served as the positive control assay for group-specific confirmation of BTV positive samples. Additionally, a novel seg-1-based Pan-BTV-Classic-S1-RT-qPCR assay was developed, which specifically detects the classical BTV serotypes 1–24 and does not react with the atypical BTV serotypes of small ruminants. All newly designed primers and probes were selected by the in silico analysis of published sequence data using the bioinformatics software Geneious vR8 (Auckland, New Zealand). For the control of nucleic acid extraction, a published RT-qPCR assay, which amplifies a fragment of the beta-actin mRNA, was integrated in the BlueTYPE array (Toussaint et al., 2007). All selected RT-qPCR assays were labelled with 6-carboxyfluorescein (FAM) at the 5′ end and a black hole quencher 1 (BHQ-1) at the 3′ end. For in-process control of all individual wells of the BlueTYPE array, a heterologous internal control system was used. The EGFP Mix 1 with a HEX-BHQ1 labelled probe was added to each array well and used for co-amplification of a heterologous in vitro RNA (IC2-RNA) (Hoffmann et al., 2006). Oligo’s were synthesised by Metabion GmbH (Planegg, Germany) and stored at −20 °C until use. All primers and probes in BlueTYPE are shown in Table 1. The concentration of EGFP-Mix 1 was for the forward primer: 5 µM, for the reverse primer: 5 µM, and for the probe: 5 µM, and the concentration in all other primer-probe mixtures was; for the forward primer: 20 µM; for the reverse primer: 20 µM, and for the probe: 5 µM.

2.2. Viruses and RNA

The BTV reference serotypes 1–24 were obtained from the EURL for BT at TPI (UK). Viral RNA of the reference strains was extracted using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany). For some BTV serotypes both the major eastern and western topotype RNAs were available. Therefore, for each of the eastern serotypes 1, 2, 4, and 9 and the western serotype 16, a 560bp long GeneArt™ Strings™ DNA Fragment was synthesised (ThermoFischer, Darmstadt, Germany) using sequence data from representative BTV isolates form Australia, India, and Nigeria. The DNA fragments consisted of the BTV Seg-2 nucleotide sequence parts, targeted by the respective serotype-specific RT-qPCR assay (Accession numbers: MF384473.1; AJ585152.1; KY947343.1; JF443167.1; AJ585150.1). The GeneArt™ Strings™ DNA Fragment including a T7 promotor side was used for the generation of the respective in vitro RNA (T7 Riboprobe® in vitro Transcription system of Promega Corporation, Madison, USA). The transcripts were purified using Trizol and the RNeasy Mini Kit (Qiagen, Hilden, Germany). For serotype 25, a full-length in vitro transcript of Seg-2 of TOV from Switzerland was available (kindly provided by IVI Mittelhäusern, Switzerland). For serotypes 26, 27, and 28, virus isolates were available, kindly provided by TPI (UK), ANSES (France) and the Kimron Veterinary Institute (Israel). The three atypical Mongolian BTV strains (so-called Mongolia 1, 2, and 3) were isolated on BSR cells in our lab. The viral RNAs of all virus isolates were extracted manually using the

Table 1
Primers and probes used in the BlueTYPE array.

Location in array (well)	Assay Name and product size (bp)	Oligo names	Oligo Sequence (5'-3')	Source
	Controls			
1	Beta Actin Mix FAM (131)	ACT-1005-F ACT-1135-R ACT-1081-FAM	CAGCACAATGAAGATCAAGATCATC CGGACTCATCGTACTCCTGCTT FAM-TCGCTGTCCACCTTCCAGCAGATGT-BHQ1	Toussaint et al. (2007)
2	Pan-BTV-S10-RT-qPCR FAM	BTV_IVI_F BTV_IVI_R BTV_IVI_FAM	TGGAYAAAGCRATGTCAAA ACRTCATCACGAAACGCTTC FAM-ARGCTGCATTCGCATCGTACGC-BHQ1	OIE terrestrial Manual (2018)
3	Pan-BTV-Classic-S1 FAM (111)	cBTV-S1 – 17F cBTV-S1 – 127R cBTV-S1 – 35FAM	TCGCAATCACCGTGCAAGGT GAAAACTTATATATRTAACATGCYCC FAM-CACAGCTYATCAARCGAGTGGTYGA-BHQ1	New designed
1 – 29	EGFP Mix 1 limit (5) HEX (132)	EGFP-1-F EGFP-2-R EGFP-Probe 1 HEX	GACCACTACCAGCAGAACAC GAACTCCAGCAGGACCATG HEX-AGCACCCAGTCCGCCCTGAGCA-BHQ1	Hoffmann et al. (2006)
	BTV 1			
4	BTV1-VP2-Mix2-FAM (120)	BTV1-VP2 – 2407F BTV1-VP2 – 2526R BTV1-VP2 – 2438FAM	CCTCAAAGGCGATTCGATTTAGC TCACGACGTTGTAGTTGACTCC FAM-TGAAGCGCAGCCCAAGATTGCACG-BHQ1	Hoffmann et al. (2009)
5	BTV-1e-add-Maan-FAM (122)	BTV-1/1590eF BTV-1/1711eR BTV-1/1630eFAM	ATGTTTAAYGCYAARTTRCGAATYAA GTTARCCTCTGCAAYACAATAGG FAM-CASAACGACRGAATGAYGAYCCRATGGTGAAAC-BHQ1	Maan et al. (2016)
5	BTV-1w-add-Maan-FAM – 79	BTV-1/2575wF BTV-1/2653wR BTV-1/2604wFAM	GTATTTCTGAYGGTATYGTYTGG TCATCAGAYACCTCGATCGCY FAM-CCGATCACACATCCGAACAAATGC-BHQ1	Maan et al. (2016)
	BTV 2			
6	BTV-2w-add-Maan-FAM (128)	BTV-2/1401-wF BTV-2/1528wR BTV-2/1455wFAMas	GATGAYRYAARTAYTCTGAG GYATCYYTTTCGAARTCRATTGTRAG FAM-CATTCCATCCACCATCTATAATTTCCCC-BHQ1	Maan et al. (2016)
6	BTV-2e-add-Maan-FAM (111)	BTV-2/60eF BTV-2/170eR BTV-2/116eFAM	GAGCATTTGTTGAAARGTTATG GATATCRAAYGCGTACATYTCTG FAM-CCAAGATGGCCGACATGACGTATC-BHQ1	Maan et al. (2016)
	BTV 3			
7	BTV-3w-Maan-FAM (100)	BTV-3/619wF BTV-3/718wR BTV-3/656wFAM	GARCGGTTRTCRACGGAWGARG TATCRTAAGCGTTATCTCCTARCYG FAM-CYCCRCAGTTTCAYACAATACAGAGGAACCATC-BHQ1	Maan et al. (2016)
	BTV 4			
8	BTV-4w-add-Maan-FAM (88)	BTV-4/S2/2470 – 2488F2 BTV-4/S2/2557 – 2532R2 BTV-4/S2/2502 – 2529P2	GAACACGAAGATATCGCAG GCATARAGAAGCTARATGTATCTTCA FAM-TACCTGTTGTGACRTCCAAGTTGGACAC-BHQ1	Maan et al. (2016)
8	BTV-4e-add-Maan-FAM (126)	BTV-4/1379eF BTV-4/1504eR BTV-4/1454eFAMas	TTGTGTAAAGTGGATGAGGAGA GAAGTCTATCGTCAAAAGGTTAGGGGCT FAMas-CCGCTCTTGATCCCACCCACCTTGA-BHQ1	Maan et al. (2016)
9	BTV4-Assay 2 (104)	BTV4 – 2226-F BTV4 – 2329-R BTV4 – 2263-FAM	TTTCTGCCGTTAYTATTTCTTRTTCA TCCAACYTCAACCGGWATCAA FAM-CTTATTGGCATAGGCARTGGTCRGT-BHQ1	FLI-in house
	BTV 5			
10	BTV-5w-Maan-FAM (94)	BTV-5/08wF BTV-5/101wR BTV-5/36wFAM	GCTTCTCAGGATGGATGAG CARRTCRAYCTTAAYRTCRTAYC FAM-CCGATWTTKCGRTCGAGCCAAGTTCC-BHQ1	Maan et al. (2016)
10	BTV5-Mix1-FAM (113)	BTV5-VP2 – 1F BTV5-VP2 – 1R BTV5-VP2 – 1FAM	AGAAGGGCAAGGGTTGACATG GATCCATCTCGCTACGTATATCGG FAM-CCTGGGTATCCGCTTTTCCGCGCA-BHQ1	FLI-in house
	BTV 6			
11	BTV-6w-Maan-FAM (112)	BTV-6/2001wF BTV-6/2112wR BTV-6/2086wFAMas	GTCGATGTYACACAGTTGATCGT TAGCACGTCTAATCGTTTCTATG FAMas-CACCTTGAYTCATCCACACTACGAAC-BHQ1	Maan et al. (2016)
11	BTV6-VP2-Mix2-FAM (89)	BTV6-VP2 – 1056F BTV6-VP2 – 1144R BTV6-VP2 – 1081FAM	TATAATGGCAGAATATGGTGGAC CAGTAAACATCGCCCAACCT FAM-ATCCGTACCCTTGCTTGCGTGGAG-BHQ1	Hoffmann et al. (2009)
	BTV 7			
12	BTV-7w-Maan-FAM (97)	BTV-7/1608wF BTV-7/1704wR BTV-7/1635wFAM	AGTATGTGAGACGTCAATCTCAGA GTCTAATAGGTCCGCAGCTTTAG FAM-CCACAATCTAGACCCGGCAATATCGC-BHQ1	Maan et al. (2016)
	BTV 8			
13	BTV-8w-Maan-FAM (88)	BTV-8/72wF BTV-8/159wR BTV-8/132wFAMas	GATGGRTATGATTACATCATTG GAATTYCTGTYACATCGTGTCG FAM-CGGGCTCATCACCTTCCTCTTCAACAC-BHQ1	Maan et al. (2016)
13	BTV8-VP2-Mix1-FAM (86)	BTV8-VP2 – 1604F BTV8-VP2 – 1689R BTV8-VP2 – 1631FAM	GTTACGCATTACCGAGGTTGTG GATCATGTGTGAACGCCTTCG FAM-AACGGCTCACACCGACGATCCAGC-BHQ1	Hoffmann et al. (2009)
	BTV 9			
14	BTV-9w-Maan-FAM (107)	BTV-9/1673wF BTV-9/1779wR BTV-9/1703wFAM	GGTTATGCTTCAATTACGAACG GGGTCTTATGTAGGGATGTCTGTG FAM-CTTATATGACACTCGCCCTGCCATC-BHQ1	Maan et al. (2016)

(*continued on next page*)

Table 1 (*continued*)

Location in array (well)	Assay Name and product size (bp)	Oligo names	Oligo Sequence (5'-3')	Source
14	BTV-9e-Maan-FAM (98)	BTV-9/1706eF	GTATGATACCAGGCCAGCG	Maan et al. (2016)
		BTV-9/1803eR	GTTCATTTTGAGGATCATCCA	
		BTV-9/1735eFAM	FAM-CAACCCTATCAATGAGACAACGCCAGAC-BHQ1	
	BTV 10			
15	BTV-10w-Maan-FAM (108)	BTV-10/1470wF	TATTRACWACWGAACCAAACCT	Maan et al. (2016)
		BTV-10/1577wR	GYGARTTRATCCRTTTGTCAT	
		BTV-10/1519wFAM	FAM-YCTTGGYNCGCGYTCTGAATTAGTATTYCCRCCY-BHQ1	
	BTV 11			
	BTV-11w-Maan-FAM	BTV-11/1510wF	GGATGCGYAYYTGAATATTAG	Maan et al. (2016)
16	(108)	BTV-11/1617wR	ATCTCTCCATGAGTTATTCGCA	
		BTV-11/1540wFAM	FAM-YGTGCTCCCAAGTTATTTCGATCAATGGATCTAC-BHQ1	
	BTV 12			
17	BTV-12w-add-Maan-FAM (138)	BTV-12/999wF	ATACAATYCAGGCYATCMRGA	Maan et al. (2016)
		BTV-12/1136wR	CAATGATYGTTCCTCGTAAGC	
		BTV-12/1101wFAMas	FAM-CTCCACCATATGCGCCARCGATAGC-BHQ1	
	BTV 13			
18	BTV-13w-Maan-FAM (79)	BTV-13/1147wF	GGTGACGTYTATTATAAATTGCG	Maan et al. (2016)
		BTV-13/1225wR	GGCGATCCARATCYCGWGG	
		BTV-13/1206wFAMas	FAM-CTTATATCCCTCACGTACGCTCCAYTCATACC-BHQ1	
	BTV 14			
19	BTV-14w-Maan-FAM (143)	BTV-14/2616wF	GCCATTGARTTTTCTGAYGAYAG	Maan et al. (2016)
		BTV-14/2758wR	TCWGTATAYGCCTTAACYGCTCT	
		BTV-14/2663wFAM	FAM-CCGGCTTCGCGCGAGRTTYCC-BHQ1	
19	BTV14-Mix6-FAM (90)	BTV14 – 2640F	GTYGATGCAGCYCACAGGTG	FLI-in house
		BTV14 – 2729R	AGCTATYGAAAGAATCACTATTCCT	
		BTV14 – 2662-FAM	FAM-ACCGGCTTCGCGCGAGATTYCCC-BHQ1	
	BTV 15			
20	BTV-15w-Maan-FAM (149)	BTV-15/29wF	CCTGTGAGCGTGATCGAAC	Maan et al. (2016)
		BTV-15/177wR	CTTACACCTATGTTTCGCACTC	
		BTV-15/130wFAMas	FAM-CCCTCCCGATAAAGCGACCATATTCC-BHQ1	
20	BTV15-Mix1-FAM (108)	BTV15-VP2 – 2645 F	TGTGAGACGGCGCTTCGTAGA	FLI-in house
		BTV15-VP2 – 2752R	ACCGCGCTTCCAAACTTGCT	
		BTV15-VP2 – 2728FAMas	FAM-CTCACCCGGCCTCCCAACCTGG-BHQ1	
	BTV 16			
21	BTV-16w-Maan-FAM (117)	BTV-16/1221wF	GCGAGAGCAAGAGAAGTATATCG	Maan et al. (2016)
		BTV-16/1337wR	GATGTTCGATACGTCTGGG	
		BTV-16/1291wFAMas	FAM-CCTTCGTTGCTGGCTCTCCCTCTAGATC-BHQ1	
21	BTV-16e-Maan-FAM (128)	BTV-16/1193eF	GACCTGAATATAAACCGCGAG	Maan et al. (2016)
		BTV-16/1320eR	ATTAATCAATTCGTACTCCCAGTG	
		BTV-16/1291eFAMas	FAM-CCTTCGTTGCRGGCTCTCCTTCTAAGTC-BHQ1	
	BTV 17			
22	BTV-17w-Maan-FAM (138)	BTV-17/2178wF	TGCTRAAAGAGATCAAATTTGTRCGG	Maan et al. (2016)
		BTV-17/2315wR	ACTTGATCGTATCGTCAAACA	
		BTV-17/2224wFAM	FAM-CCTCCCTCTGATGTTCCTTGTTCATGATAAC-BHQ1	
	BTV 18			
	BTV-18w-Maan-FAM	BTV-18/357wF	GATTATCAACCACTTAAGGTCGACG	Maan et al. (2016)
23	(95)	BTV-18/451wR	GCTCTCTTTGCGTGTAACCTTACCGTG	
		BTV-18/387wFAM	FAM-CATGTACCATCACGGATAAGCCACGCCC-BHQ1	
	BTV 19			
24	BTV-19w-Maan-FAM (98)	BTV-19/2313wF	AGTGTTGRTATCRCATAAATTACG	Maan et al. (2016)
		BTV-19/2410wR	GGAAAGTYAGATGCGAAATYARRGAAGTCAAT	
		BTV-19/2378wFAMas	FAM-CCAAACCTATTATARTACGCACCRAGCTCAACC-BHQ1	
	BTV 20			
25	BTV-20w-Maan-FAM (91)	BTV-20/1838wF	GCAATATGTCCGCATGCTG	Maan et al. (2016)
		BTV-20/1928wR	GCTCCGGGCTTAATTTTTCG	
		BTV-20/1876wFAM	FAM-CCGTAAAACCGCTTTGATGCTGATGGC-BHQ1	
	BTV 21			
26	BTV-21e-Maan-FAM (76)	BTV-21/2562eF	ACTGAGGGATTAGTTTGG	Maan et al. (2016)
		BTV-21/2637eR	GRTCATCRCAAATTTCAATSG	
		BTV-21/2582eFAM	FAM-CCTCCCAATAACGCATCCGC-BHQ1	
	BTV 22			
27	BTV-22w-Maan-FAM (112)	BTV-22/1013wF	ATCTCAAGCGGTCAAACAGA	Maan et al. (2016)
		BTV-22/1124wR	CCATTTCACAYGCTATTATAGTTCC	
		BTV-22/1101wFAMas	FAM-CTCCACCAGATACGCCACCGATAAC-BHQ1	
	BTV 23			
28	BTV-23e-Maan-FAM (89)	BTV-23/60eF	GCGGARYTGTTAGATGGCTATG	Maan et al. (2016)
		BTV-23/148eR	GGAATTTGWGYRACRTCATGACG	
		BTV-23/92eFAM	FAM-CGAYGTAAGCACACGYATCGATGAACC-BHQ1	
28	BTV23-Mix 2 FAM (110)	BTV23-S2 – 2531F	CTTGCTATATGAAACACACTTAG	New designed
		BTV23-S2 – 2640R	CCACYTCRAAGGCAACTAAAC	
		BTV23-S2 2589FAM	FAM-TGGTATGTTCCTATAACGCACCCGTC-BHQ1	
	BTV 24			

(*continued on next page*)

Table 1 (*continued*)

Location in array (well)	Assay Name and product size (bp)	Oligo names	Oligo Sequence (5'-3')	Source
29	BTV-24w-Maan-FAM (116)	BTV-24/1901wF BTV-24/2016wR BTV-24/1944wFAM	GAACTAYGAGAAGCTTAYR GCGAAAARTCYYTCATATCTA FAM-CATCAGACTTACAYGCACCCGAARATAAAY-BHQ1	Maan et al. (2016)
29	BTV-24-Mix 8 FAM (142)	BTV24-S2−1027F BTV24-S2−1168R BTV24-S2−1074FAM	GATTCTACGCTTTAWTCGCRAT ACCTGACGCARYGTRAAATAC FAM-AGGGTGTGGAGGATGAATCCGTATC-BHQ1	New designed

Table 2
The newly developed PanClassic BTV assay was compared to the Pan-BTV-S10-RT-qPCR by using the "Classical Panel" and the "Atypical Panel". All classical serotypes were recognised by the PanClassic-BTV-assay with a mean deviation of 0.5 Ct-values compared to the Pan-BTV-S10-RT-qPCR. The cross reactivity with the only atypical BTV strain (Mongolia 3) deviated from the Pan-BTV-S10-RT-qPCR of 14.1 Ct-values.

	Pan-BTV-S10-RT-qPCR	Pan Classic-BTV-assay	|△ ct|
BTV 1	21.0	22.7	1.7
BTV 2	19.4	19.8	0.4
BTV 3	19.8	20.6	0.8
BTV 4	20.2	19.6	0.6
BTV 5	20.0	20.0	0
BTV 6	20.8	20.6	0.2
BTV 7	20.9	21.8	0.9
BTV 8	19.8	19.4	0.4
BTV 9	21.0	22.0	1.0
BTV 10	21.0	21.5	0.5
BTV 11	21.1	21.8	0.7
BTV 12	22.5	22.8	0.3
BTV 13	21.0	21.4	0.4
BTV 14	21.1	20.2	0.9
BTV 15	21.4	21.9	0.5
BTV 16	21.1	22.1	1.0
BTV 17	21.7	22.5	0.8
BTV 18	21.1	21.0	0.1
BTV 19	20.7	20.9	0.2
BTV 20	21.8	23.0	1.2
BTV 21	22.1	24.2	2.1
BTV 22	22.0	22.1	0.1
BTV 23	22.6	22.1	0.5
BTV 24	20.6	21.0	0.4
BTV 25	21.2	–	–
BTV 26	22.3	–	–
BTV 27	20.6	–	–
BTV 28	20.7	–	–
Mongolia 1	20.7	–	–
Mongolia 2	21.2	–	–
Mongolia 3	21.4	35.5	14.1

QIAamp Viral RNA mini Kit (Qiagen, Hilden, Germany). Finally, for each RNA of the BTV serotypes, 10-fold dilution series in BTV-negative cattle-RNA were produced for the evaluation of the BlueTYPE assays. Additionally, we created two RNA Panels ('Classical Panel' with serotypes 1–24 and the 'Atypical Panel' with BTV serotypes 25-Mongolia 3) with Ct- values of approximately 20 in the Pan-BTV-S10-RT-qPCR. These panels were used for further evaluation of the novel Pan-BTV-Classic-S1-RT-qPCR assay.

2.3. RT-qPCR master mix and amplification temperature profile

For evaluation of the individual RT-qPCR assays in the single-plex format, 2.25 µl of RNase-free water, 6.25 µl of 2x RT-PCR buffer, 0.5 µl of RT-PCR Enzyme Mix and 1 µl of BTV specific primer-probe-mix were combined. After heat denaturation of 2.5 µl viral RNA at 95 °C for 5 min, the template RNA was cooled in liquid nitrogen or ice-water immediately and afterwards the 10 µL master mix was added.

The composition of the BlueTYPE RT-qPCR reactions with the different FAM- and HEX-labelled assay(s) per well was 1.0 µl (0 µl, if two serotype-specific assays were combined in one well) of RNase-free water, 6.25 µl of 2x RT-PCR buffer, 0.5 µl of RT-PCR Enzyme Mix, 1 µl (1 µl + 1 µl, if two serotype-specific assays were combined in one well) of primer-probe-mix-FAM, 1 µl of EGFP-mix1-HEX, and 0.25 µl of internal control template (IC2-RNA).

All RT-PCRs were run on the CFX 96 real-time PCR cycler (Bio-Rad, Hercules, CA, USA) with the AgPath-ID™ One-Step RT-PCR Reagents of Applied Biosystems™ (Waltham, USA). The temperature profile used for BlueTYPE and all individual RT-qPCR runs was 10 min at 45 °C (reverse transcription), 10 min at 95 °C (inactivation of the reverse transcriptase/activation Taq polymerase) followed by 42 cycles of 15 s at 95 °C (denaturation), 20 s at 56 °C (annealing), and 30 sat 72 °C (elongation). Fluorescence values (FAM, HEX) were collected during the annealing step.

3. Results

3.1. Evaluation of the BTV group and serotype specific RT-qPCR assays

All 10-fold-RNA and in vitro transcript dilution series were first tested in the Pan-BTV-S10-RT-qPCR (Hofmann et al., 2008), and with the respective serotype-specific assays selected for the BlueTYPE array. The Pan-BTV-S10-RT-qPCR and all selected individual serotype-specific assays recognised the respective 10-fold dilution series. The serotypes 1–22 did not deviate from the Pan-BTV-S10-RT-qPCR results by more than 2.5 Ct-values on average. For BTV-23 and BTV -24 the deviation amounted to 5.7 and 3.2 Ct-values on average. Therefore, new serotype-specific assays for serotypes 23 and 24 were developed and combined with the published assays for BTV-23 and BTV-24 (supplementary material, S1). The combination of the published and the newly designed serotype-specific assays for serotypes 23 and 24 in duplex format deviated by 0.3 and 0.5 Ct-values on average from the Pan-BTV-S10-RT-qPCR result. The combination with the universal internal control system (heterologous IC2-RNA and EGFP-Mix1 (Hoffmann et al., 2006) was tested using the Pan-BTV-S10-RT-qPCR and all serotype-specific assays or assay combinations selected for BlueTYPE. Thereby, a HEX-fluorescence signal of the EGFP-Mix1 based on the successful amplification of the internal control IC2-RNA was obtained in each RT-qPCR. This internal control process confirms the addition of the master mix and the inhibition-free amplification in each well.

The newly developed Pan-BTV-Classic-S1-RT-qPCR assay was also combined with the universal internal control system IC2-RNA/EGFP-Mix1 assay, and the performance was evaluated in comparison to the Pan-BTV-S1-RT-qPCR and the serotype-specific assays. The results were compared to the Pan-BTV-S10-RT-qPCR (Table 2). Furthermore, inclusivity and exclusivity were analysed using both panels based on classical and atypical BTV serotypes.

The Pan-BTV-Classic-S1-RT-qPCR assay recognised all classical BTV serotypes of the "Classical Panel" with a mean deviation of 0.5 Ct-values from the Pan-BTV-S10-RT-qPCR result. No serotype of the "Atypical Panel" was recognised by the Pan-BTV-Classic-S1-RT-qPCR assay except for the Mongolian strain 3. The deviation compared to the Pan-BTV-S10-RT-qPCR was 14.1 Ct-values for this Mongolian strain 3. This slight cross reactivity of less than factor 10^4 can be neglected.

For the design of the BlueTYPE array, the different serotype-specific assays were combined. For serotypes 3, 7, 10, 11, 12, 13, 17, 18, 19, 20, 21, and 22 only one serotype-specific assay was chosen based on the published assays from Maan et al. (2016). For serotypes 1, 2, 4, 5, 6, 8, 9, 14, 15, 16, 23, and 24 two serotype-specific assays were combined in one well. For serotypes 1 and 4, three serotype-specific assays were selected by published in-house and the newly developed assays and applied to two different wells. The selected and combined FAM-labelled serotype specific assays were tested together with the HEX-labelled universal IC2/EGFP-Mix1 internal control system in a duplex format, and compared to the respective results of the Pan-BTV assays and the serotype-specific assays without co-amplification of the internal control.

In conclusion, all selected FAM-labelled assays could be combined with the heterologous universal internal control system without any substantial loss of sensitivity and specificity.

3.2. BlueTYPE array

3.2.1. Plate layout and preparation

The BlueTYPE array in its current layout consists of 29 wells for each sample. In detail, well 1 was used for the beta-actin extraction control assay (Toussaint et al., 2007), well 2 for the Pan-BTV-S10-RT-qPCR (Hofmann et al., 2008), well 3 for the Pan-BTV-Classic-S1-RT-qPCR (this study), and wells 4–29 for the serotype-specific assays of the 24 classical BTV serotypes. For serotypes 1 and 4, two wells were in use for the serotyping assays. It is possible to run three samples in parallel on a 96-well PCR plate (layout shown in Fig. 1).

The setup of BlueTYPE with one (or two) selected target assay(s) per well started with prefilling of the 96-well PCR plates with the respective primer and probes per well. The preparation of PCR plates depends on the real-time PCR machine used for this diagnostic approach. In the respective wells of the PCR plate, 1.0 µl (1 µl + 1 µl in case of two target assays) of each FAM-labelled primer-probe-mix, 1.0 µl of the HEX-labelled EGFP Mix1, and 1.0 µl (0 µl, if two target assays were used) of RNase-free water were mixed. For simultaneous preparation of several BlueTYPE PCR plates, large scale mixing of primer-probes in a 96 deep-well master plate is recommended. Using an 8-channel multi-pipette and transfer of 3 µl primer-probe-mixes in the respective wells, the BlueTYPE PCR plates can be pre-filled very quickly. Pre-prepared plates can be stored at −20 °C for several months until usage without a substantial loss of sensitivity (data not shown).

3.2.2. BlueTYPE run setup

After thawing of the pre-filled BlueTYPE array plate, 75 µl of the extracted viral RNA (mostly from EDTA blood samples) were mixed with 7.5 µl internal control IC2-RNA (Hoffmann et al., 2006) and 2.5 µl of this template/IC2-RNA were filled into each well of the BlueTYPE array plate. The plate was heat denatured for 5 min at 95 °C followed by the immediate cooling step in liquid nitrogen or ice water. Afterwards, 7 µl of the master-mix consisting of 6.25 µl 2x RT-PCR buffer, 0.5 µl of RT-PCR Enzyme Mix, and 0.25 µl of RNase-free water was added into each well prior to starting the amplification according to the temperature profile described above. The final results of serotyping were obtained in less than two hours starting with the BTV suspicious specimen (procedure shown in Fig. 2).

3.2.3. Controls

To evaluate a BlueTYPE array run as valid, several criteria had to be fulfilled. First, positive HEX fluorescence signals are required for each well and the respective Ct-values should be very consistent. The common location of the primer and probes for the internal control and the target system in the prefilled PCR plate, was combined with the mixed RNA template (BTV target RNA and IC2-control RNA) and amplified with the pipetted master mix in each well; a full internal process control was ensured. In cases of missing HEX fluorescence signals in one or more wells, the respective target result has to be considered as invalid and should be repeated.

Successful sample extraction prior to the PCR array run is reflected by the positive FAM fluorescence signal of the beta-actin well. Subsequently, if the beta actin signal is missing and no BTV specific assay reacted as positive, the sample extraction and the BlueTYPE run must be repeated.

Another important control element of the BlueTYPE array is the Pan-BTV-S10-RT-qPCR (Hofmann et al., 2008). This assay detects all classical and atypical BTV and will be used for confirmation and verification of a BTV positive sample in general. Thus, this assay serves as a "positive control system".

The Pan-BTV-Classic-S1-RT-qPCR detects the presence of classical serotype(s) of BTV in the sample. Consequently, a negative result in this assay in context with a positive result in the Pan-BTV-S10-RT-qPCR will deliver first indications for the presence of atypical BTV in the analysed sample.

All serotype-specific assay combinations were run in parallel in separate wells. If one or more of the 26 wells representing the classical BTV serotypes give a FAM fluorescence signal, the sample can be evaluated as positive for these respective serotype(s).

It should be noted, that the typically used standard controls like 'no template control' (NTC) and 'positive control' (PC) are not necessary to run the BlueTYPE array. Based on the other internal process control

BlueTYPE 1				BlueTYPE 2				BlueTYPE 3			
ß-Actin	BTV 4	BTV 12	BTV 20	ß-Actin	BTV 4	BTV 12	BTV 20	ß-Actin	BTV 4	BTV 12	BTV 20
Pan NS3 IVI	BTV 5	BTV 13	BTV 21	Pan NS3 IVI	BTV 5	BTV 13	BTV 21	Pan NS3 IVI	BTV 5	BTV 13	BTV 21
Pan Classic	BTV 6	BTV 14	BTV 22	Pan Classic	BTV 6	BTV 14	BTV 22	Pan Classic	BTV 6	BTV 14	BTV 22
BTV 1	BTV 7	BTV 15	BTV 23	BTV 1	BTV 7	BTV 15	BTV 23	BTV 1	BTV 7	BTV 15	BTV 23
BTV 1	BTV 8	BTV 16	BTV 24	BTV 1	BTV 8	BTV 16	BTV 24	BTV 1	BTV 8	BTV 16	BTV 24
BTV 2	BTV 9	BTV 17		BTV 2	BTV 9	BTV 17		BTV 2	BTV 9	BTV 17	
BTV 3	BTV 10	BTV 18		BTV 3	BTV 10	BTV 18		BTV 3	BTV 10	BTV 18	
BTV 4	BTV 11	BTV 19		BTV 4	BTV 11	BTV 19		BTV 4	BTV 11	BTV 19	

Fig. 1. Current layout of a BlueTYPE array plate with 29 wells in use. Plates are pre-filled with 1.0 µL (2 µL) of the serotype-specific assay (s), 1.0 µL of EGFP-Mix1 and 1.0 µL (0 µL) of H2O. The pre-filled plates are storable at −20 °C for 9 months without the loss of sensitivity. Three sample runs in parallel are possible. The three empty wells (here in grey) can be used for integration of the respective real-time RT-PCR assays of relevant differential diagnosis like FMDV or PPRV.

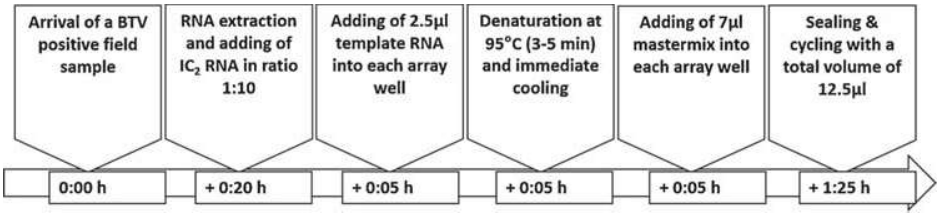

Fig. 2. BlueTYPE run procedure presented on a timeline.

systems described above, the NTC and PC must not be used in the routine diagnostic application of the BlueTYPE array. Nevertheless, RNase free water as NTC identifies contaminations in the used primer-probe-mixes and should be run after preparation of a new batch of the prefilled array plates. For evaluation of the functionality of all used RT-qPCR assays in the low-density array testing, a PC containing BTV RNA of all serotypes is necessary. Testing of this multi-serotype PC is required after a new batch production of BlueTYPE, as well as when the stored pre-filled array plates have not been in use for several months. Combined testing of NTC and multi-serotype PC in one array run is possible. In addition, the Ct-values of this regularly used multi-serotype PC can be used for the evaluation of test robustness over months and years.

3.2.4. Diagnostic performance

All selected serotype-specific real-time RT-PCR combinations in the BlueTYPE array were tested with the reference RNA of all 24 BTV-serotypes as template. All serotype-specific assay combinations recognised the respective homologous target RNA. A slight cross reactivity was observed for serotypes −3, −6, and −16 only. Thereby, the Ct-value difference between homologous target serotype and heterologous serotype(s) was in all cases > 14 Ct-values (shown in Table 3). The combination of two serotypes of BTV-3, -6 and -16 in array runs revealed the same cross-reactivities as with the single serotype template (data not shown). Besides, the specificity of BlueTYPE was tested with a mixed template consisting the nucleic acid of peste des petites ruminants' virus (PPRV), parapox virus, lumpy skin disease virus (LSDV), foot and mouth disease virus (FMD), sheep pox virus (SPPV), bovine parainfluenza virus 3, ovine herpesvirus 2 and bovine herpesvirus 1. The BlueTYPE run was negative for all wells except for the beta-actin well (data not shown).

Furthermore, the BlueTYPE array was tested in the routine diagnostic work of the NRL for Bluetongue in Germany. Pan-BTV-positive field samples from the federal states Saarland, Rhineland-Palatinate, and Baden-Württemberg were screened with BlueTYPE. With this method, the first BTV-8 serotype outbreaks of 2018/19 in Germany were confirmed and infections with multiple other serotypes could be excluded (Fig. 3). Furthermore, the BlueTYPE array was successfully used for analysing the EURL proficiency test for BTV of 2018. Here, a sample with a mixture of BTV-1 (MOR2007/01) and BTV-4 positive blood (MOR2009/07) diluted in negative ovine was successfully identified in the BlueTYPE array. In addition, the BlueTYPE array was used for free testing of the monospecific BTV serotype 1–24 virus stocks propagated in cell culture. The identification of contaminating BTV strains and the definition of clean, mono-serotype-specific virus stocks is essential for the generation of serotype-specific sera and the qualified establishment of serotype- specific neutralisation assays, the standard method for serotyping. In some cell culture virus stocks, multiple serotypes could be identified unexpectedly (data not shown) and the BlueTYPE array delivered an easy tool for the ongoing cleaning procedure to generate monospecific stocks also for these BTV serotypes. Moreover, 5 organ and 5 blood samples of former animal experiments performed at the FLI were tested with BlueTYPE. Four spleens and one lymph node of four sheep individual infected with BTV-4, BTV-8, BTV-12 and BTV-24, were successfully analysed. In addition, the EDTA blood of sheep infected with BTV-8, BTV-12 and BTV-24 as well as the EDTA blood of a BTV-4 infected goat and a cattle were successfully identified (data not shown).

4. Discussion

Several single and multiplex real-time RT-PCR systems with specific primer and probe systems for the different BTV serotypes have been developed over the years (Harding et al., 1995; Aradaib et al., 2003; Hoffmann et al., 2009; Vandenbussche et al., 2009; Feng et al., 2015; Reddy et al., 2016), but so far there is no tool for a complete serotyping in only one PCR run. Recently, TaqMan' fluorescence-probe based serotype-specific real-time RT-PCR assays for up to, now all, 28 known BTV serotypes have been developed (Maan et al., 2016). These TaqMan assays were validated with a broad panel of BTV isolates from the Orbivirus reference collection, representing all known BTV serotypes and further Orbivirus strains from different areas of the world. No evidence for cross-amplification of RNA from heterologous serotypes by any of their type-specific assays was reported (Maan et al., 2016).

In our study, a low-density RT-qPCR array, called BlueTYPE, which can run 29 TaqMan-real-time RT-PCR assays in parallel for identification of all the 24 classical BTV serotypes, was developed. The BlueTYPE array profits from the integration of two control systems, on the one side is the extraction control (Toussaint et al., 2007) and on the other side is the process control for each separate well (Hoffmann et al., 2006).

The real-time RT-PCR of Hofmann et al. (2008) is the OIE- recommended first line assay for detection of BTV and is capable of detecting all known BTV serotypes and strains currently circulating (OIE terrestrial Manual 2018). The integration of this OIE-listed Pan-BTV-S10-RT-qPCR as positive control into the BlueTYPE array increases the reliability of the results immensely.

Furthermore, the newly developed Pan-BTV-Classic-S1-RT-qPCR assay, which targets segment 1, helps enormously in the interpretation of the results, especially for the identification and distinction of atypical BTV serotypes (≥ BTV25). The Pan-BTV-Classic-S1-RT-qPCR assay detects the presence of classical serotype(s) of BTV in the sample; consequently, the negative result in this assay in combination with a positive result in the Pan-BTV-S10-RT-qPCR will deliver first indications for atypical BTV in the sample. It has to be mentioned that the Pan-BTV-Classic-S1-RT-qPCR assay was tested experimentally with the available RNA reference strains 1–24 only, and not with the broad panel of the ORC. Nevertheless, the Pan-BTV-Classic-S1-RT-qPCR assay represents an adaptation of the well-established S1-pan-BTV real-time RT-PCR assay published by Toussaint et al. (2007) and was optimised by integrating the BTV segment 1 sequences of the BTV serotypes 1–24 published in the last years.

In the BlueTYPE array, the extensively validated TaqMan real-time RT-PCR assays from Maan et al. (2016) were combined with published and non-published in-house assays used at the German NRL for BT for selected BT serotypes. All these FLI in-house assays were developed and evaluated in context with the molecular analysis of diagnostic samples with the focus on high sensitivity. Extensive testing of the analytical specificity (inclusivity, exclusivity) could not be performed based on the limited Orbivirus sample panel. Therefore, some cross amplifications cannot be excluded, and were up to now only observed for the BTV4-VP2-Mix2 and BTV1-VP2-Mix2 with serotypes 17 and 24 during

Table 3
Cross reactivity of the selected serotype-specific assay combinations used in BlueTYPE, with the reference RNA strains as template. Cross reactivity was observed for only serotypes -3, -6, and -16.

	BTV 1 RNA	BTV 2 RNA	BTV 3 RNA	BTV 4 RNA	BTV 5 RNA	BTV 6 RNA	BTV 7 RNA	BTV 8 RNA	BTV 9 RNA	BTV 10 RNA	BTV 11 RNA	BTV 12 RNA	BTV 13 RNA	BTV 14 RNA	BTV 15 RNA	BTV 16 RNA	BTV 17 RNA	BTV 18 RNA	BTV 19 RNA	BTV 20 RNA	BTV 21 RNA	BTV 22 RNA	BTV 23 RNA	BTV 24 RNA
well1-BTV1	**31.3**	–	–	–	–	**30.6**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
well2-BTV1	**21.2**	–	–	–	–	**31.2**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 2	–	**20.0**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 3	–	–	1902	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
well1-BTV 4	–	–	–	**20.9**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
well2-BTV 4	–	–	–	**29.8**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 5	–	–	–	–	**22.6**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 6	–	–	–	–	–	**16**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 7	–	–	–	–	–	–	**24.0**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 8	–	–	–	–	–	–	–	**19.3**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 9	–	–	–	–	–	–	–	–	1498	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 10	–	–	–	–	–	–	–	–	–	**15.9**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 11	–	–	–	–	–	–	–	–	–	–	**22.2**	–	–	–	–	–	–	–	–	–	–	–	–	–
BTV 12	–	–	–	–	–	–	–	–	–	–	–	**19.2**	–	–	–	–	–	–	–	–	–	–	–	–
BTV 13	–	–	–	–	–	**31.6**	–	–	–	–	–	–	**18.3**	–	–	–	–	–	–	–	–	–	–	–
BTV 14	–	–	–	–	–	–	–	–	–	–	–	–	–	**11.2**	–	–	–	–	–	–	–	–	–	–
BTV 15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**14.4**	–	–	–	–	–	–	–	–	–
BTV 16	–	–	**35.7**	–	–	–	–	–	–	–	–	–	–	–	–	**13.3**	–	–	–	–	–	–	–	–
BTV 17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**28.4**	–	–	–	–	–	–	–
BTV 18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**17.1**	–	–	–	–	–	–
BTV 19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**13.1**	–	–	–	–	–
BTV 20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**12.72**	–	–	–	–
BTV 21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**21.3**	–	–	–
BTV 22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**14.5**	–	–
BTV 23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**24.9**	–
BTV 24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**22.4**

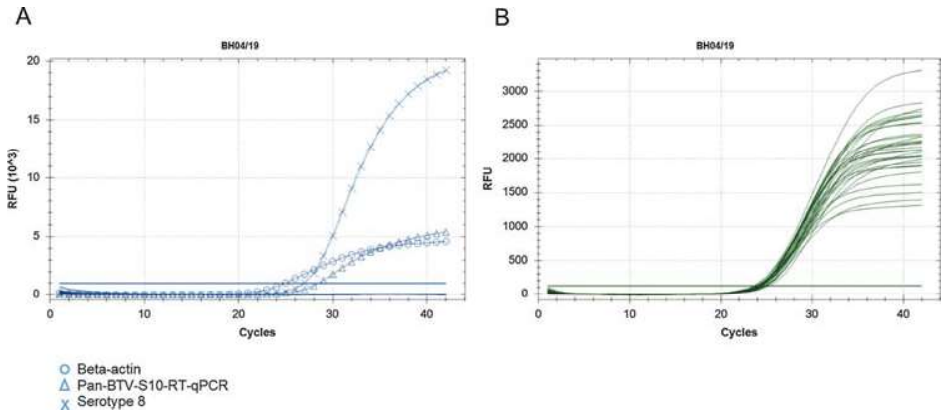

Fig. 3. Confirmation of the BTV-8 outbreak in Germany with the BlueTYPE array. A) shows the FAM amplification plots of the valid BlueTYPE run. B) shows all HEX amplification plots. BlueTYPE is only valid with HEX plots for each well.

cell culture testing. The positive BTV4-VP2-Mix2 and BTV1-VP2-Mix2 results should be considered as questionable, when in the same run the Maan et al. (2016) assays for serotypes 1 and 4 were negative, as they profit from a higher specificity. Furthermore, we could observe cross-amplification of RNA of serotypes −3, −6, and −16 as well as with the mixed templates of these serotypes. However, the differences in Ct-values between RNA amplified with the homologous assay and the heterologous assays were in all cases > 14, which means that these false-positive amplicons were usually detected with a lower sensitivity. Thus, they can easily be identified as false positive. These tendencies for the co-amplification of non-targeted RNA were also described for RITA (Hoffmann et al., 2016). The parallel application of several BTV real-time RT-PCR assays in one run offers the possibility for a substantial plausibility check and a final decision regarding the BTV serotype included in the test sample. Here, the qualitative results of the different pan-BTV and serotype-specific RT-PCR systems and the semi-quantitative data by comparing the Ct-values of positive assays can be evaluated. If the generated data does not deliver a clear result, alternative PCR methods can be used for a final decision (Eschbaumer et al., 2011). If no serotype could be identified in a valid BlueTYPE run, it is either a "so far unknown" serotype or mutations/variations of the segment 2 genome were not covered by the oligo's used for the respective serotype. In this case, alternative strategies can be used for serotype/strain identification e.g. partial or complete sequencing of the VP2 gene or the whole genome. In this context, it should be noted that the BlueTYPE array is very flexible for updates and the newly developed assays for novel strains or mutants can easily be integrated. This is an important attribute of this low-density real-time RT-PCR array format as the probability of mutations, variations, or reassortment events of BTV are high, (Maan et al., 2010; Shaw et al., 2013; Nomikou et al., 2015) and several new serotypes have been discovered recently (Hofmann et al., 2008; Maan et al., 2011; Zientara et al., 2014). In addition, the flexibility and simplicity of array design adaption allowed the integration of relevant non-BTV real-time RT-PCR assays in the array format. For countries with an endemic situation of pathogen-relevant differential diagnosis, e.g. Peste des petits ruminants virus (PPRV) or Foot-and-mouth disease virus (FMDV), the integration of the respective real-time RT-PCR assays in the BlueTYPE array can deliver further information in the case of clinical outbreaks and mixed infections. Therefore, the extension of the BlueTYPE array is of particular value e.g. in north African countries dealing with both diseases, FMDV and BTV (Kardjadj, 2018) or countries dealing with even concurrent BTV and PPRV infections in the same animal, as reported in India (Maan et al., 2018).

In conclusion, the BlueTYPE array allows the identification of single or multiple serotypes within one PCR run using a standard real-time PCR cycler. It can be a helpful tool for virus characterisation e.g. in a primary BTV outbreak and for an efficient outbreak control, especially in countries and regions with multiple circulating BTV serotypes. In addition, BlueTYPE can be used for easy "free-testing" of cell culture propagated BTV and the generation of monospecific stocks of BTV serotypes. The handling of BlueTYPE is simple by using pre-filled primer-probe PCR plates stored at −20 °C. The adaptation of single BTV assays based on novel sequence information or the extension of the panel of assays in the array for epidemiologically relevant pathogens is quickly possible and permits the flexible design of an optimised diagnostic tool.

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CRediT authorship contribution statement

Christina Ries: Validation, Formal analysis, Investigation, Methodology, Writing - original draft, Visualisation. **Martin Beer:** Conceptualization, Formal analysis, Resources, Writing - review & editing, Project administration, Funding acquisition. **Bernd Hoffmann:** Conceptualization, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2020.113881.

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viruses

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Article

Isolation and Cultivation of a New Isolate of BTV-25 and Presumptive Evidence for a Potential Persistent Infection in Healthy Goats

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Abstract: Recently, several so-called "atypical" Bluetongue virus (BTV) serotypes were discovered, including BTV-25 (Toggenburg virus), in Switzerland. Most "atypical" BTV were identified in small ruminants without clinical signs. In 2018, two goats from a holding in Germany tested positive for BTV-25 genome by RT-qPCR prior to export. After experimental inoculation of the two goats with the BTV-25 positive field blood samples for generation of reference materials, viremia could be observed in one animal. For the first time, the BTV-25-related virus was isolated in cell culture from EDTA-blood and the full genome of isolate "BTV-25-GER2018" could be generated. BTV-25-GER2018 was only incompletely neutralized by ELISA-positive sera. We could monitor the BTV-25 occurrence in the respective affected goat flock of approximately 120 goats over several years. EDTA blood samples were screened with RT-qPCR using a newly developed BTV-25 specific assay. For serological surveillance, serum samples were screened using a commercial cELISA. BTV-25-GER2018 was detected over 4.5 years in the goat flock with intermittent PCR-positivity in some animals, and with or without concomitantly detected antibodies since 2015. We could demonstrate the viral persistence of BTV-25-GER2018 in goats for up to 4.5 years, and the first BTV-25 isolate is now available for further characterization.

Keywords: Bluetongue virus; BTV; atypical BTV; serotype 25; persistent infection; goats

1. Introduction

Bluetongue virus (BTV) is a double stranded and segmented RNA virus within the family *Reoviridae*, genus *Orbivirus*, that causes bluetongue disease in ruminants [1]. The first approaches of serotyping BTV strains according to the neutralization capabilities of strain-specific sera were made in the 1960s in South Africa [2]. Since then, the virus neutralization test (VNT) has become the gold standard for serotype identification, and up to now 24 classical BTV serotypes are known (Mertens et al. 2004; OIE terrestrial manual). Nevertheless, with the rapid progress in genomics in recent decades, more and more BTV sequence data have become available, and the idea of typing BTV according to its genotype arose. In 2011, a working group suggested levels of maximum and minimum nucleotide (nt) and amino acid (aa) identities in segment-2 of the BTV genome as an alternative to the traditional typing methods [3]. A remarkably increasing number of novel serotypes have been

described since the discovery of BTV-25 (Toggenburg Virus, TOV) in 2008 [4]. This group of newly discovered BTV-strains differs in several viral characteristics, but also at the molecular level from the classical BTV serotypes 1–24. Consequently, non-classical BTV serotypes are referred to as the group of "atypical" BTVs, distinct from the classical and notifiable BTV serotypes 1–24 [5,6]. Nevertheless, the OIE recommended the Pan-BTV-segment 10 RT-qPCR [7,8] in order to detect all BTV serotypes, including the known atypical BTVs. Recently, we established the Pan-BTV-Classic-S1-RT-qPCR assay, targeting BTV segment 1 for distinction between classical and atypical serotypes [9]. The discovery of TOV was followed by the description of BTV-26 in samples from symptomatic sheep in Kuwait [3]. In addition, BTV-26 antibody circulation was discovered in cattle and dromedaries in the Islamic Republic of Mauritania [10]. Interestingly, horizontal contact transmission could be demonstrated for BTV-26 [11,12], which is in sharp contrast to the insect vector dependent transmission dynamics of classical BTV serotypes. Furthermore, three variants of BTV-27 were detected in asymptomatic goats on Corsica [13]. The two putative novel serotypes, BTV XJ1407 from China [14] and BTV-X ITL2015 from Italy [6], were serologically and molecularly characterized, but still require assignment to a new serotype. For another BTV strain—isolated from a contaminated sheep pox vaccine in Israel—full-length sequence data are available, and an experimental infection of sheep was conducted [15,16]. The most recent BTV-strain description was the Tunisian BTV-Y TUN2017 strain in sheep [17].

The initially described BTV-25 (Toggenburg Virus—TOV) was detected in two different asymptomatic goat flocks in the Toggenburg region in Switzerland [4]. Similarly, to naturally infected goats, experimentally TOV-infected goats did not develop clinical signs typical for BTV, even though they exhibited a high virus replication rate [18]. Experimentally TOV-infected sheep also presented a very mild clinical disease consisting of minor BTV characteristic symptoms [18]. Horizontal transmission of TOV seems unlikely, as contact control animals did not get infected, and all swabs as well as milk and saliva samples revealed negative results [19]. It should be also mentioned that the systemic spread of TOV in infected goats was described as being rather slow [19]. Nevertheless, the high seroprevalence rate of naturally infected goat flocks in combination with an extremely low vector activity in Switzerland provided some indication for the presence of an efficient alternative transmission route [18,19]. Furthermore, there are indicators for transplacental infection, but additional studies were suggested for confirmation [18,20]. TOV RNA could be detected for up to 25 months and the infectivity of blood during that period was demonstrated [21]. The antibody response of experimentally infected animals was described as slow and weak [19]. All attempts of cell culture-based virus isolation of TOV remained unsuccessful [19]. Thus, for the use in virus neutralization tests (VNTs), a chimeric classical BTV/TOV virus was generated by reverse genetics [20]. In 2018, another TOV-related BTV strain (BTV-Z ITA2017) was described in the Piedmont region in Italy, and it could also not be cultivated [22]. This TOV-related strain was found in healthy goats and showed a high identity with TOV, both on the nucleotide (nt) and the amino acid (aa) level. Nevertheless, the serotype remained undefined, due to the failure of cELISA-positive sera to neutralize the reference and the atypical (including chimeric strains) BTV serotypes by serum neutralization (SN) [22]. For BTV-25 detection, two specific real-time RT-qPCR systems targeting segment 2 have been developed over the years [23,24].

Concerning the BTV situation in Germany, BTV-8 played a major role and was present from 2006 to 2009. Eradication was successful with the application of an obligatory BTV-8 vaccination program [25]. In February 2012, Germany was declared officially free of BTV until the re-emergence of BTV-8 in December 2018 [26]. In our study, the novel BTV-25 related virus (BTV-25-GER2018) detected in healthy goats in the southern part of Germany is further characterized. Full genomes were generated and phylogenetically analyzed, and for the first time a BTV-25-related virus could be propagated in cell culture. For consistent virus detection, a BTV-25-specific RT-qPCR assay targeting segment 2 and adapted to the new BTV-25-GER2018 strain was developed. Furthermore, the infected goat flock was monitored over a 14-month period with a series of blood and retrospective serum samples.

2. Materials and Methods

2.1. RNA Extraction and RT-qPCR

Viral RNA of all EDTA blood samples was extracted either manually using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) or the NucleoMagVET kit (Macherey-Nagel, Düren, Germany) with the help of a half-automated KingFisher platform (King-Fisher Flex magnetic particle processor, Thermo Fisher Scientific, Waltham, MA, USA). The RNA was amplified using the Pan-BTV-S10-RT-qPCR recommended by the OIE [7] and was considered positive when quantification cycle (Cq) values were <40. For initial serotype identification, two published available RT-qPCRs targeting segment 2 were used [23,24]. For further screening, an RT-qPCR (BTV-25-Mix13 assay) was developed based on all available sequence information of BTV-25-related strains. The forward primer BTV-25-2434-F (5'-GGT TCR ATT TGT TAT CGC TAC TAT A-3') and the reverse primer BTV-25-2609-R (5'-ACA AGR CAC TTC TCT GGA TGT G-3') were used in a 20 µM concentration, whereas the probe BTV-25-2494FAM (6-FAM-CCG GTT ATC ACT ACA AAG TTG GAC AC-BHQ1) was used in a 5 µM concentration for preparation of the primer–probe mixture. For process control, a heterologous control system was implemented and co-amplified in all PCR runs using the HEX channel [27]. The final composition of the RT-qPCR reactions was 1.25 µL of RNase-free water, 6.25 µL of 2× RT-PCR buffer, 0.5 µL of RT-PCR Enzyme Mix, 1 µL of primer-probe-mix-FAM, 1 µL of EGFP-mix1-HEX and 2.5 µL of the heat denatured template RNA. All RT-qPCRs were run on the CFX 96 real-time PCR cycler (Bio-Rad, Hercules, CA, USA) with the AgPath-ID™ One-Step RT-PCR Reagents of Applied Biosystems™ (Waltham, MA, USA). The temperature profile used was 10 min at 45 °C (reverse transcription), 10 min at 95 °C (inactivation of the reverse transcriptase/activation Taq polymerase) followed by 42 cycles of 15 s at 95 °C (denaturation), 20 s at 56 °C (annealing), and 30 s at 72 °C (elongation). Fluorescence values (FAM, HEX) were collected during the annealing step. The specificity of the BTV-25-Mix13 assay was tested in silico by BLAST search (https://blast.ncbi.nlm.nih.gov) and in vitro using available viral RNAs of all 24 classical BTV serotypes and further atypical BTV serotypes (BTV-26, three variants of BTV-27 and BTV-28). At all 5 blood sampling time points, EDTA blood was analyzed with the BTV-25 specific RT-qPCR (BTV-25-Mix13 assay). Furthermore, individual EDTA blood samples were tested in the BlueTYPE array as described previously [9].

2.2. Sequence Analysis

The sequences of the ten segments of BTV-25-GER2018 were generated using the HTS-SISPA technology [28] on the Illumina platform. In the first step, the viral RNA was extracted from the BTV-25 infected cell culture material using the MasterPure Complete DNA and RNA Purification Kit (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). The cDNA first strand synthesis using the SISPA K8N random primer was performed by the qScript Flex cDNA synthesis Kit (Quanta Biosciences, Beverly, MA, USA). The heat denatured and immediately cooled down extracted RNA (10 µL) mixed with 0.5 µL of the 100 µM concentrated SISPA-K8N primer served as template. After including the 9.5 µL master mix preparation, reverse transcription was run with a total reaction volume of 20 µL using the temperature profile of 10 min 25 °C, 45 min at 42 °C, 5 min at 85 °C and cooling of 10 °C. For the second strand synthesis, the Second Strand cDNA Synthesis Kit-dNTP based (Applied Biological Materials Inc. (abm), Richmond, Canada) was used according to the supplier's instructions. Briefly, the 20 µL cDNA template was heat denatured for 3 min at 95 °C and cooled down on ice for 5 min. Together with the 30 µL mastermix preparation the total amount of 50 µL reaction mix was incubated for 2.5 h at 16 °C. The double stranded cDNA was amplified using the K primer and the Phusion High Fidelity PCR Polymerase (New England Bio labs, Ipswich, USA). Therefore, 5 µL of purified double stranded (ds) cDNA was used as template in a 50 µL total reaction mix. The temperature profile used was 30 s at 98 °C followed by 35 cycles of 10 s at 98°C, 30 s at 60 °C and 30 s at 72 °C and in the end 5 min at 72 °C before permanent cooling at 10 °C. The generated double stranded cDNA was purified before and after the Phusion PCR with the sparQ PureMag

Beads Kit (Quanta Biosciences, Beverly, MA, USA) by adding the 50 µL of double stranded cDNA to 40 µL of beads. After the procedure and according to the manufacturer's instructions, the beads were re-suspended in 35 µL of 10 mM TRIS-HCL (pH 8.0). The amplified and purified ds cDNA was sent to Eurofins Genomics (Ebersberg, Germany) for sequencing on an Illumina platform. Raw data as fastq files were trimmed and assembled by mapping to the BTV-25 TOV reference sequences with the following accession numbers: GQ982522 (Seg-1), EU839840 (Seg-2), GQ982523 (Seg-3), GQ982524 (Seg-4), EU839841 (Seg-5), EU839842 (Seg-6), EU839843 (Seg-7), EU839844 (Seg-8), EU839845 (Seg-9), EU839846 (Seg-10) using the Geneious software v2019.2.3 (Biomatters Ltd., Auckland, New Zealand). For phylogenetic analyses, a multiple alignment of BTV sequences was performed by using the MAFFT alignment feature in the Geneious software. We included the identical BTV strain selection, representing known BTV serotypes as used in the publication of BTV-X-ITL2015 [6]. Phylogenetic trees of each of the 10 segments were created with MegaX [29] using the genetic distinction model Tamura–Nei and tree-built method UPGMA and for modification of the layout of the segments 2 and 6 FigTree v1.4.4 [30]. To assess the robustness of individual nodes on the phylogenetic trees, we performed a bootstrap analysis with 1000 replications. Furthermore, the consensus sequences of each of the ten segments of BTV-25-GER2018 were blasted against the nt/aa database of the NCBI for identifying the nearest molecular neighbors. The BTV-25-GER-2018 sequences obtained in this study were submitted to NCBI with the following accession numbers: LR798441 (Seg-1), LR798442 (Seg-2), LR798443 (Seg-3), LR798444 (Seg-4), LR798445 (Seg-5), LR798446 (Seg-6), LR798447 (Seg-7), LR798448 (Seg-8), LR798449 (Seg-9) and LR798450 (Seg-10).

2.3. Goat Flock in Bavaria

Two individual goats from a holding in the southern part of Bavaria (Germany) were initially tested for the presence of BTV genomes prior to export and tested BTV RNA positive. Subsequently, this goat flock was monitored and bled five times from August 2018 to October 2019. EDTA blood and serum samples were taken from the goats present at the time point of sampling. Two German breeds ("White German Edelziege" and "Colourful German Edelziege") were present in the goat flock originating from Switzerland (n = 31), Baden-Württemberg (n = 51), Bavaria (n = 4) and their offspring (n = 52), respectively. The flock composition changed slightly over the different bleeding time points. In total, 23 goats were removed and another 20 were newly introduced over the monitoring period (July 2018—October 2019). The overview of the goat flock is given in Table 1. Furthermore, individual retrospective serum samples from February 2015, stored at −20°C, were available for investigation.

Table 1. Closest neighbors of Bluetongue virus (BTV)-25GER2018 (CDS) following BLAST analyses. The three highest identities/similarities are presented.

	BLAST Best Hits				
Segment/ Protein (Accesion No.)	**Serotype (nt/aa) #**	**Strain (nt/aa)**	**Accession No. (nt/aa)**	**Identity Level % (nt/aa)**	**Query Cover %**
1/VP1 (LR798441)	BTV-25/BTV-25	BTV-Z ITL2017/TOV	MF673720.1/ACY02806.1	96.55/92.17	88/100
	BTV-25/unknown	TOV/V196-XJ-2014	GQ982522.1/ASW41946.1	83.77/91.01	99/100
	BTV-27/BTV-27	BTV-27-FRA-2014-v02/BTV-27-FRA-2014-v01	KU760987/CEK41871.1	82.97/90.78	99/100
2/VP2 (LR798442)	BTV-25/BTV-25	BTV-Z ITL2017/BTV-Z ITL2017	MF673721.1/AVA16289.1	92.53/88.99	79/79
	BTV-25/BTV-25	TOV/TOV	EU839840.1/ACJ06702.1	83.48/82.79	100/100
	BTV-27/unknown	BTV-27-FRA-2014-v02/BTV-XJ1407	KU760988.1/AMM44543.1	74.54/75.18	100/100
3/VP3 (LR798443)	BTV-25/BTV-27	BTV-Z ITL2017/BTV-27-FRA2014-v03	MF673722.1/AMQ36829.1	96.74/96.00	63/100
	BTV-27/unknown	BTV-27-FRA-2014-v02/XJ1407	KU760989.1/AMM44545.1	85.18/95.89	100/100
	BTV-27/unknown	BTV-27-FRA-2014-v01/V196-XJ-2014	LN713672.1/ASW41948.1	85.18/95.89	100/100
4/VP4 (LR798444)	BTV-25/BTV-25	BTV-Z ITL2017/BTV-Z ITL2017	MF673723.2/AVA16291.2	96.13/99.66	90/91
	BTV-25/BTV-25	TOV/TOV	GQ982524.1/ACY02808.1	90.81/96.58	100/100
	Unknown/unknown	V196-XJ-2014/XJ1407	KX695173.1/AMM44546.1	80.88/93.79	100/100
5/NS1 (LR798445)	BTV-25/BTV-25	BTV-Z ITL2017/TOV	MF673724.1/ACJ06703.1	94.63/82.58	58/99
	BTV-25/BTV-28	TOV/ SPvvvv-02	EU839841.1/QGW56799.1	78.36/80.80	99/100
	BTV-8/BTV-28	CYP2016-04/BTV-28-1537-14	MN710167.1/QDH76488.1	75.12/80.62	99/100
6/VP5 (LR798446)	BTV-25/BTV-25	BTV-Z ITL2017/TOV	MF673725.1/ACJ06704.1	96.53/91.83	32/100
	BTV-25/BTV-27	TOV/BTV-27-FRA-2014-v01	EU839842.1/CEK41875.1	82.92/86.12	100/100
	BTV-27/BTV-28	BTV-27-FRA-2014-v01/BTV-28-1537-14	LN713675.1/QDH76491.1	78.24/82.70	100/100
7/VP7 (LR798447)	Unknown/unknown	V196-XJ-2014/V196-XJ-2014	KX695176.1/ASW41952.1	84.76/97.71	100/100
	Unknown/unknown	BTV-XJ1407/BTV-XJ1407	KR085416.1/AMM44548.1	83.90/97.42	100/100
	BTV-27/BTV-28	BTV-27-FRA-2014-v01/SPvvvv-02	LN713676.1/QGW56801.1	81.71/97.13	100/100
8/NS2 (LR798448)	BTV-25/BTV-25	TOV/TOV	EU839844.1/ACJ06706.1	98.21/98.30	100/100
	BTV-25/BTV-27	BTV-Z ITL2017/BTV-27-FRA-2014-v01	MF673726.1/CEK41877.1	97.28/86.69	58/100
	BTV-27/BTV-27	BTV-27-FRA-2014-v03/BTV-27-FRA-2014-v03	KU761004.1/AMQ36834.1	82.86/86.69	100/100
9/VP6 (LR798449)	BTV-25/BTV-25	TOV/TOV	EU839845.1/ACJ06707.1	85.35/82.37	100/100
	Unknown/unknown	BTV-XJ1407/V196-XJ-2014	KR085418.1/ASW41954.1	80.34/75.76	100/100
	Unknown/unknown	V196-XJ-2014/BTV-XJ1407	KX695178.1/AMM44550.1	79.16/75.68	100/100
10/NS3 (LR798450)	BTV-27/BTV-27	BTV-27-FRA-2014-v02/ BTV-27-FRA-2014-v02	KU760996.1/AMQ36826.1	88.84/95.63	100/100
	BTV-25/BTV-25	TOV/TOV	EU839846.1/ ACJ06708.1	87.97/94.32	100/100
	Unknown/unknown	BTV-X ITL2015/ BTV-X ITL2015	KX234087.2/ APC23697.2	82.87/93.89	99/100

(nt = nucleotide, aa = amino acide)

2.4. Experimental Inoculation of Goats

Two male, 6-month-old Thuringian goats were kept in the vector-free high containment buildings of the FLI, Isle of Riems, for diagnostic inoculation. The two goats (#19, #20) were inoculated with two EDTA blood samples from naturally BTV-25-GER2018 infected goats. Goat #19 was inoculated with the first (BH66/18_1; Cq-value = 26.1), and goat #20 with the second EDTA blood sample (BH66/18_2; Cq-value = 33.6). Then, 700 µL PBS-washed blood and 500 µL of unwashed blood were injected subcutaneously at two different injection sites. Both goats were monitored daily for clinical symptoms. EDTA blood and serum were taken regularly throughout the whole experiment. Goat #19 was kept until 31 dpi and goat #20 until 46 dpi. The respective experimental protocols were reviewed by the state ethics commission and approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany; Ref. LALLF M-V/TSD/7221.3-1-048/19 from 07.11.2019).

2.5. Production of Antisera in Rabbits

Two rabbits were immunized with binary ethyleneimine (BEI)-inactivated BTV-25 full-virus cell culture material. Binary ethylenimine (BEI) was prepared freshly by cyclization of 0.1 M 2-bromoethylamine hydrobromide in 200 mM sodium hydroxide (NaOH) solution at 37 °C for 60 min [31]. Before inactivation, the two different BTV-25 GER2018 virus preparations had a titer of 10^3 and $10^{3.75}$ $TCID_{50/mL}$. Next, 2.7 mL of virus preparation was mixed with 0.3 mL of 0.1 M BEI and transferred into a new falcon after overnight incubation at 28 °C. After another incubation for 24 h at 28 °C the reaction was stopped by adding 0.3 mL of 200 mM sodium thiosulfate solution. The antigen preparation was aliquoted and stored at −70 °C until usage. The success of the inactivation procedure was confirmed by decreasing RT-qPCR Cq values of the cell culture material during 3 serial cell culture passages. Rabbits were inoculated subcutaneously three times at two-week intervals with 1 mL BTV-25-GER2018 inactivated antigen mixed with 100 µL of Polygen as adjuvant (MVP Adjuvants, USA). The final serum was collected at 56 dpv. The respective experimental protocols were reviewed by the state ethics commission and approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany; Ref. LALLF M-V/TSD/7221.3–2-042/17).

2.6. Isolation of BTV-25-GER2018

Blood samples from naturally and experimentally infected goats were processed identically for the virus isolation experiments: 500 µL of EDTA blood was centrifuged (8000 rpm) for 2 min and the red blood cells were washed twice in 1 mL PBS and finally diluted in 500 µL PBS prior to lysis by 20 s ultrasound treatment at 30W (Sonifier 450, Branson Ultrasonics, USA). Additionally, unwashed blood of the experimentally infected goat was lysed by ultrasound treatment and used for virus isolation experiments as well. BHK-21 (BSR/5) cells (FLI cell culture collection number RIE0194) in T25 cm^2 cell flasks were incubated initially for three hours at 37 °C using the cultivation medium MEM with essential amino acids (FLI intern medium number ZB5d) supplemented with 10% FCS (fetal calf serum). Afterwards, the cells were inoculated with either 200 µL of washed blood cells from the BTV-25 positive non-experimental blood samples or with 200 µL of washed/unwashed blood from the experimentally infected goat #19 at 17 dpi (Cq-value 25.1) for two hours. Afterwards, the blood inoculum was removed, and flasks were refilled with medium supplemented with 10% FCS and antibiotics in double standard concentration (20,000 µg/mL Penicillin, 20,000 units/mL Streptomycin, 10 mg/mL Gentamicin, 250 µg/mL Amphotericin B). After 3 to 4 days of incubation at 37 °C, the infected BSR cell monolayer was split by using 1 mL of trypsin and mixed with 5 mL of the supernatant. In the next step, 3 mL of the cell–trypsin–supernatant suspension was transferred to a new T75 cm^2 cell flask with fresh BSR cells grown for 3 h. Three passages were performed, and the success of the virus replication was confirmed by the genomic load estimated by RT-qPCR. Furthermore, the virus presence was confirmed by the

positive signal in the immune fluorescence test. Therefore, BSR cells were incubated for 4 h in 96-well cell culture plates and infected with the BTV-25 virus suspension. After 4 days of incubation at 37 °C and 5% CO2 a partial cytopathic effect (CpE) was visible and infected and non-infected BSR cells were fixated with 100 µL ice-cold Acetone-Methanol 1:1 for 10 min. After adding 100 µL of the 1:200 diluted BTV-25 rabbit immune serum, BSR cells were blocked with 100 µL ROTI®Block solution (Roth Chemie GmbH, Karlsruhe, Germany) for 30 min to reduce non-specific reaction. For the secondary antibody reaction, Goat anti-Rabbit IgG (Alexa Fluor® 488, Abcam, UK) was prepared at a dilution of 1:1000 in ROTI®Block solution and 100 µL was added to each well. Fluorescence signaling was analyzed using an Axio Vert.A1 microscope (Zeiss, Oberkochen, Germany) with an HXP 120 V fluorescent light source.

2.7. ELISA

All serum samples were screened for BTV-group-specific antibodies using a cELISA (ID Screen® Bluetongue Competition, ID-Vet, France) according to the manufacturer's instructions. Samples with ≤50% of negativity compared to the negative control (S/N) were considered as positive, samples with ≥50% S/N as negative.

2.8. Virus Neutralization Test

A virus neutralization test was performed for the detection of serotype-specific neutralizing antibodies. BTV-25-GER2018 was used after 12 passages on BSR cells. In the last passage, the content of a T1700 cm^2 cell roller flask was pelleted via centrifugation and re-suspended in 60 mL medium. For this VNT stock virus a titer of $10^{5.83}$ $TCID_{50}/_{mL}$ could be defined. VNTs were run with cELISA positive rabbit sera, sera of the experimentally infected goat, and all cELISA positive field samples of the goat flock. Furthermore, reference sera of classical BTV serotypes 1–24 (generated in guinea pigs or rabbits), and sera reactive against BTV-26, BTV-27v1 and BTV-28 were available for the VNT. A cELISA positive BTV-8 serum and a negative reference serum were used as positive and negative controls. Briefly, the serum was diluted in log2 steps starting from 1:10 to 1:280 and titrated against 100 $TCID_{50}$ of BTV-25-GER2018 per 96 well. Plates were incubated for 1 h at 37 °C before overnight incubation at 4 °C. The following day, 100 µL of a BSR cell suspension of approximately 30,000 cells/100 µL was added per well. After incubation for 3–5 days at 37 °C, all wells were scored for a cytopathic effect (CpE). The neutralization titer was determined as the dilution of serum giving 100% neutralization. The calculations according to the Spearman and Kärber method were used.

3. Results

3.1. Genome Analysis

The sequences of all 10 segments of the BTV-25-GER2018 strain were established and used for phylogenetic analyses (Figure 1) including BTV strains representing the known BTV serotypes [6]. For segment 2, the nt identities for the BTV strains used in the phylogenetic tree varied from 40.9% (BTV-12) up to 60.8% (BTV-10) for the classical serotypes 1–24. The identity of the atypical serotypes started from 57.5% with BTV-28/Sheep pox vaccine derived BTV to up to 83.4% with TOV. For segment 6, identities for the classical serotypes from 58.0% (BTV-15) up to 72.2% (BTV-4 and BTV-24) were revealed. The identities for the atypical BTV serotypes varied from 68.5% for BTV-26 to the highest identity for TOV with 82.9%. In comparison, segment 10, a more conserved BTV segment, showed identities of 76.7% (BTV-18) up to 79.9% (BTV-21) with the classical BTV 1–24, and from 79.7% (SP vaccine derived BTV = BTV-28) up to 88.0% in comparison with atypical BTV (TOV).

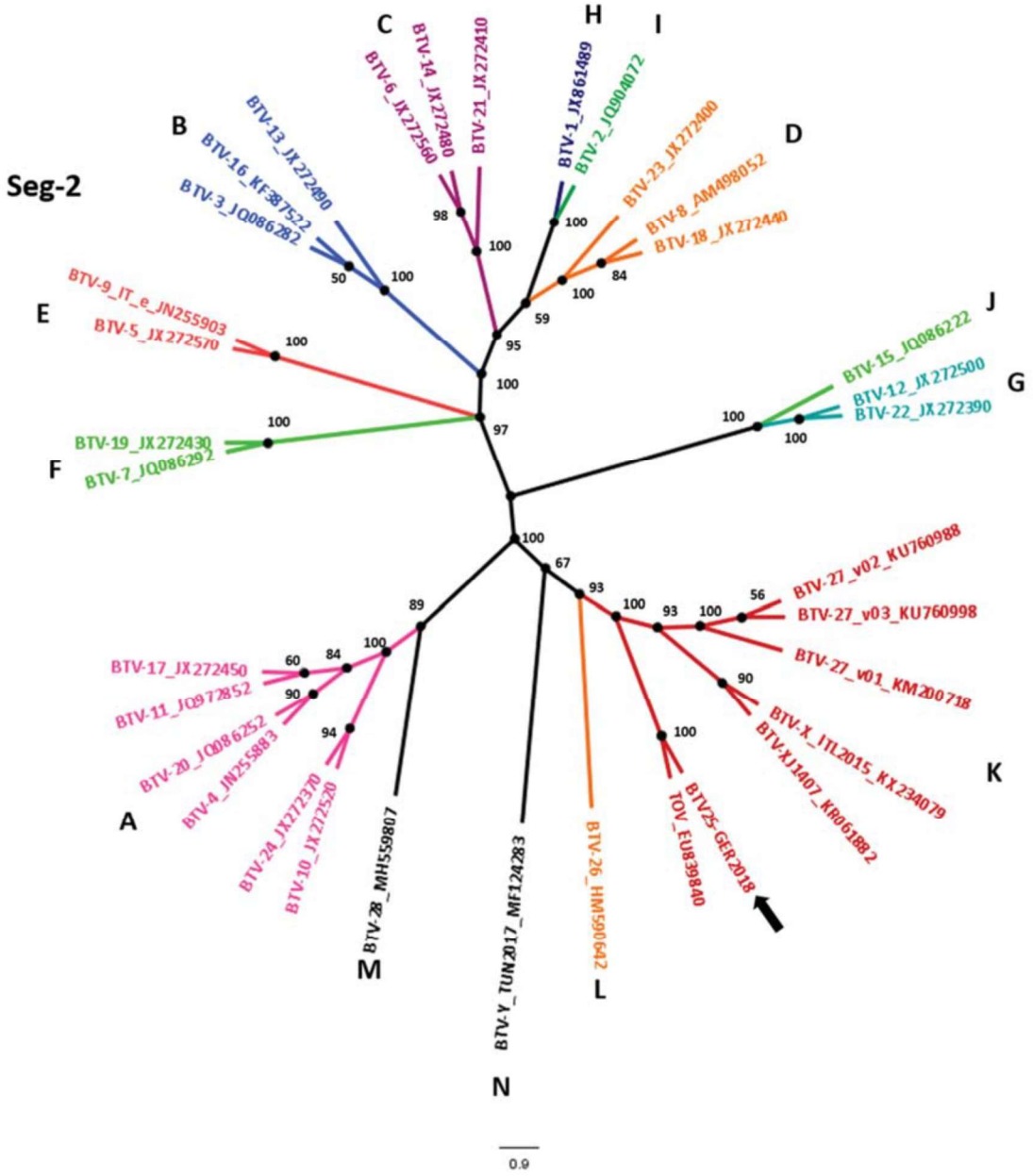

Figure 1. *Cont.*

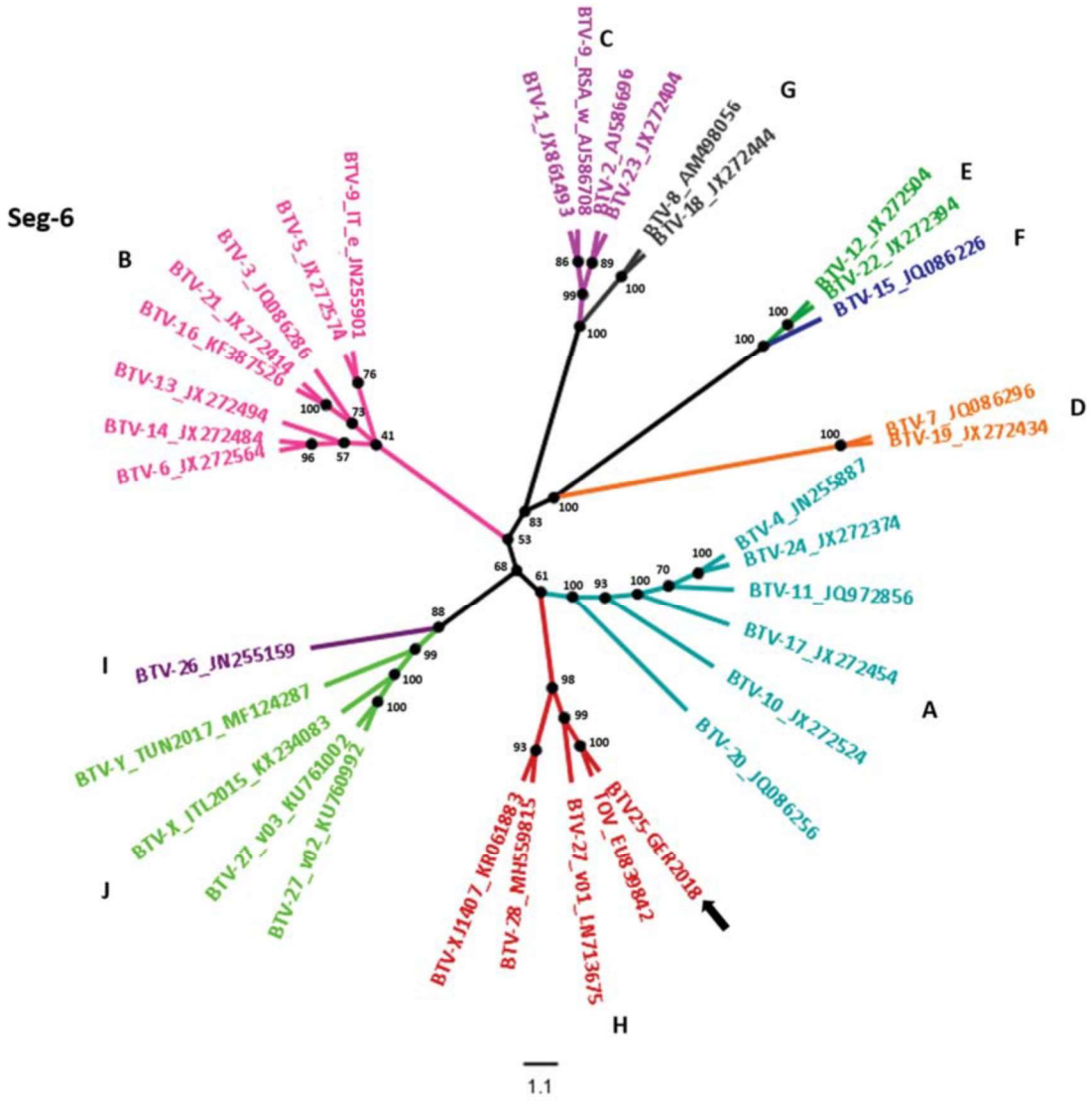

Figure 1. *Cont.*

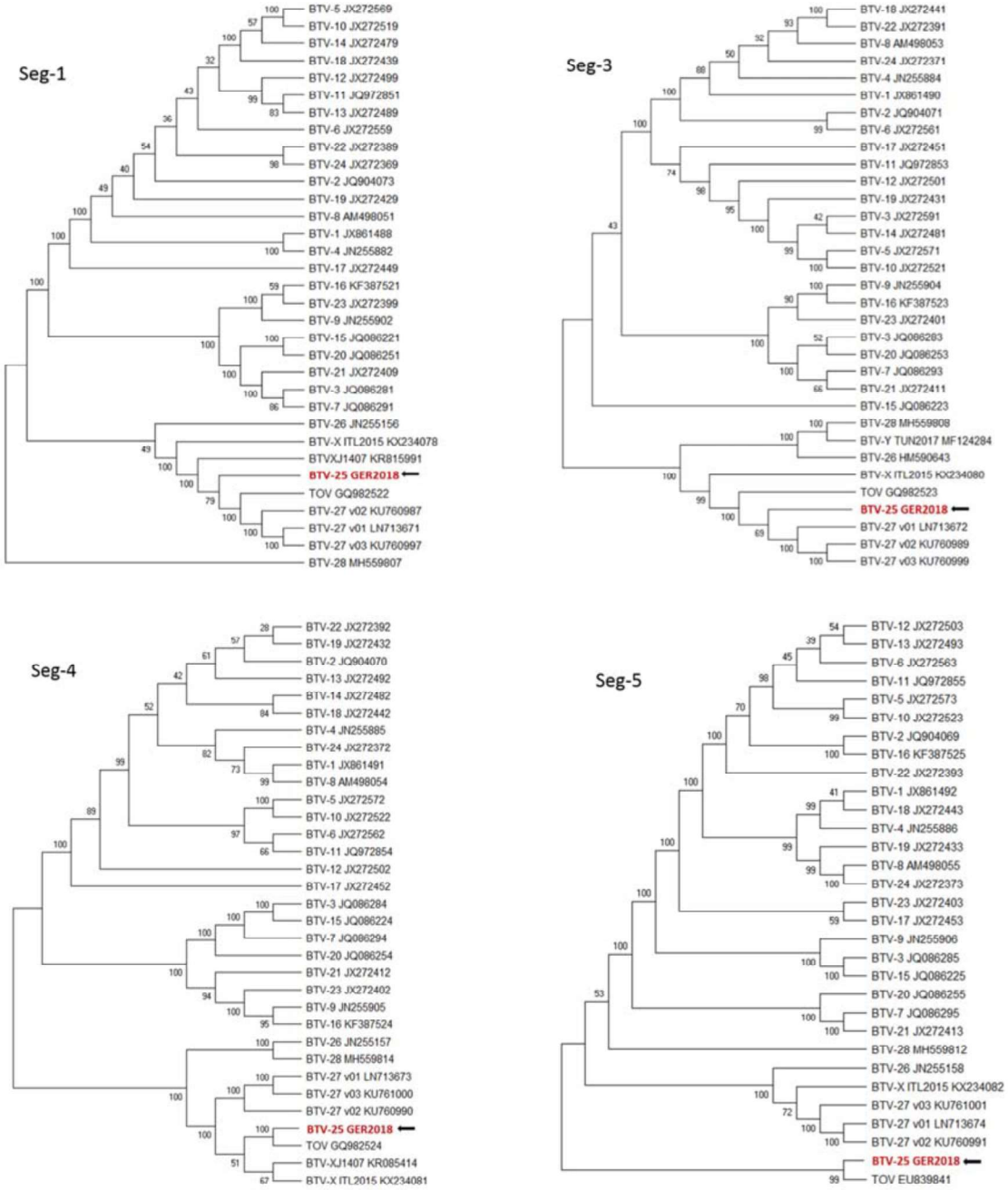

Figure 1. *Cont.*

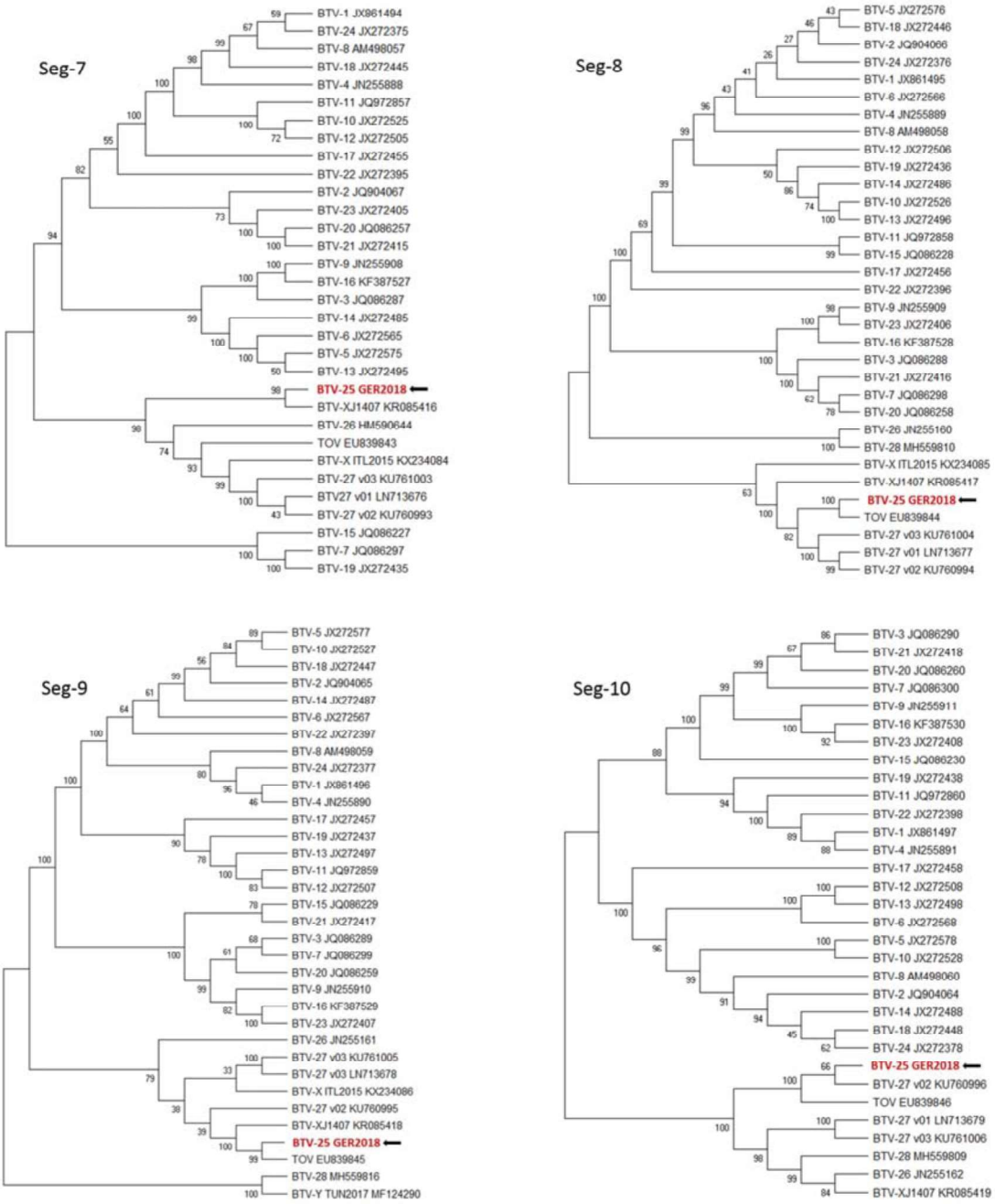

Figure 1. Phylogenetic analyses of the BTV-25-GER2018 genome. The phylogenetic trees of each of the 10 segments were created with MegaX using the genetic distinction model Tamura–Nei and tree-built method UPGMA including BTV strains representing the known BTV serotypes published [6]. We performed a bootstrap analysis with 1000 replications. The colors of the phylogenetic trees of segment 2 (Seg-2) and segment 6 (Seg-6) represent the different nucleotype groups [32] and trees were modified with FigTrees. For easier identification of the different nucleotypes A-N, the unrooted tree layout was chosen for Seg-2 and Seg-6. The arrows point at the BTV-25-GER2018 sequence.

The BLAST results of the nucleotide and amino acid sequences of the complete coding sequence of the BTV-25-GER2018 segments are shown in Table 1. The most related strains for all segments were found to be solitary representatives of atypical BTVs, which is consistent with the phylogenetic trees. The nearest neighbor (nt-based) for segment 1 was BTV-Z ITL2017 with 96.6%, and aa-based with 92.2% TOV. Segment 2 also matched with BTV-Z ITL2017 in nt and aa with 92.5% and 89.0%

(query cover 79%), and as the second nearest neighbor with TOV in nt and aa with 83.5% and 82.8% (query cover 100%), respectively. The nearest neighbor of segment 3 was BTV-Z ITL2017 with 96.7% (nt) and the French BTV-27 variant 3 with 96.0% (aa). Segment 4 showed the highest identity in nt and aa with BTV-Z ITL2017 (96.1%/99.7%). The closest relatives for segment 5 were again BTV-Z ITL2017 (94.6%, nt level) and TOV (82.6%, aa level). Segment 6 matched to BTV-Z ITL2017 at 96.5% on the nt level (query cover 32%), whereas the second nearest neighbor was TOV reaching 82.9% (query cover 100%). Based on aa, segment 6 showed 91.8% identity with TOV. Segment 7 showed the highest identity with a Chinese BTV strain named "V196/XJ/2014", both on the aa and the nt-level with 84.8% and 97.7%, respectively. For segment 8, the highest identities with 98.2% (nt) and 98.3% (aa) could be ascertained again with TOV. Similarly, for segment 9 the highest identity was found for TOV (85.3%/82.4%). For segment 10, the BTV-27/FRA/2014 variant 2 showed the highest identity on both the nt and aa level (88.8%/95.6%).

BTV serotypes can be also divided into nucleotypes representing distinct evolutionary lineages [32]. In accordance with their serological cross-reactions and nucleotide identities, each BTV serotype clusters in a nucleotype group for segment 2 and segment 6, respectively. For the nucleotype classification of segment 2 their identity must be higher than 66.9% [33,34]. Currently 12 nucleotypes "A–L" are known for segment 2 with a putative 13th new nucleotype "M" involving BTV-28/Sheep pox vaccine derived BTV and a 14th nucleotype "N" involving BTV-Y TUN2017. Segment 2 of BTV-25-GER2018 belongs to nucleotype "K" together with TOV, BTV-X ITL2015, BTV-XJ1407 and the three variants of BTV-27. For segment 6, members of the same nucleotype need to show a >76% nt identity [34]. Currently, 10 nucleotypes "A–J" are known with the newest nucleotype "J" including BTV-27/FRA2014/v02, v03 and BTV-X ITL 2017 [6,35]. For segment 6, the newly described BTV-25-GER2018 strain is part of the nucleotype "H" together with TOV, BTV-27/FRA2014/v01, BTV-28/Sheep pox vaccine and BTV-XJ1407. Different BTV isolates are defined as serotypes by using the virus neutralization test, however, molecular typing is also possible. Within the same serotype, the minimum levels of Seg-2/VP2 sequence identities were defined as 68.4% nucleotide (nt) / 72.6% amino acid (aa) [24,34]. Segment 2 of BTV-25-GER2018 matched to 92.5% with BTV-Z ITL2017 as the closest neighbor, and with TOV to 83.5% as the second closest neighbor. Both are representatives of serotype 25. On the amino acid level, BTV-25-GER2018's closest relatives are BTV-Z ITL2017 with 89.0%, and TOV with 82.8%. Overall, based on the sequence data analysis, it can be concluded that BTV-25-GER2018 belongs to the Seg-2 nucleotype grouping K for which TOV is the prototype isolate for BTV-25.

3.2. Goat Flock Monitoring

Results of the goat flock monitoring during the sampling period is shown in Table 2. Eleven goats were positive in the cELISA at all five bleeding time points, and four of those were continuously positive in the RT-qPCR as well, and one goat was continuously negative in the RT-qPCR. In total, 55 goats were negative in the cELISA at all five bleeding time points, five thereof were continuously positive in the RT-qPCR, and 33 were constantly negative. All data collected over the time are shown in detail in the Supplementary Materials (Table S1).

Figure 2 shows the median of all samples in the serogroup antibody-specific cELISA and the BTV-25 Seg-2 specific RT-qPCR analysis during the five bleeding time points. The median Cq-values developed from 35.96 to 34.48, 33.03, 33.58 and 35.72 at the fifth bleeding time point. For the cELISA, the median S/N% developed from 74 to 75, 77, 98 and 93, whereas regarding only the cELISA-positive results the S/N% values ranged from 25 to 24, 27, 27 and 14.

Table 2. Dynamics within the goat flock over the different sampling time points. The total numbers of positive goats in the BTV-25 specific RT-qPCR (quantification cycle (Cq) <40) and the cELISA (less than 50% negativity compared to the negative control (S/N)) are shown.

	Bleeding 1 07/08/2018	**Bleeding 2 04/09/2018**	**Bleeding 3 03/12/2018**	**Bleeding 4 16/05/2019**	**Bleeding 5 08/10/2019**
N° goats (EDTA/Serum)	118 (118/118)	117 (117/117)	116 (116/115)	121 (120/120)	115 (115/115)
New introduced	-	-	2	13	5
Removed	-	1	3	8	11
N° goats	118	117	115	120	115
PCR positiv	37 (31%)	45 (38%)	43 (37%)	44 (37%)	39 (34%)
cELISA positiv	27 (23%)	26 (22%)	27 (23%)	21 (18%)	24 (21%)

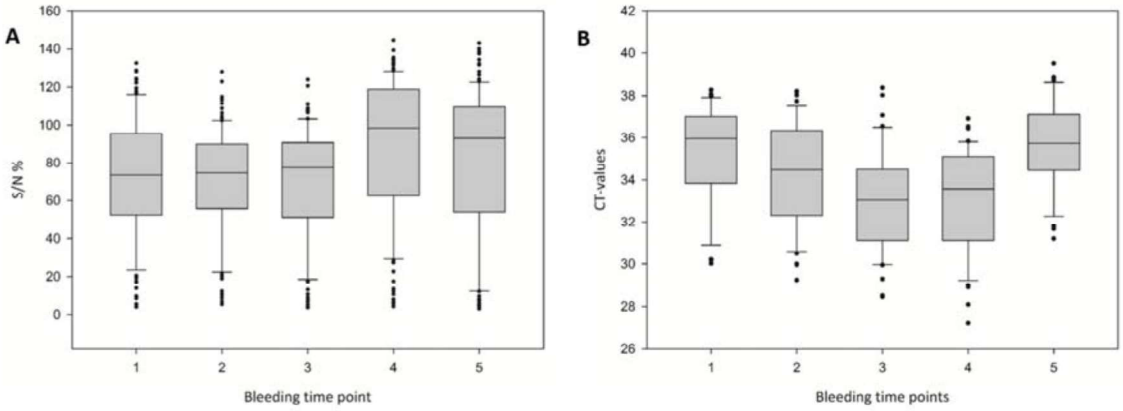

Figure 2. ELISA and PCR results of the 5 bleeding time points of the goat herd. The median values of the (**A**) reactivities of the ID.Vet cELISA in percent of negative control (≥50% is negative according to the manufacturer) and (**B**) the Cq values of the BTV-25 Mix13 Cq-values during the 5 bleeding time points of the goat flock are shown. The box and whisker plots show the median (broad central line), the interquartile range (box), the range of values (bars) and outliers (points).

Results of the RT-qPCR results and the cELISA assays for 20 new-born kids or goats newly added to the flock are shown in Table 3. The two individuals that arrived before December 2018 were negative in both the RT-qPCR and the cELISA at bleeding time points 3 and 4. Two of the 15 (13.3%) goats that arrived during spring 2020 were positive in the RT-qPCR for BTV-25 at bleeding 4, goat #127 with a positive cELISA result and goat #129 with a negative cELISA result. Interestingly, goat #127 was negative in both RT-qPCR and cELISA at bleeding time point 5, whereas goat #129 stayed positive in the RT-qPCR without seroconversion at bleeding 5. At this bleeding time point, 12 of 20 (60%) newly introduced goats were positive for BTV-25 viral RNA in RT-qPCR with no detection of group-specific antibodies. Only two of the goats—new at bleeding time point 5—were clearly positive in the cELISA, but negative in the RT-qPCR.

The analysis of the retrospective serum samples revealed the presence of BTV genomes in the goat flock since February 2015. In serum samples from three goats, evidence of BTV-25 nucleotype genomes could be detected in 2015 and viral RNA was present during the samplings in 2018/2019. In detail, the three goats originated from Switzerland, and all were present in the goat flock from February 2015 until the fifth bleeding in October 2019. Two goats were constantly positive for BTV-25 genome in RT-qPCR from 2015 until October 2019, one goat thereof was constantly negative in the cELISA, and one varied in the cELISA starting and ending with a negative result. The course of the RT-qPCR results of the third goat started with a positive test for the 2015 sample with variations over the different bleeding time points in 2018/2019 ending at bleeding 5 with a positive result again. The corresponding cELISA results of the third goat started with a negative result in 2015, followed by positive results during bleeding 1 to 3, and ending with a negative cELISA result again in 2019.

The data are summarized in Supplementary Materials S1. The BlueTYPE array runs were negative for other serotypes than BTV-25-GER2018.

Table 3. BTV-25 specific RT-qPCR and cELISA results of the newly introduced individuals (offspring and newly introduced into the farm). Goats were considered positive for BTV-25 specific RT-qPCR with Cq <40 could be defined. The cut-off for the cELISA was 50% and serum samples with an S/N% ≤50% were determined as positive (in bold). "−"represents "no sample available" (not present at that time point in the goat flock).

	Bleeding 3 03/12/2018		**Bleeding 4 16/05/2019**		**Bleeding 5 08/10/2019**	
Goat ID	**RT-qPCR**	**cELISA**	**RT-qPCR**	**cELISA**	**RT-qPCR**	**cELISA**
	Cq-Value	**S/N%**	**Cq-Value**	**S/N%**	**Cq-Value**	**S/N%**
#119	no Cq	74	no Cq	115	32.8	93
#120	no Cq	106	no Cq	122	36.3	70
#121	–	–	no Cq	123	35.2	67
#122	–	–	no Cq	114	36.5	95
#123	–	–	no Cq	133	31.2	118
#124	–	–	no Cq	134	35.6	93
#125	–	–	no Cq	135	34.0	110
#126	–	–	no Cq	123	31.8	61
#127	–	–	32.1	28	no Cq	77
#128	–	–	no Cq	98	32.3	99
#129	–	–	30.8	84	31.7	102
#130	–	–	no Cq	132	34.4	70
#131	–	–	no Cq	98	no Cq	100
#132	–	–	no Cq	114	36.7	80
#133	–	–	no Cq	92	no Cq	79
#134	–	–	–	–	no Cq	68
#135	–	–	–	–	no Cq	92
#136	–	–	–	–	no Cq	107
#137	–	–	–	–	no Cq	16
#138	–	–	–	–	no Cq	20

3.3. Animal Experiments

Diagnostic inoculation of goats with two BTV-25-GER2018 RNA positive blood samples led to successful infection in one of the two animals. Viral RNA detection by RT-qPCR started on 7 dpi and peaked on day 17 with a Cq value of 25.1. The sera of the positive goat from 7, 14, 21 and 31 dpi increased in antibody titers measured by cELISA but did not reach the cut-off point to be classified as positive. The second experimentally infected goat remained BTV-genome negative throughout the animal trial and did not react with an antibody titer either. The respective data are shown in Table 4. Both goats did not show any clinical symptoms or fever at any time point during the trial.

Table 4. Results from experimental inoculation of goats. RT-qPCR (Cq-value) and cELISA (S/N%) results after diagnostic inoculation of two goats (#19 and #20) are shown.

Goat		**dpi**	**0**	**3**	**5**	**7**	**10**	**11**	**12**	**14**	**17**	**18**	**21**	**24**	**31**
#19	RT-qPCR		noCq	noCq	noCq	36.1	33.5	30.9	28.7	27.7	25.1	26.4	27.3	28.4	28.6
#19	cELISA					108				93			79		70
#20	RT-qPCR		noCq	noCq	noCq	noCq	noCq	noCq	noCq	noCq	noCq	noCq	noCq	noCq	noCq
#20	cELISA					101				107			106		108

A polyclonal BTV-25-GER2018 antiserum was generated from the two immunized rabbits. The rabbit sera were clearly positive in the cELISA for group-specific BTV antibodies (log2 cELISA titers of up to 1:16). Nevertheless, no neutralizing titer could be determined for the rabbit sera due to an incomplete neutralization in the VNT assay.

3.4. BTV-25-GER2018 Isolation in Cell Culture

BTV-25-GER2018 was successfully isolated on BSR cells from washed blood samples of the experimentally infected goat #19 only. After the third cell culture passage, a CpE was observed and successful propagation of the virus could be confirmed with decreasing Cq-values in the RT-qPCR in higher cell passages. A maximum titer of $10^{7.0}$ $TCID_{50}$ /mL was achieved after pelleting the cell culture material of passage 6, whereas the cleared supernatant fraction reached only $10^{4.33}$ $TCID_{50}$/mL. The Pan-S10-RT-qPCR delivered a Cq value of 11.2 for the cell pellet and a Cq value of 16.3 for the cleared cell culture supernatant.

3.5. Virus Neutralization

A VNT using BTV-25-GER2018 was performed with the cELISA positive sera of the rabbit immunization trial, sera from the experimentally infected goats and all cELISA positive goat samples originating from the five bleeding time points. All these sera lead to the same result of an incomplete neutralization of BTV-25-GER2018. At only the 1:10 dilution step of the serum dilution, a CpE was observed in a part of the cell monolayer, and the CpE increased with the higher dilution steps until a 75–100% CpE was seen at dilution steps 1:80 or 1:160. The BTV-25-GER2018 negative reference serum showed a 100% CpE starting from dilution 1:10. Moreover, the reference sera of BTV serotypes 1–24 failed to neutralize BTV-25-GER2018, as well as the BTV-26, BTV-27x and BTV-28 specific sera. Figure 3 shows microscopy pictures of the partial virus neutralization effect of strain BTV-25-GER2018 in comparison to BTV-8, a representative of classical BTV. In contrast, the BTV-8-specific positive control serum showed the expected neutralization titer of 1:320 against the used BTV-8 strain.

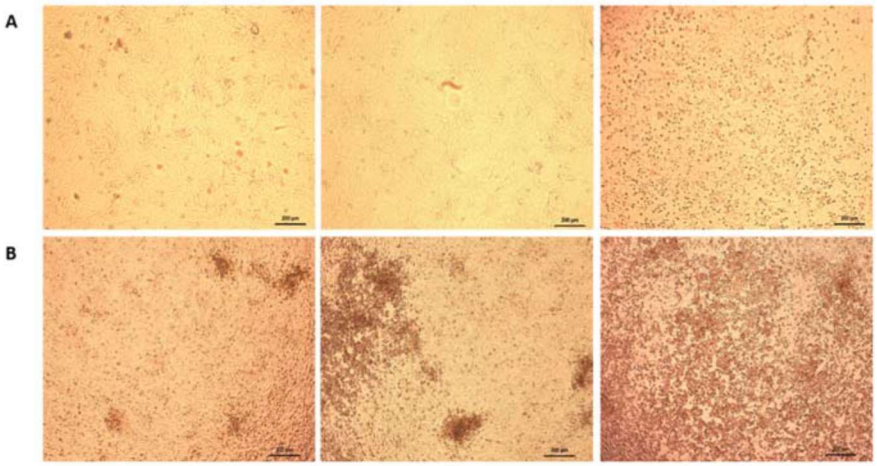

Figure 3. Comparison of BTV-8 and BTV-25-GER2018 virus neutralization. Virus neutralization results of (**A**) methodical control with BTV-8 virus and BTV-8 positive serum (1:10, 1:80 and 1:1280) (**B**) cELISA positive serum of a BTV-25-GER2018 field infected goat (1:10, 1:80 and 1:280).

4. Discussion

Here, we present the first isolation and characterization of a novel atypical BTV strain "BTV-25-GER2018", detected in clinically healthy goats in a farm in Bavaria, southern Germany. Molecular analyses showed the highest identities on both the nucleotide and the amino acid sequence level with the group of atypical BTV-25 strains, which are clearly distinct from the canonical "classical" serotypes 1–24. Most importantly, BTV-25-GER2018 is the first BTV-25 strain, which could be efficiently propagated in cell culture. BTV-25-GER2018 did not cause any BTV typical clinical signs, neither in the goat flock nor in the experimentally inoculated goats. Infection with atypical BTV is characteristically not associated with clinical disease or only with very mild clinical signs [6,7,12,35,36]. BTV-25-GER2018 may have circulated in this flock for at least four years, as shown by retrospective analysis of serum

samples. Serum is not recommended for BTV genome detection and only positive PCR results based on hemolytic sample materials can be accepted as true-positive. Furthermore, other serotypes than BTV-25-GER2015 present in the goat flock could have been excluded with the help of the BlueTYPE array [9] and are also unlikely from the epidemiological point of view. Germany was free of BTV from February 2012 until December 2018 and only <60 cattle tested positive since the BTV-8 re-emergence in the country [25,26]. No BTV-8 case in goats was reported in the area of sampling.

A closer monitoring of BTV occurrence in the flock during five sampling time points in 2018/2019 revealed variations in the RT-qPCR and cELISA results from individual goats in the different consecutive samples. During the sampling period (from August 2018 until October 2019), 31% up to 38% of the goat flock tested positive for BTV genomes. Interestingly, all possible combinations of positive or negative RT-qPCR and cELISA results could be identified. Furthermore, the positive and negative results of individual goats varied with similar frequencies (Table S1). A long-lasting RNA-positivity for up to 25 months of goats infected with TOV was observed previously in a goat flock in Switzerland [21]. Furthermore, TOV was found to be present in archived serum samples as early as 1998 [37]. Like in our study, no efficient viral clearance was observed in the Swiss goat flock. Possible re-infections with BTV, but above all the long-term viral persistence of BTV-25-GER2018 should be also considered as a possible scenario [21]. Unfortunately, no bleeding data between 2015 and 2018/19 were available for underlining the persistent infection model.

We observed a certain number of animals with only a weak antibody response, and goats with low BTV RNA levels during the monitoring period—both had also been reported for the Swiss BTV-25 related strain. These findings may be common for atypical BTV strains in contrast to the classical BTV 1–24 showing high RNA levels during the peak of viremia and long-lasting high-level antibody responses [21]. In the Bavarian goat flock, 18% to 23% of the goats were positive for group-specific antibodies in the cELISA during the monitoring period. In contrast to our findings, the BTV-25-infected Swiss goat flock showed a high in-herd seroprevalence of 97% in the cELISA [21]. In another seroprevalence study performed in Switzerland, the observed in-herd seroprevalence for goats was 75% in 2008 in the Swiss Alps (Valais), whereas the mean estimated in-herd prevalence in Ticino ranged from 33.5% to a maximum of 100% [37].

In the BTV-25-GER2018-positive goat flock, it seemed that goats had very variable genome loads (positive to weak positive to negative and positive again) independently of their antibody level. The "re-positivity" of several goats during the surveillance period could be interpreted as a re-infection, but more likely, the virus persisted in the goats without permanent viremia. Viral persistence mechanisms of BTV, in light of a "non-arthropod-based" overwintering, are an ongoing debate. One study suggested that BTV might persist in GammaDelta-T-cells [38], but this finding could not be reproduced, and it is currently not assumed that BTV could cause a persistent infection in ruminants [39,40]. Therefore, further research is needed to explore the potential for the long-term infection mechanisms of goats infected with atypical BTV strains including the novel BTV-25-GER2018.

The high variation in cELISA titers observed for the different sampling time points ranging even from seropositivity to seronegativity in several individuals has not been reported for BTV so far. In contrast, for classical BTV a long-lasting antibody response was observed in both sheep and cattle for at least 7.5 years [41,42]. In healthy individuals, true physiological fluctuations of antibody titers can result from polyclonal activation or depression of B-cells as observed for measles virus [43]. Long-term viral RNA persistence in cattle is known for Vesicular stomatitis virus, with fluctuations of IgG antibodies as shown in endemic areas such as Costa Rica. Reinfection of seropositive animals occurred as well [44]. The immune response of goats to atypical BTV strains needs to be further investigated to understand the observed antibody level variations. Nevertheless, a lower avidity of the VP7 (origin is a classical viral strain) used in the commercial cELISA towards the group-specific BTV antibodies produced to atypical viral strains cannot be fully excluded and may also contribute to a reduced sensitivity leading to false-negative results. The development of a cELISA using the VP7 of BTV-25 could illuminate the antibody reaction towards BTV-25-GER2018 in the goat flock.

Neutralizing antibody levels are parameters of protective immunity towards the respective serotype [39,45]. For our studies no anti serato alternative atypical strains such as BTV-25 or BTV-Z-ITL2017, BTV-X ITL2015, BTV-Y TUN2017 and the Chinese BTV-XJ1407 isolate were available. Nevertheless, for sera specific for BTV-1 to 24, BTV-26, BTV-27 FRA2014-v01 and BTV-28, a VNT with BTV-25 GER2018 was performed and no cross-neutralization was observed. Interestingly, even the strong cELISA positive rabbit antiserum did not exhibit complete neutralization. In addition, all 125 ELISA-positive sera from the affected goat flock likewise did not show complete neutralization. It is rather unlikely that the other EDTA blood samples negative in cELISA show improved neutralization capacities. The lack of absolute neutralizing antibodies in those sera may contribute to the fact that BTV-25-GER2018 infections perpetuate for years in the goat flock without viral clearance and might be a part of the mechanisms supporting viral persistence of this atypical BTV. Nevertheless, prolonged viremia is known for BTV despite the presence of neutralizing antibodies due to its affinity to cell membranes and erythrocytes [39]. Unfortunately, as for the other BTV-25-related strains, virus isolation results have been unsuccessful so far [18,22], and only a recombinant BTV-25 chimera with a BTV-1 backbone (BTV-$1^{VP2/VP5\ BTV25}$) was available for neutralization assays as a positive control virus [20]. This recombinant BTV-25 virus could be neutralized by the TOV positive serum, which is in contrast to the here observed incomplete neutralization of BTV-25-GER2018 [18]. Nevertheless, the recombinant chimeric BTV-25 virus failed to be neutralized by the BTV-Z-ITL2017 positive serum [22]. For a better understanding of the pathogenesis and immune reactions of BTV-25-GER2018 and related strains, further research is necessary.

The detailed analysis of samples from 15 newly introduced animals to the goat flock revealed that 13.3% of these were BTV-RNA positive in May 2019 at bleeding time point 4. In October 2019, at bleeding time point 5, the number of infected goats that had newly arrived increased to a level of 60%. The seasonality of competent vectors leads to the seasonality of BTV infections throughout the year with infection peaks during the late summer and autumn months [46] and might be the rationale for the increased infection rate in the newly introduced goats. In contrast, the number of BTV-25-GER2018 infections did not increase on a whole herd level in the autumn months. Therefore, alternative transmission ways and mechanisms of viral persistence relevant for BTV-25 related strains should be studied in the future.

Previously BTV-25 strains could not be propagated in cell culture until now [6,18,22]. For BTV-25-GER2018 virus propagation was successful on the mammalian cell line BSR, and we could show that BTV-25-GER2018 replication is cell-associated. This is in agreement with experiences from classical BTVs [47].

The genome sequence of BTV-25-GER2018 differed from other atypical BTV-25 strains. A small number of nucleotide exchanges within the BTV sequence detected in two genetically related strains could have a strong impact on virus characteristics, as demonstrated for two BTV-8 strains [48], and it is therefore very likely that differences on the molecular level have a major impact on virus isolation attempts. The difficulties of virus propagation in cell culture for BTV-25 and related strains, as well as the observed lack of neutralization for BTV-25-GER2018 and BTV-Z-ITL2015, lead to non-typeable phenotypes at least using traditional serotyping via VNT. For the atypical BTV strains, "genotyping" based on molecular data could be a practical solution. Finally, applying the criteria from Maan et al., 2016, BTV-25-GER2018 was genotyped as serotype 25.

In conclusion, an atypical BTV, isolate BTV-25-GER2018, was found to circulate in southern Germany. It was identified as a member of the BTV-25 serotype group using sequence data and phylogeny. Retrospective samples confirmed the likely prolonged presence of BTV-25-GER2018 RNA within the studied goat flock. Furthermore, it was possible for the first time to propagate a BTV-25 related virus efficiently in cell culture. The analysis of consecutive samples from the affected goat flock suggests a persistent BTV-25-GER2018 infection in goats. This hypothesis is affirmed by the observation of mainly non-neutralizing antibodies against BTV-25 GER2018. Nevertheless, our findings are not conclusively proving the concept of persistent infection of goats with BTV-25 related viruses

and further research on the biology of atypical BTV strains is necessary for a better understanding of their epidemiology and pathogenesis in comparison to the well-studied classical BTVs.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/9/983/s1, Table S1: All BTV-25 specific RT-qPCR (Cq value) and cELISA (S/N%) results of the goat herd over time.

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5. Discussion

5.1. Longevity of vaccine derived antibodies and the effect on vaccination and surveillance programs

For the control and eradication of the BTV-8 epidemic from 2006-2009 in Europe, compulsory large-scale vaccination campaigns were implemented using different inactivated BTV-8 vaccines [95, 98, 164]. The commercially available, inactivated whole-virus vaccines were tested to be safe in application and efficient in protective immunity in short [233] and long term studies [233, 234]. Presence of neutralizing antibodies post BTV-8 vaccination was shown for up to 6 years in cattle [184] and 7.5 years in sheep [185]. However, further data are necessary e.g. for a better understanding of the duration of seropositivity following BTV vaccination. From 2009 until the end of our study, no new BTV infection was reported in Germany. We analysed 157 cattle born after 2009 with a recorded BTV-8 vaccination history to ensure the vaccine origin of circulating BTV-8 antibodies. Thus, we were able to analyse the longevity of vaccine-derived group- and serotype specific antibodies of cattle during a time span of 5 to 8 years post BTV-8 vaccination with a variable number of re-vaccinations. Therefore, we grouped the cattle in groups 5 to 8, representing the approximated time spans of 5 to 8 years between the last BTV vaccinations and the sampling time points. With the VP7-specific cELISA we could determine group-specific, and with the SNT serotype specific antibodies.

In our study, the cELISA showed a specificity of 100%, whereas several of the SNT positive samples were tested negative in cELISA. This is in line with previous studies [235]. The two methods target two different types of antibodies, directed towards VP7 in the case of the cELISA and VP2 in the case of the SNT. The group-specific antibodies directed against VP7 can only neutralize the BTV core particles, but not the intact virus particle.

Our study revealed the presence of neutralising BTV-8 antibodies in more than 50 % of the analysed cattle (n= 14) 8 years post BTV-8 vaccination. Since the presence of neutralising antibodies strongly correlates with protection of the animals [235], this is also an indication of a long term protection of those animals against BTV-8 infection. Thus, a large part of BTV-8 vaccinated cattle might have acquired protection against BTV-8 infection that lasts at least 8 years. However, the level of neutralising antibodies does not always correlate with protection and both antibodies as well as T-cell -mediated responses contribute to an efficient protection

[154, 163, 236]. Nevertheless, the long-term benefit of BTV-8 inactivated vaccines in cattle is remarkable and should be considered in vaccination and prevention campaigns particularly as BTV-8 has been able to re-emerge in Northern Europe [105]. In comparison, the protection period of vaccines for other disease can be quite a bit shorter, like for Influenza A, where conventional vaccines are only effective for a short period of time and even uncertain from year-to-year [237]. For inactivated FMD vaccines protection periods of 4 to 12 months were reported and revaccinations at regular intervals of six months are necessary to sustain protective immunity [238]. For African Swine Fever still no vaccine is available [239].

In all four groups 5, 6, 7 and 8, we found seroprevalences in both the cELISA and the SNT of ≥50 % independently of the number of vaccinations received. Thus, even after only a basic immunisation against BTV-8 (one initial application and one subsequent booster), cattle can develop a long-lasting humoral response to BTV-8 (see Paper I). However, the number of booster vaccinations significantly increased the percentage of seropositive cattle to up to 90% with already one revaccination. For the long-term perspective of controlling the spread of BTV-8 and eradicating the disease, the FAO recommended 5 years of vaccination of 95% of susceptible cattle and sheep [240]. Nevertheless, the cost-benefit calculations are the critical point particularly for livestock owners to decide whether to vaccinate their livestock or not, but also for the choice of surveillance strategies [241]. The vaccine costs per dosage alone was calculated with 0.40 Euro and cattle require two doses for primary immunisation [242]. The benefit of vaccination can reduce the economic impact of treatment and production losses particularly in dairy cattle [243]. Nevertheless, on the basis of our data, the yearly re-vaccination scheme recommended by the manufacturer should be revisited, as it might further limit the acceptance of vaccination by livestock owners. Our data might positively influence the acceptance of BTV vaccination among the farmers by showing that a reduction of necessary booster vaccinations is possible.

We observed less than 20 % of “none” or “poor responders” despite BTV-8 vaccination. Faulty entries of BTV vaccination in the HIT database cannot be excluded, however, there are several factors which influence the success of vaccination outcome (see Paper I). Thus, the choice of the vaccine itself can increase the success of vaccination, as it was shown for BTVPUR® AlSap 8 (Merial, France) in contrast to Zulvac® 8 Bovis (Zoetis, Belgium). The difference of the two vaccines might be explained by its intrinsic qualities like the administration route (subcutaneous for BTVPUR® AlSap 8 and intramuscular for Zulvac® 8

Bovis) or the composition of adjuvants and excipients [244]. Furthermore, animals vaccinated by a trained veterinarian had a 2-fold higher probability to seroconvert than those vaccinated by untrained veterinarians [244]. Moreover, young animals had a 5-fold higher probability to seroconvert in comparison to older animals, because the immune response is stronger in younger animals [244]. In addition, animals boosted with a heterologous vaccine had higher neutralizing antibody titers than those boosted with the vaccine already used for primary immunization [236]. Finally, an important factor for the success of BTV-vaccination might be the genetic background of the host, as potential single nucleotide polymorphism (SNPs) and toll like receptors (TLRs) were connected with disease resistance and humoral and cell-mediated immune response [245].

Overall, vaccination with inactivated BTV-8 vaccines proved to be a highly effective tool in BTV-8 control. Furthermore, high safety was proven for all commercially available vaccines and the protection period of the inactivated BTV-8 vaccines is remarkably long in comparison to e.g. vaccines for Influenza A or FMD. These findings might positively influence the acceptance of BTV vaccination by showing that a reduction of necessary booster vaccinations is possible, despite the yearly revaccination schedule recommended by the producers.

5.2. The “BlueTYPE array” as novel diagnostic tool for high-performance BTV serotyping

Real-time (RT)-PCR ((RT)-qPCR) based on TaqMan technology has become the most common qPCR technique [193], and numerous RT-PCR assays for the identification of BTV serotypes and strains have been developed over time. Fast serotype identification, as well as the detection of eventually multiple serotypes in one sample are important for efficient disease control, but also for a well-controlled laboratory work. Serotype identification up to now can be performed mainly by running numerous single serotype-specific RT-PCRs or by serological assays. Therefore, we developed a novel low-density RT-qPCR array named “BlueTYPE”, which comprises a single well combination of 29 TaqMan-real-time-RT-PCR assays for the identification of all known 24 classical BTV serotypes in only one PCR run (see Paper II). The advantage is that BlueTYPE identifies single or multiple serotypes present in a BTV positive sample within a single PCR run. Furthermore, when PCR plates are pre-filled with the primer-probe mixes and stored at−20 °C, the handling of BlueTYPE becomes very simple. Thus, after adding the extracted RNA, results are achieved in less than 2 hours. Moreover, we equipped the BlueTYPE array with an extraction control by adding a beta-actin assay in a separate well and with a heterologous spike-in process control realized in each well [246, 247].

A similar system was recently reported for the typing of influenza A viruses [248]. For result interpretation, the different pan-BTV and serotype-specific RT-qPCR systems can be analysed not only qualitatively, but also semi-quantitatively regarding the viral genome loads, which could be very helpful or even essential for plausibility checks. Altogether, these features of the BlueTYPE array increase the reliability of diagnostic test results immensely.

In detail, we integrated the OIE-listed Pan-BTV-S10-RT-qPCR as broad-range control assay (detecting all classical and atypical BTV serotypes) into the BlueTYPE array to generally confirm the presence of BTV genomes in the PCR template. The Pan-BTV-S10-RT-qPCR detects all currently circulating BTV serotypes including the atypical BTV strains. This is of high importance since the number of discovered atypical BTV strains is increasing worldwide. However, atypical BTV have a very different status and are not notifiable in the EU. Hence, the newly developed Pan-BTV-S1-RT-qPCR assay of this study is of great interest for BTV diagnostics, as it can differentiate between classical and atypical BTV strains. It is an adaption of the assays already published by Toussaint et al. in 2007 [247], and was mainly modified based on the BTV segment 1 sequence data of the classical BTV serotypes 1–24 published in the last years. By integration of the novel Pan-BTV-S1-RT-qPCR assay into the BlueTYPE array, we ensure that a BTV positive sample is first analysed specifically for the presence of classical BTV strains. A negative result in the Pan-BTV-S1-RT-qPCR combined with a positive result in the Pan-BTV-S10-RT-qPCR subsequently suggests the presence of an atypical BTV strain in the sample of interest. Then, serotype specific RT-qPCRs for the existing atypical BTV strains as well as partial or complete sequencing of segment 2 is used to characterize the putative atypical BTV strain.

However, if no serotype could be identified in a valid BlueTYPE array run, mutations/variations of the segment 2 genome may not be covered by the oligo's selected for the respective classical serotypes. In this case, alternative strategies can be used for serotype/strain identification like partial or complete sequencing of the VP2 gene or even whole genome sequencing. Nevertheless, the here developed BlueTYPE array is the first-choice diagnostic tool for analysing BTV positive sample, as sequencing methods are much more labour and cost intensive.

The high flexibility and simplicity of the BlueTYPE array is of remarkable importance regarding the genetic variability of BTV, particularly the serotype-defining segment 2. Both genetic shifts due to reassortment and continuous genetic drifts lead to changes of the BTV

genome [70]. However, necessary changes of single BTV assays within the array due to novel sequence information can easily be integrated by adding a new or adapted primer/probe combination. In order to ensure a high sensitivity and specificity, we based the array on the serotype-specific TaqMan assays developed by the Pirbright institute in 2016, since those assays were broadly tested with a representative *Orbivirus* reference collection [127] [74]. For developing novel RT-qPCR assays with high sensitivity and specificity as well as for keeping the RT-qPCR assays for BTV detection up to date, it is of utmost importance to publicly share all available Bluetongue and *Orbivirus* virus sequences. In addition to previously validated PCRs, several RT-qPCR assays developed in this thesis were integrated into BlueTYPE to further improve the diagnostic performance of molecular BTV-serotyping. In the case of novel strains or serotypes, the array panel can easily be extended. Furthermore, relevant non-BTV real-time RT-PCR assays can be also integrated in the array format. Thus, the concept of BlueTYPE is of particular value in countries dealing with diseases of differential-diagnostic importance like FMD and PPR [249]. In addition, the identification of mixed infections like with PPRV in sheep can easily be realized [250]. In conclusion, thanks to its flexible design, the BlueTYPE array is a state-of-the art diagnostic tool suitable for use in different epidemic situations and adaptable in the future.

5.3. The atypical Bluetongue virus serotype 25

Several novel “atypical” Bluetongue virus (BTV) serotypes were discovered in mostly clinically healthy small ruminants worldwide. In 2018, we detected BTV-25 genomes by RT-qPCR in connection with an export investigation in two goats from a holding in southern Germany. After experimental inoculation of two goats with BTV-25 positive blood samples, viremia could be ascertained in one goat. And for the first time, a BTV-25-related virus could isolated in cell culture from the fresh EDTA blood of the infected goat and a full genome of the unique isolate “BTV-25-GER2018” could be generated. Interestingly, strain BTV-25-GER2018 was only incompletely neutralized by ELISA-positive sera. Fortunately, we could further monitor the affected goat flock of approximately 120 goats over several years. The EDTA blood samples were screened with RT-qPCRs and serum samples were tested using a commercial BTV cELISA. Overall, the presence of BTV-25-GER2018 was detected over 4.5 years in the affected goat flock with intermittent PCR-positivity in some animals, and with or without concomitantly detected antibodies since 2015.

This study allowed for the first time a detailed long-term view on the occurrence of BTV-25, and it was for the first time possible to propagate a BTV-25-related strain in cell culture. However, it remains unclear why BTV-25-GER2018 could grow in cell culture in contrast to the closely related two other BTV-25 strains TOV [215] and BTV-Z ITL2017 [212]. BTV-25-GER2018 was propagated on BSR cells, which is in line with previous reports that BSR cells support a productive BTV infection with a clear CpE [251]. However, future studies should target, whether BTV-25-GER2018 is able to replicate in insect cell lines like in KC or C6/36 cells, as this was not part of the current study. BTV-26 could only be propagated in mammalian cells, suggesting that BTV-26 might be unable to initiate infection in KC cells. With reverse genetics the segments Seg-1/VP1, Seg-2/VP2, Seg-3/VP3 and Seg-7/VP7 were identified to possibly play a role in the inability of BTV-26 to replicate in KC cells [213]. However, TOV and BTV-Z ITL2017 failed to be propagated in both, mammalian cell lines and insect-derived cells [212, 215]. Similar studies with the different BTV-25 strains could reveal genome segments restricting the growth of both BTV-Z ITL2017 and TOV in cell culture and should be a major aim in future studies. Nevertheless, differences in the handling and isolation procedure between the different laboratories cannot be excluded to play a role either. It has been known for a long time, that the conserved terminal ends of BTV play a role in virus replication [252]. However, only a 'serotyped' TOV strain has been available for in vitro studies so far, where the ORFs of the TOV genome segments were inserted into a BTV-1 backbone [221]. Hence, the NTR of TOV was that of a classical viral strain. Only this recombinant BTV-TOV virus has been available for VNTs until now. Our BTV-25 isolate for the first time enables *in vitro* and *in vivo* studies with a '100%' representative BTV-25 genome.

Furthermore, we have robust evidence for persistent BTV-25 infection based on the goat flock monitoring over several years. The lack of neutralising antibodies is a further hint for persistent infections. Unfortunately, we can only suspect a 4.5-year persistence, because further consecutive yearly samples were not available for our study. In general, *Orbiviruses* are not known for causing persistent infection [140]. However, persistent infection of γδ T cells was suggested as overwintering strategy for Bluetongue virus [55]. But no other working group could reproduce the results of Takamtsu et al, and the current opinion is therefore that BTV infection might be prolonged but not persistent. Interestingly, for *Middle Point Orbivirus* (MPVO) another species of *Orbiviruses,* an apparent persistent infection in naturally infected sentinel cattle has also been shown recently [253]. Based on our results and the results

reported for TOV-infected goat flocks [222], the possible persistence mechanisms of atypical BTV infections should be further investigated.

Interestingly, goats in the BTV-25-GER2018-positive goat flock, showed variable genome loads independently of their antibody level. The renewed positive virus detections of several goats during the surveillance period could be also interpreted as re-infections. On the other hand, the virus might persist in the goats without permanent viremia, and a kind of "inactive carrier goats" might exist. Several animals showed only a weak antibody response and low BTV RNA levels during the monitoring period. All these findings are in strong contrast to infections with classical BTV strains 1-24. Therefore, also alternative transmission pathways should be further studied.

Atypical BTV strains do not only differ in their biological characteristics, but also concerning the genome sequence level. For BTV-25-GER2018, the nt identities for segment 2 varied from 40.9% (BTV-12) to 60.8% (BTV-10) for the classical serotypes 1–24. On the other hand, the identity to the atypical serotypes started from 57.5% with BTV-28/Sheep pox vaccine derived BTV to up to 83.4% with TOV. However, the atypical BTV cluster together, clearly distinct from the classical BTV strains 1 to 24. Moreover, the atypical BTV strains seem to be very well adapted to their small ruminant host by not causing any clinical disease and with evidence for persistent infection without viral clearance. Goats can be seen as the natural host for atypical BTV, and the atypical BTV strains are perfectly adapted to their host. The different pathogenesis and the phylogenetic distance to the classical BTV strains need to be considered for further determining the role of atypical BTV strains in Germany, the EU or even globally. This raises the question who was first, atypical or classical BTV, and whether they are able to reassort despite the molecular discrepancies.

5.4. Conclusion

With this thesis, it could be demonstrated that commercially available inactivated BTV-8 vaccines used for disease control during the large 2006 to 2009 BTV-8 epidemic induced a long-lasting antibody response with detectable group specific and serotype specific neutralising antibodies for up to 8 years. Furthermore, the booster effect after re-vaccination significantly increased the percentage of long-term seropositive cattle; however also the basic immunisation (initial immunisation and one booster application) alone led to a high number of seropositive cattle 5 to 8 years post vaccination. These results need to be considered e.g.

in ongoing serological surveillance studies on one hand, and support on the other hand the use of BTV-8 vaccines, as the long-lasting vaccine antibody response might efficiently support to remain or regain a BTV-8 free country status after an BTV outbreak.

While vaccination is one of the most important tools for BTV control and the long-term benefit was described in our study for inactivated BTV-8 vaccines, diagnostics is the cornerstone for early detection and supports outbreak control and trade. In this thesis, molecular serotyping was therefore also a major focus. The newly developed BlueTYPE real-time RT-PCR array enables both the single or the multiple serotype identification rapidly within one PCR run. The BlueTYPE array is not only a fast-diagnostic tool with easy handling, but also highly accurate. As multiple serotypes are circulating currently in Europe, and BTV continues to spread, a flexible diagnostic tool as the BlueTYPE array is ideal for samples of the first outbreak. Also, in laboratory's dealing with different BTV strains in cell culture, the BlueTYPE array can help to generate monospecific stocks of BTV and to detect possible contaminations. Diagnostics and typing of all 24 classical BTV serotypes is crucial for the detection, typing and control of notifiable outbreaks. However, there are also so-called atypical BTVs, which are less studied, mainly detected in sheep and goats, in most cases avirulent and different to classical BTV e.g. concerning host spectrum and immune response. Therefore, the BlueTYPE array is equipped for differentiation between the classical BTV serotypes and the atypical BTV strains.

With this work, we described in addition an atypical BTV strain occurring in Germany and were able to monitor a clinically healthy goat flock in southern Germany infected with atypical BTV-25 (strain BTV-25-GER2018) over a longer period. The low RNA levels during viremia, the fluctuations in antibody titers, the lack of neutralizing antibodies, the observation of BTV reinfections and no viral clearance on the flock level suggested very clear differences in transmission and pathogenesis in comparison to the classical strains BTV 1 to 24. Most importantly, here we describe the first BTV-25 cell culture isolate, which therefore enables now for the first time further in vitro and in vivo studies.

6. Summary

Bluetongue virus (BTV) is mainly located in regions with a tropical and subtropical climate, however since 1998, several serotypes are endemic in Southern Europe, and since the BTV-8 incursion in 2006 also in Northern Europe. Several years after the German vaccination program stopped, serum samples from cattle, which received their last vaccination dose 5 to 8 years ago, were available for serological analysis. The commercially available inactivated BTV-8 vaccines induced a long-lasting antibody response with detectable group specific and serotype specific neutralising antibodies for up to 8 years post-vaccination. Moreover, the basic immunisation alone led to a high number of seropositive cattle post vaccination. These findings encourage the usage of inactivated BTV-8 vaccines as a powerful tool for eradication with a long-term benefit. Nonetheless, before any serotype specific vaccination can be performed, it is crucial to identify the BTV serotype or eventually multiple serotypes involved in an outbreak. Here, our novel developed diagnostic tool "BlueTYPE real-time RT-PCR array" identifies fast and reliable single or multiple serotypes within one PCR run and facilitates BTV diagnostics enormously. Furthermore, the BlueTYPE array can specifically distinguish between typical and atypical BTV strains, which is necessary, as the number of atypical BTV strains are raising worldwide, but the mostly avirulent atypical BTVs are not necessary to be controlled. We could here report the occurrence of an atypical BTV-25 strain in a healthy goat flock in Germany with great differences in transmission and pathogenesis in comparison to the classical strains BTV 1 to 24. Most importantly, BTV-25-GER2018 is the first BTV-25 cell culture isolate, which therefore enables now for the first time future *in vitro* and *in vivo* studies.

The studies summarized in this thesis produced major recent contributions to BTV research. We were able to demonstrate the great long-term benefit of inactivated vaccines used for BTV-8 eradication during the BTV-8 epidemic in Northern Europe. Furthermore, the novel BlueTYPE array extended BTV diagnostics as a fast and reliable diagnostic tool for molecular serotyping and particularly allows the differentiation between classical and atypical BTV strains. Moreover, this work contributes to an improved understanding of the pathogenesis of these atypical and mostly avirulent BTV strains and enables future work with the first BTV-25 cell culture isolate.

7. Zusammenfassung

Das Blauzungenvirus (BTV) kommt hauptsächlich in Regionen mit tropischem und subtropischem Klima vor, jedoch sind seit 1998 mehrere Serotypen in Südeuropa und seit den BTV-8 Ausbrüchen im Jahr 2006 auch in Nordeuropa endemisch. Nun, einige Jahre nach Beendigung des deutschen BTV-8 Impfprogramms, standen uns Serumproben von Rindern, die vor 5 bis 8 Jahren ihre letzte Impfdosis erhalten hatten, zur Verfügung. Dabei konnten wir eine langanhaltende Antikörperantwort mit nachweisbaren Gruppen- und Serotyp-spezifischen neutralisierenden Antikörpern für bis zu 8 Jahre nach Erhalt der letzten Impfung feststellen. Schon die Grundimmunisierung alleine hatte zu einer hohen Anzahl seropositiver Rinder geführt. Damit konnte der hohe langfristige Nutzen der inaktivierten BTV-8-Impfstoffe zur BTV Bekämpfung gezeigt werden. Bevor jedoch ein Impfprogram starten kann, ist es entscheidend, den BTV-Serotyp oder eventuell mehrere an einem Ausbruch beteiligte Serotypen zu identifizieren. Dabei erleichtert das im Rahmen dieser Doktorarbeit neu entwickelte Diagnostiktool, der „BlueTYPE Array“, die BTV-Diagnostik enorm, da er schnell und zuverlässig einzelne oder mehrere Serotypen innerhalb nur eines PCR-Laufs identifiziert. Darüber hinaus kann der BlueTYPE Array zwischen klassischen und atypischen BTV-Stämmen unterscheiden, was zwingend erforderlich ist, da die Anzahl der atypischen BTV-Stämme weltweit zunimmt. Diese meist avirulenten BTV-Stämme sind jedoch nicht bekämpfungswürdig. Auch in Deutschland konnten wir vom Auftreten eines atypischen BTV-Stammes in einer gesunden Ziegenherde berichten. Dieser BTV-25 Stamm zeigt große Unterschiede hinsichtlich der Übertragung und Pathogenese im Vergleich zu den klassischen BTV Stämmen 1-24. Erstmalig ist es in dieser Studie gelungen, ein BTV-25 Zellkulturisolat zu generieren, so dass nun weitere *In-vitro*- und *In-vivo*-Studien möglich werden.

Diese Arbeit vereint somit wichtige aktuelle Beiträge in der BTV-Forschung. Wir konnten den großen langfristigen Nutzen inaktivierter BTV-8 Impfstoffe demonstrieren. Darüber hinaus erweitert der BlueTYPE Array die BTV-Diagnostik als ein schnelles und zuverlässiges Diagnostikwerkzeug zur molekularen Serotypisierung. Der BlueTYPE Array kann zudem zwischen klassischen und atypischen BTV-Stämmen unterschieden. Mit der Charakterisierung des deutschen BTV-25 Strammes, trägt diese Arbeit zum Verständnis der Pathogenese dieser atypischen BTV-Stämme bei und ermöglicht zukünftiges Arbeiten mit diesem ersten BTV-25 Zellkultur-Isolat.

8. References

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9. Abbreviations

aa	Amino acids
AHSV	African Horse Sickness virus
Arbovirus	Arthropod borne virus
BEI	Binary ethylenimine
BHK	Baby hamster kidney
BSR	A clone of baby hamster kidney cells, BHK-21 (BSR/5)
BMELV	Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz
BT	Bluetongue
BTV	Bluetongue virus
cDNA	Complementary desoxyribonucleic acid
cELISA	Competitive immune sorbent assay
CDS	CoDing sequence
CLP	Core-like particle
CTL	Cytotoxic T-lymphocyte
CpE	Cytopathic effect
Cq	Cycle threshold
DB	Dissemination barrier
DISA	Disabled infectious single animal
DISC	Disabled infectious single cycle
DIVA	Differentiating infected from vaccinated animals
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
Dpi	Days post infection
ECE	Embryonated chicken eggs
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
EGFP	Enhanced green fluorescent protein

FMD	Foot and mouth disease
IFN	interferon
IC	Internal control
IV	intravenous
(m)RNA	(Messenger) ribonucleic acid
(k)bp	(Kilo) base pair
KC	*Culicoide*s *sonorensis*
(k)Da	(Kilo) Dalton
Mab	Monoclonal antibody
MAPK/ERK	mitogen-activated protein kinases/ extracellular signal-regulated kinases
MEB	mesenteron infection barrier
MLV	Modified-live virus
N	North
NGS	Next generation sequencing
NS	Non-structural protein
Nt	nucleotide
NTC	No template control
NTR	Non translated region
OIE	World Organisation for Animal Health
ORF	Open reading frame
PBS	Phosphate buffered saline
PC	Positive control
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real time quantitative polymerase chain reaction
S	South
SA	South African
S.c.	subcutaneous
SCAHAW	Scientific Committee on Animal Health and Animal Welfare
SISPA	Sequence-Independent, Single-Primer-Amplification
Seg	Segment
SGIB	salivary gland infection barrier

ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
spp.	*Species pluralis*, several species
TCID	Tissue culture infectious dose
Tsg101	Tumor susceptibility gene 101
TOV	Toggenburg orbivirus
TPI	The Pirbright Institute
US, U.S.	United States
Vero	African green monkey kidney
VLP	Virus-like particle
VNT	virus neutralization test
VP	Virion protein

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