CHARACTERIZATION OF EMERGING BLUETONGUE VIRUS SEROTYPE 3 STRAINS IN THE MEDITERRANEAN BASIN BY NEXT GENERATION SEQUENCING

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LIST OF ABBREVIATIONS

AA – *Aedes albopictus* clone C6/36

AHSV – African horse sickness virus

AGID – Agar Gel Immunodiffusion Assay

BHK-21 – baby hamster kidney

BT – Bluetongue

BTV – Bluetongue virus

CaPV – Capripoxvirus induced disease

c-ELISA – competitive ELISA

cDNA – complementary DNA

CPE – a cytopathic effect

DNA – deoxyribonucleic acid

dsDNA – double-stranded deoxyribonucleic acid

dsRNA – double-stranded ribonucleic acid

ECE – embryonated chicken eggs

EDTA – ethylenediamine tetraacetic acid

EHDV – Epizootic hemorrhagic disease virus

ELISA – antigen capture enzyme-linked immunosorbent assay

FMD – Foot and mouth disease

ICTV – International Committee for the Taxonomy of Viruses

IZS – Istituto Zooprofilattico Sperimentale

IZSAM – Istituto Zooprofilattico Spermentale dell'Abruzzo e del Molise, Teramo, Italy

KC – *Culicoides* sonorensis

MAbs – monoclonal antibodies

NGS – Next-generation sequencing

OIE – The World Organisation for Animal Health

PAN – Permanent Account Number

PBS – phosphate buffered saline

PPR – Peste des petits ruminants disease

PCR – polymerase chain reaction

qPCR – quantitative polymerase chain reaction

RNA – ribonucleic acid

RT – reverse transcriptase

RT-PCR – reverse transcriptase-polymerase chain reaction

qRT-PCR – quantitative reverse transcriptase-polymerase chain reaction

SISPA – sequence-independent single-primer-amplification

SN – serum neutralization

SNT – serum neutralization test

TCID50 – 50% tissue culture infective dose

VERO – African Green monkey (*Cercopithecus aethiops*) kidney

VP – viral protein

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1. INTRODUCTION

Bluetongue (BT) is a vector-borne viral OIE-listed disease of wild and domestic ruminants caused by bluetongue virus (BTV). The disease is generally characterized by inflammation of mucous membranes, oedema and haemorrhages which can be responsible for up to 75% mortality in livestock. BT has severe economic repercussions for the livestock industry (VELTHUIS et all, 2010) due to direct losses caused by the infection but also due to indirect losses as a result of restrictions on animal trade (DAL POZZO et al., 2009.; MÉROC et al., 2009.; ZIENTARA et PONSART, 2014.; TAGO et al.,2014.).

BTV is the prototype of the genus *Orbivirus*, within the family *Reoviridae* and possess a linear double stranded segmented (Segment 1 to Segment 10) RNA genome coding for structural and no-structural proteins. BTV exists in multiple serotypes and serotype-specificity is given by the antigenic relatedness of the major outermost structural protein of the virion, VP2, encoded by Seg-2 and for a less extent by VP5, encoded by Seg-6.

The virus is transmitted between hosts by several species of biting midges of the *Culicoides* genus, critical for the geographical range of the disease (CONTE et al., 2003.). BT was first described in South Africa in Merino sheep in the late 18th Century (WALTON, 2004.) as “malarial catarrhal fever” and “epizootic catarrh of sheep” (HUTCHEON, 1902.) and considered, for decades, endemic in the tropical areas of the world comprised between latitudes 35°S and 40°N with epidemic incursions in temperate zones where the presence of the vectors allowed its transmission. However, in the last 20 years epidemiology of BT changed drastically in Europe and the regions historically free of BT have been experiencing major outbreaks in sheep and cattle.

In this thesis, we will focus upon epidemiology of BT in Europe with particular emphasis on the recently emerged BTV-3, which represents a great concern for the entire European livestock industry.

2. BACKGROUND

2.1. FAMILY REOVIRIDAE

The family *Reoviridae* is one of the most complex in all of virology, currently comprising 15 recognized genera within two subfamilies of viruses (Fig 1) with genomes composed of multiple (10–12) segments of double-stranded RNA (dsRNA). Individual viruses within the family infect a remarkable variety of hosts, including mammals, birds, reptiles, amphibians, fish, mollusks, crustaceans, insects, plants and fungi. A prefix "reo-" is derived from respiratory enteric orphan viruses. The term "orphan virus" refers to the fact that some of these viruses have been observed not associated with any known disease. Reoviruses are transmitted also in different routes. Some of them replicate only in certain vertebrate species (Orthoreoviruses and Rotaviruses) and are transmitted between hosts by the respiratory or fecal–oral route. Other vertebrate viruses (Orbiviruses, Coltiviruses and Seadornaviruses) replicate in both arthropod vectors (e.g. biting midges, mosquitoes or ticks) and vertebrate hosts. Plant viruses (Phytoreoviruses, Fijiviruses and Oryzaviruses) replicate in both plants and arthropod vectors (leafhoppers). Viruses that infect insects (Cypoviruses) are transmitted by contact or fecal–oral routes.

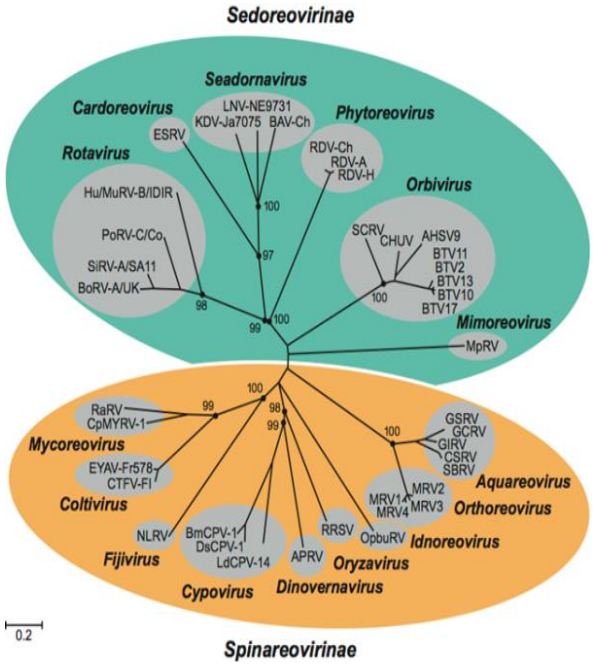


Figure 1. Taxonomy of *Reoviridae* family.
(source: https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsrna-viruses-2011/w/dsrna_viruses/190/reoviridae-figure)

Genus Orbivirus

The genus *Orbivirus* is the largest one as it includes 22 species (representing 22 distinct virus serogroups) recognized by the International Committee for the Taxonomy of Viruses.

The genus *Orbivirus* is named for their doughnut-shaped capsomeres (*orbi* means ring in Latin). They are icosahedral, non-enveloped dsRNA viruses which have a wide host range that includes domestic and wild ruminants, equines, marsupials, sloths, bats, birds and humans (MERTENS, 2005.; ATTOUI, 2009.).

The genus *Orbivirus* comprises several viral species, and some of them can cause economically important diseases of domestic and wild animals. These include BTV, African horse sickness virus (AHSV), equine encephalitis virus (EEV) and epizootic hemorrhagic disease virus (EHDV).
The *Orbivirus* genome consists of ten linear segments of dsRNA (Seg–1 to Seg–10 in order of decreasing molecular weight), which are covered within a triple layered icosahedral protein capsid (MERTENS, 2005.). The genome segments encode seven structural (VP1 to VP7) and five non-structural (NS1, NS2, NS3/NS3a, NS4, and S10-ORF2) proteins (SCHWARTZ-CORNIL et al., 2008.; RATINIER et al., 2011.; STEWART et al., 2015.). VP7 protein, one of the structural proteins, is a major determinant of serogroup specificity, and most of the serological assays to detect BTV are based on detecting anti-VP7 antibodies (ROJAS, 2019.).

On contrast, the outer capsid layer of the *Orbiviruses* interacts with cellular components of the mammalian immune system and with the neutralizing antibodies. Neutralizing antibodies are produced after infection, in response to virus infection in mammal host. The outer capsid proteins of each *Orbivirus* specie interact differently with antibodies of the host. That means that the variations in the outer capsid proteins determine the specificity of interaction between virus capsid and neutralizing antibodies. In other words, variations in the outer capsid proteins define the identity of the different virus serotype within each *Orbivirus* species.

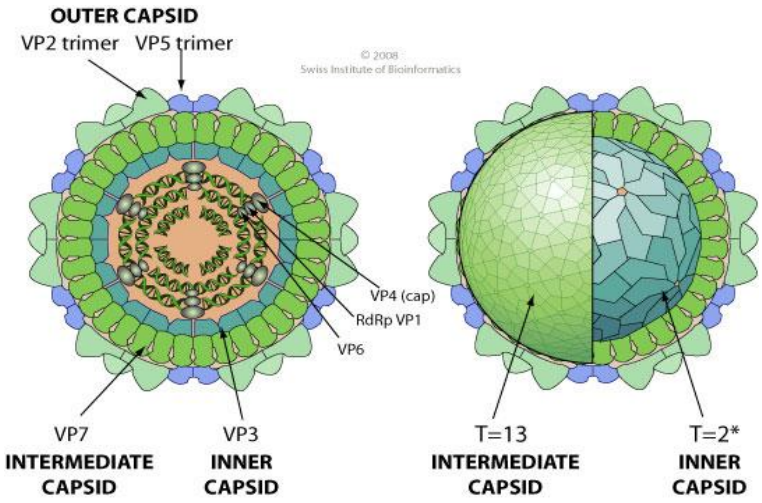


Figure 2. Structure of the Orbivirus.
(source: https://viralzone.expasy.org/106?outline=all_by_species)

Bluetongue (BT) virus has been studied in the most profundity of al *Orbiviruses*. Up to 2008, 24 serotypes of BTV were officially recognized (MAAN et al., 2008.). However, in the last years new and generally asymptomatic BTV serotypes have been identified in the field (LORUSSO at all, 2018.). These involve BTV-25 (TOV strain) from Switzerland, BTV-26 from Kuwait, BTV-27 (variants 01, 02 and 03) viruses from Corsica (France), BTV-XJ1407 from China, a BTV strain isolated from a sheep pox vaccine (SP vaccine derived BTV) and BTV-X ITL2015 from Sardinia, Italy (HOFMANN et al, 2008.; MAAN et al, 2011.; ZIENTARA et PONSART 2014.; SCHULZ et al, 2016.; SUN et al, 2016.; BUMBAROV et al, 2016.; SAVINI et al, 2017.; LORUSSO et al, 2018.). BT serotypes are originally identified and distinguished by a combination of their biological host and vector, clinical signs and specific serological assays. Beside serotypes, there are many different “topotypes" (groups reflecting the geographical origins of each virus isolate) and “nucleotypes” (lineages). These terms have been used even though they do not have a formal taxonomic status (MELLOR et al., 2008.).

BTV strains have notably variations in the nucleotide sequence in their RNA genome, which reflect their geographic origins. Considering this, we can divide BTV into “Eastern” and “Western” groups –topotypes (MAAN et al., 2007.; MERTENS et al., 2007.) even within the same BTV serotype. Viruses belonging to the Western topotype group are present in Africa, Europe and Americas, while those from Eastern topotype group are endemic in Asia, Indonesia and Australia (GOULD and HYATT, 1994.; CARPI et all, 2010.). The ability of each strain to cause a BTV disease varies, in meaning that each serotype may not cause bluetongue disease in each host (WALTON, 2004.).

In addition, different BTV strains (including the different serotypes and topotypes) in the same genus *Orbivirus* can reassort and potentially create large numbers of different progeny virus strains. Indeed, the number of possible reassortments in the case of BTV, which has 10 segments, increases with the number of co-circulating serotypes (e.g., 1024 for 2 serotypes, 59049 for 3 serotypes (DUNGU et al., 2004.; SAEGERMAN et al, 2008.). Moreover, the phenomenon of reassortment has already been demonstrated during the BTV outbreaks in Europe.

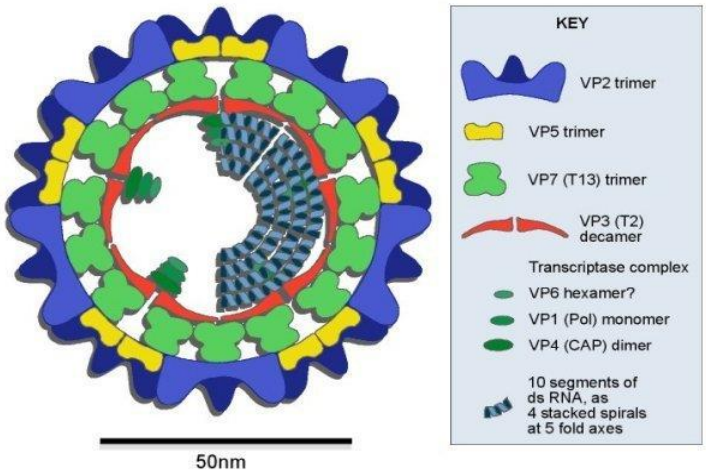


Figure 3. The structure of the bluetongue virus.
(source: http://www.fao.org/ag/againfo/programmes/en/empres/gemp/avis/A090-bt/tools/0-diag-BTV-core-organisation.html)

2.2. BLUETONGUE DISEASE

Despite the fact that BTV has been characterized both structurally and genetically, the life cycle of BTV is not clearly understood. However, basic mechanisms of infection and pathogenesis of a BT disease have been described. Bluetongue disease infects ruminants and camelids (COETZEE, 2014.) causing severe morbidity and mortality in sheep (DE KOEIJER, 2007.) and in white tail deer (HOWERTH, 1988.). However, infection can be asymptomatic in some domestic and wild ruminants (ROJAS, 2019.). The severity of disease varies among different species; however symptoms are most serious in sheep. In highly susceptible ruminants morbidity can be as high as 100%. Mortality averages from 2-30% but it may rise up to 70%. Cattle and goats are typically subclinically infected and they are often considered as amplifying hosts in endemic regions (COETZEE, 2012.). Even subclinical, BTV typically cause significant economic losses in all livestock populations.

Culicoides bite the ruminant host, small amount of midge saliva are injected into the skin of a ruminant. In most cases the viral amount in the infected insect saliva is very low. However, a single bite from a transmission-capable insect will accurately infect a susceptible animal. When inside the ruminant blood stream, the virus travels to the regional lymph node where initial replication occurs. After the initial replication, a virus spreads to various tissues and tissue cells, however, replication occurs primarily in mononuclear phagocytes and endothelial cells. BTV has an organ tropism to the endothelial cells, in which it replicates causing cell injury and necrosis. In vivo

experiments, BTV can also infect ovine and bovine endothelial cells, monocytes, lymphocytes and skin fibroblasts primary culture cells (MELLOR et al., 2008.). Viraemia in BTV-infected ruminants is highly cell associated. Because of the short lifetime of platelets, virus is most associated with erythrocytes. Ruminants, after infection with BTV develop a prompt and high titer antibody response to a variety of viral proteins. Serotype-specific neutralizing antibodies are directed against viral VP2 protein (MACLACHLAN, 2004.).

Clinical signs and lesions in BTV infected ruminants are reflecting this endothelial injury due to the virus replication (HOWERTH, 2015.). Endothelial injury at that time develops alterations in the vascular permeability. Extremely increased vascular permeability leads to the vascular damage and oedema in tissues causing lung (pulmonary oedema), vascular thrombosis, hemorrhages and tissue infarctions. A white-tailed deer, the specie which is highly susceptible to BTV, develop harmful coagulopathy as a consequence of BTV-induced destruction to endothelial cells.

Early signs of BT are, however, unspecific and are also present in many other infectious diseases (OIE, 2018.). This non-specific signs include pyrexia, lack of appetite, depression and milk production loss in lactating animals. Its severity and range may be influenced by numerous factors such as species, age of animals, virus strains and immune status of the host.

In sheep, specie in which clinical disease is more evident, disease usually manifest with symptoms including: fever, conjunctivitis, lacrimation, congestion of nasal and oral mucosa and edema of the face and lips. In rare cases, clinical signs may not develop further from this ones, but in most cases these signs progress to more severe clinical forms such as salivation, mucopurulent nasal discharge, severe facial edema, different localized ulcerations of the oral and nasal cavity, hemorrhages in the nose, lips and tongue. Described lesions are typically edematous, erosive, and hemorrhagic. These signs can sometimes lead to cyanosis of the tongue, the symptom which gives a BT originated name. However, a swollen cyanotic tongue is rarely detected. In most cases, pharyngeal and esophageal paresis and respiratory distress are observed. Most sheep also develop coronitis. The symptom begins with mild coronitis, developing into a cronical lameness. Clinical signs such as intensity and diffused redness in the hoof coronary band occur in 8 to 14 days post-infection. In the most severe cases, serum exudate around the coronary band can be seen. This hyperemia at the coronary bands of the feed is apparently painful, causing inability to move and even to eat. Animals with this kind of symptoms often develop characteristic knee-walking. Weakness and fatigue can lead to the respiratory distress and bacterial complications. Acute infection may also lead to a dead within 14 days post-infection.

However, sheep that are native to tropical and subtropical regions of the world where BTV is endemic are usually resistant to BT, while finewooled European breeds such as Merino are highly susceptible. Nutritional and immune status, age, presence of additional pathological stressors also influences the intensity of BT in individual sheep. Also, BTV cross-reacts with many antigenically related viruses including viruses that cause African Horse sickness and epizootic haemorrhagic disease of deern (ROJAS, 2019.).

In cattle signs are usually milder than in sheep. However, circulation of several BTV serotypes in the same region may increase clinical signs in cattle. Despite not showing noticeable clinical signs, cattle have prolonged viaremia (BARRATT-BOYES et MACLACHLAN, 1994.). Since their viraemic phase may elapse without showing any clinical signs, cattle play an important role in the epidemiology of BT disease.

Despite the fact that the bite of infected *Culicoides* remains the main source of BTV infection, it is mandatory to be aware of other possible routes of BTV transmission. In addition to being spread between ruminants by *Culicoides* insects, evidence suggests that at least some strains of BTV can be transmitted directly from host to host by one or more secondary mechanisms (BELBIS, 2013.), (VAN DER SLUIJS, 2016.; ROJAS, 2019.) including transplacental, iatrogenic and oral transmission, as well as potentially being transmitted mechanically between hosts on the mouthparts of biting flies. During the First International Symposium on Bluetongue and related *Orbiviruses* (BARBER, 1985.) it was confirmed that BTV infection of bovine and ovine fetuses could produce developmental defects that resulted in death or deformities in offspring and poor viability of the newborn. Virus may also be able to persist in either vector or host for longer than a normal transmission cycle via long-lived infected *Culicoides*, persistently infected ruminants or other methods.

2.3. BLUETONGUE DIAGNOSING

A positive diagnosis of BTV usually involves detection and identification of BTV specific antigens, antibodies or RNA. Biological samples are taken from animals that are potentially infected using virus isolation and serological or molecular assays to identify the virus serogroup and serotype. Serological BTV tests are mainly focused on the identification of viral antibodies. Currently, the OIE manual of standard for diagnostic tests and vaccines (OIE, 2018.) cites the competitive ELISA (c-ELISA) as a prescribed test for the detection of BTV group specific antibodies. Serum neutralization test (SNT) then is regularly used to detect neutralizing antibodies that are specific for each BTV serotype in serum sample and the test is highly specific for each BTV serotype (JEGGO et al., 1986.).

Molecular assay methods are widely used to identify viral RNA of BTV from various biological samples (whole blood, spleen, lymph nodes) or from cell-culture isolates by targeting specific viral segments (ORRU et al., 2006.; ANTHONY et al., 2007.; SHAW et al., 2007.; WILSON AND MELLOR, 2009.; HOFMANN et al., 2008.) which will be described in extension.

Differential diagnosis for BT relies on the basic presentation of edema, ulcerations and epithelial lesions. BT initial signs are very similar to foot and mouth disease (FMD); however, FMD lesions are vesicular and erosive, although BT lesions are hemorrhagic, edematous and erosive. BT lesions are typically situated at the back and lateral margins of the tongue, whereas FMD lesions are situated at the tip and dorsum of the tongue. FMD lesions also rarely present on eyelids, while this clinical sign is frequent in BTV infections. Beside clinical signs, disease transmission is also an

indicator for differential diagnosis. BT presence can be sporadic due to its transmission by biting midges, whereas FMD is highly contagious which significances high morbidity in the herd. Peste des petits ruminants (PPR) is another infectious disease with similar manifestation as BT. In case of PPR, nasal discharge may also be mucopurulent with lesions appearing in the oral and nasal tract, nostrils and eyelids. However, PPR cause diarrhea but no coronitis. Moreover, like FMD, PPR is highly contagious, and thus, morbidity is notably high in in case of PPR. Beside FMD and PPR, vesicular stomatitis can also be mistaken for BT disease because of similar lesions appearance.

Although these differential characteristics of BT clinical manifestation can be useful in a field diagnosis, only serological and molecular methods can provide a definitive BT diagnosis.

2.4. VECTORS

Culicoides biting midges are one of the smallest blood sucking insects ranging in size from 1 to 3 mm in length. There are approximately 1 250 named species of *Culicoides* biting midges in the world, but only 50 of them have been shown to act as a competent vectors of BTV (WILSON et MELLOR, 2009.). The ability to act as an effective vector of BTV further requires that a species feeds mainly or wholly upon ruminants and occurs in large numbers, and as a result only a handful of *Culicoides* species are thought to be able to act as effective vectors in the field. These include *C. imicola* (the principal vector in Africa, the Middle East, parts of south Asia and parts of Southern Europe), *C. sonorensis* (the principal vector in North America) and *C. brevitarsis* (the principal vector in Australia).

Both BTV infection and BT disease usually occur during late summer and early autumn, in seasons when numbers of insect vectors are highest. Climatic factors carry an important role in the occurrence of BTV infection in host ruminants and also the effect the amount of vector populations and interval of their seasonal activity. Depending on a geographic region, different *Culicoides* species can be involved in different BTV serotype transmission. Moreover, a specific bond between the particular *Culicoides* species distribution and BTV serotypes prevalence has been demonstrated (TABACHNICK, 2004.).

Adult *Culicoides* are active in the period from sunset to sunrise (e. g. crepuscular insect). Nevertheless, studies on BTV-8 in 2006 have shown that a certain level of vector activity can be detected also during a day-light (MEISWINKEL et al., 2008.). The average lifetime of adult midges is about 3 to 6 weeks, but they can survive for up to nine weeks (EFSA, 2007.). They prefer a temperature between 25 °C and 30 °C, but *Culicoides* activity has not been recorded below approximately 13°C and above 35°C (WARD, 1995.; LISTEŠ et al., 2004.). Temperature conditions are essential not only for the survival of the vector but are crucial also in the replication cycle of the virus within the vector. Studies have shown that high mean monthly temperatures in winter support the survival of *Culicoides imicola* larvae. Also, it is known that humid areas significially accelerate Culicoides reproduction. The time required by an insect to digest a blood meal is also

reduced at higher temperatures, which increases the frequency of blood-feeding. However, the most important effect of a temperature rise is to short the generation time of the virus and to increase the possibility for transmission (WILSON et MELLOR, 2009.).

Even though biting midges are ubiquitous, warm, dump and muddy areas constitute their ideal habitat. Most *Culicoides* species of veterinary importance tend to breed in organically enriched, damp soil such as is found adjacent to their hosts in and around farm holdings (WILSON et MELLOR, 2009.). Also it is supposed that adult individuals remain around the area where they were born for all their lifetime.

Insect becomes infected by biting a viaremic ruminant and ingestion of a blood meal from infected animal. As with mosquitoes, blood-feeding is limited to female *Culicoides*, which demand protein for the production of eggs (WILLSON at MELLOR, 2009.). In order to have viral transmission from the ruminant host to the insect, it is necessary that the blood meal takes place during the viremic stage of the infection of the host -in the period up to 11 days postinfection in the sheep and 49 days postinfection in cattle (BONNEAU et al., 2002.). *Culicoides* are, however capable of transmitting the virus to a new ruminant host only in case when they take a subsequent blood-meal.

Once ingested, a virus must infect the insect cells, replicate in them, escape into the body cavity of the insect (haemocoel) and infect and replicate in the salivary glands before it can be transmitted to a new host (WILSON et MELLOR, 2009.). After passage into the lumen of the hind part of the insect mid-gut, BTV has to gain access to the epithelial cells of the insect host, before the potentially hostile environment in the gut lumen inactivates it. BTV attaches to the luminal surface of the insect cells, both infecting and replicating in the virus epithelial cells. After replication, virus particles travel through the abluminal surface, lamina lucida and lamina densa of the epithelial cells into the hemolymph circulation. From there, virus is spread into the secondary target organs including salivary glands. After secondary replication in salivary glands, virus is released within the salivary ductus. From this point, the transmission of the virus into the new host can begin. Transmission to the ruminant host is possible approximately from 10 to 14 days post infection (WITTMANN et al., 2002.). Once affected with a virus, *Culicoides* remain infected along all their life.

Culicoides imicola Kieffer (Fig. 4) is the main vector of BTV. Considering the Mediterranean region, the most important vector is *Culicoides imicola*, which is probably responsible for 90% of disease transmission (MEISWINKEL et al., 2004.). The specie is reported widely through the Mediterranean Basin from Algeria, Cyprus, Egypt, Israel, islands of Lesbos and Rhodes, Morocco, Portugal, Spain, Tunisia, Turkey, Italy and the Balearic Islands of Menorca and Mallorca (MEISWINKEL et al., 2004.).

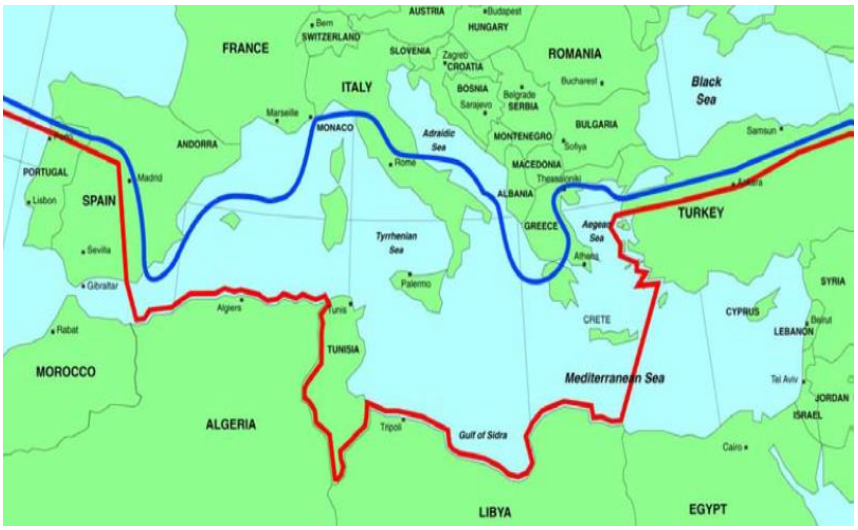
Before 1980, BTV outbreaks in the Mediterranean Basin were localised and sporadic. However, risk maps which prognosticate the distribution of *C. imicola* from climatic variables and vegetation indexes suggest that *C. Imicola* is even more widespread than it was thought. The most likely

causes of these epidemiological changes in increasing in the range and abundance of *C. imicola* are climate changes (WITTMANN et BAYLIS, 2000.).

Figure 4. *Culicoides imicola Kieffer*.
(source: https://nl.wikipedia.org/wiki/Culicoides_imicola)

BTV appears to be unable to replicate at temperatures below 12°C. As a result, in many temperate regions classical BTV transmission is almost completely interrupted for several months of the year by cold weather. However, because of insect capacity of "overwintering" (WILSON et MELLOR, 2009.) outbreaks often resume after interruptions far longer than the typical lifetime of an adult vector or the normal period of host infectiousness.

While *Culicoides* are typically capable of active flight over short distances (1–2 km), they also can be blown passively on the wind for long distances because of their small size. The transport of BTV-infected *Culicoides* on the wind, particularly over bodies of water, has been implicated as the most possibly cause of numerous introductions of BTV (WILSON et MELLOR, 2009.).



— Known northern limits of *Culicoides imicola* distribution prior to 1999
— Known northern limits of *Culicoides imicola* distribution in 2002

Figure 5. The known northernmost limits of Culicoides imicola in the Mediterranean Basin prior to 1999 and in 2002 (source: https://www.researchgate.net/figure/The-known-northernmost-limits-of-Culicoides-imicola-in-the-Mediterranean-Basin-prior-to_fig1_43343967)

2.5. BLUETONGUE IN THE EUROPE AND MEDITERRANEAN BASIN

2.5.1. A general background

The first recognized outbreak of BTV outside Africa occurred in 1943 in Cyprus (GAMBLES, 1949.). After the wave of outbreaks that affected Europe until 1979 (SELLERS and PEDGLEY, 1985.) a new wave started in 1998. Between October and December, 1998, 84 outbreaks were recorded in the four Greek islands (Rhodes, Kos, Samos and Leros). Approximately 3 000 animals (exclusively sheep) died or were culled due to BT infection and total of causative virus was identified as BTV-9. Since 1998, BTV has moved northward reaching the fifth parallel in some parts of the world (regions of Asia, North America and North Europe) where the virus had never been reported before.

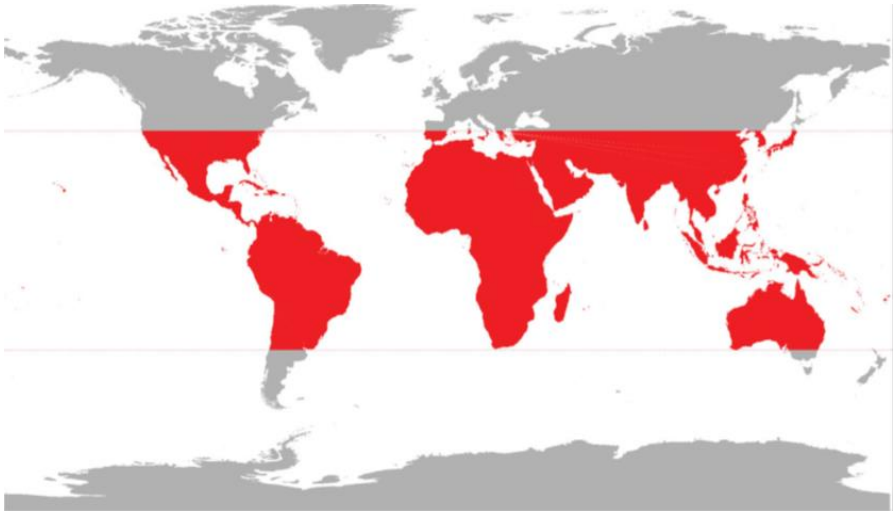


Figure 6. Map of the estimated global range of bluetongue virus prior to 1998.
(source: https://www.researchgate.net/figure/Map-of-the-estimated-global-range-of-bluetongue-virus-prior-to-1998_fig4_26748106)

Apart from the BT outbreak in Central Europe, several other outbreaks have been reported around the Mediterranean basin in the last 40 years (POLYDOROU, 1978.). Since 1998, 9 different BTV serotypes including BTV-1, BTV-2, BTV-4, BTV-6, BTV-8, BTV-9, BTV-11 and BTV-16 have been affecting countries in Mediterranean region. However, two epidemiologic systems are dominating (SAEGERMAN et al., 2008.). The first system is located in the eastern part of a basin, where serotypes 1e, 4e, 9e and 16e were identified (MELLOR, 2004.). In this system BTV strains are originated in the Near, Middle or Far East and have been spread across Turkey, Greece and Bulgaria.

In the western focus (MELLOR, 2004.) BTV was first detected in Tunisia but then infected animals were reported in Algeria and Morocco in North Africa and, subsequent in Italy, on Corsica and on two of the Balearic islands of Spain. The second epidemiological system comprises the western part of the Mediterranean Basin, where serotypes -1w, BTV-2w, BTV-4w and BTV-16w were identified.

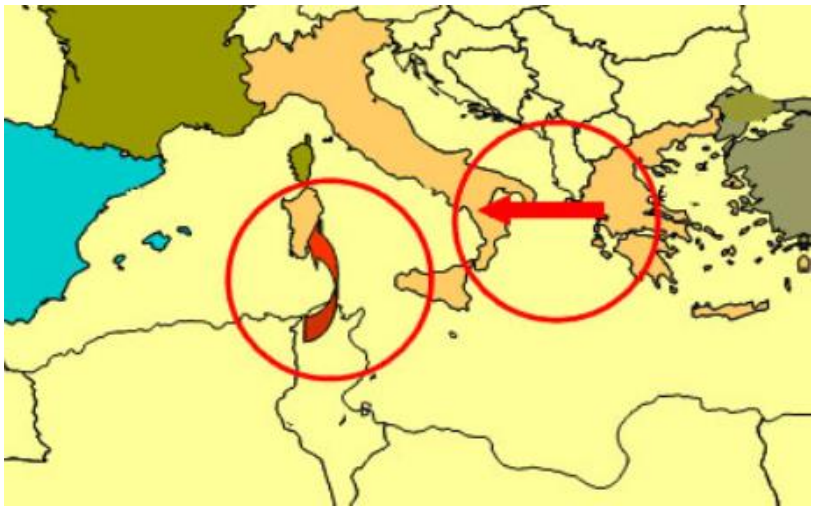


Figure 7. The two origins of the bluetongue outbreaks in Mediterranean Europe.
(source: https://www.oie.int/doc/ged/D6496.PDF)

Figure 8. Three principal routes by which bluetongue virus is introduced into Europe. A, Morocco-Spain; B, Tunisia-Sicily; C, Turkey-Greece/Bulgaria.
(source: https://www.researchgate.net/figure/The-three-principal-routes-by-which-bluetongue-virus-is-introduced-into-Europe-A_fig1_26748106)

In the summer of 1999, BTV outbreak was reported in Turkey and Bulgaria. BTV serotype 4 had been circulating through Greece, most likely coming from Turkey. In summer of 2000, the same serotype moved westward crossing the Mediterranean Sea reaching Italy (mainland and Sardinia), France (Corsica) and Spain (Balearic Islands of Menorca and Mallorca) (POTGIETER et al., 2005.; RODRIGUEZ-SANCHEZ et al., 2008.). Macedonia, Albania, Serbia and Montenegro, Bosnia and Hercegovina and Croatia were affected subsequently with the same serotype.

In this same year of 2000, BTV-16 was registered in Turkey and Israel (ERTURK et al., 2004.; SHIMSHONY, 2004.). In 2001, the disease reached not only central and north-west mainland Greece but belonging neighboring Balkan countries. After effecting Greece and Balkan countries, presence of BTV was confirmed in Tunisia and north-eastern Algeria. In these occurrences three (BTV-4, BTV-9 and BTV-16) serotypes in total were founded and diagnosed (GÓMEZ-TEJEDOR, 2004.). Epizootics of BTV were introduced in the region from two different origins: BTV-2 was introduced from the south and BTV-4, BTV-9 and BTV-16 from the east (GÓMEZ-TEJEDOR, 2004.).

BTV-1 has been also circulating through the North of Africa, Sardinia, Cyprus, Algeria, Morocco, Spain and South France in the period of 2003-2007. Shortly after the announcement of the presence of BTV-1 in the North of Spain in 2007, BTV-8 was also detected in a neighboring area (RODRIGUEZ-SANCHEZ et al., 2008.).

In 2004, a novel different strain of BTV-4 has been reported in Morocco, Spain and Portugal (BREARD et al., 2004.). During the same year, BTV-9 was reported in Serbia (RODRIGUEZ-SANCHEZ et al., 2008.) being the furthest northern outbreak reported thus far, reported before BTV-8 appeared in Central Europe. In 2004, BTV-16 was isolated for the first time in Croatia and France (Corsica Island). In October 2008, the presence of BTV-6 was also confirmed in Western Europe. A strain of BTV-11 was detected in Belgium during 2009.

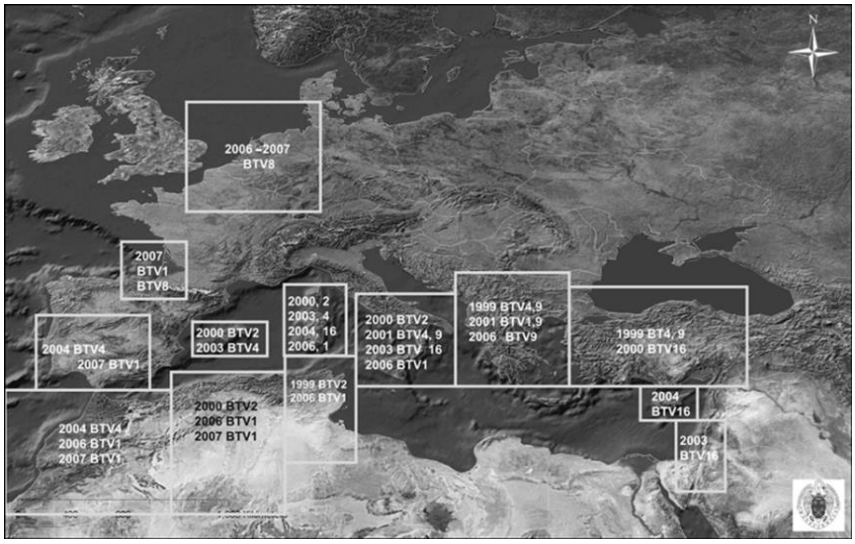


Figure 9. Distribution of BTV outbreaks detected in the Mediterranean Basin between 1999 and 2007.
(source: https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1865-1682.2008.01029.x)

The potential for BTV to enter the region of Europe occurred either by the legal and illegal movement of infected animals or by the wind dissemination of infected insects (MELLOR et al., 2008.). Trade routes which were established between Europe and Middle East in 1990's may have caused the increase of number of host animal movements and potentially increase of BTV infection occurrence in Europe (MELLOR et al., 2009.). The incursion of BTV into the Mediterranean Basin had caused great economic losses, partly due to the disease itself but mostly linked to the total ban of ruminant trade between the infected and non-infected regions.

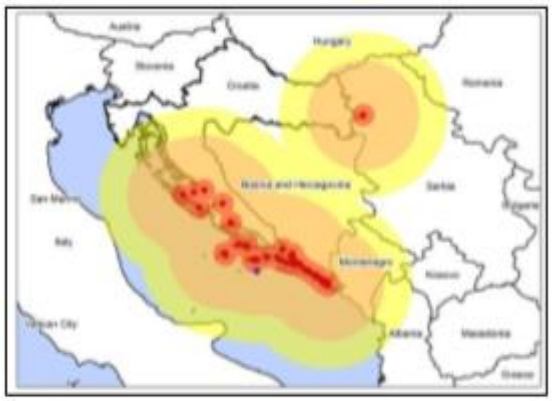
Also, recent studies on BTV have proved that particular *Culicoides* species that had never been reported before to be compatible with the transmission of BTV, now carry an important role in the transmission of BTV. Successful adaptation of the *Culicoides vectors* to overwinter in northern districts (beyond 50° N) (TAKAMATSU et al., 2003.) is most likely one of the main reason why BT disease was spread in parts of Central Europe (Belgium, France, Germany, Luxembourg and Netherlands) where the disease had never been reported before 2006 (MEHLHORN et al., 2007.; RODRIGUEZ-SANCHEZ et al., 2008.). Moreover, BTV-8 that affected Central Europe has never been detected in the Mediterranean area and its symptoms affect also cattle, which, when infected with any other ‘‘Mediterranean’’ serotype, become asymptomatic hosts.

In 2014 a novel multi - reassortment BTV-4 strain emerged in Southeast Europe (probably derived from North Africa) and moved northward reaching Central Europe (Austria) in late 2015. By the end of the summer of 2016, the same serotype was also detected in both southern and north-eastern Italy, Montenegro, Bosnia and Hercegovina.

2.5.2. Situation of BTV in Croatia

In Croatia, there are no documented evidences of BTV infection in the ruminant population prior to 2001. In December 2001, Croatia announced the suspicion of BT in three outbreaks in the region of Dubrovnik and BTV-9 was confirmed (LISTEŠ et al. 2004., LISTEŠ et al. 2011.). In October and November 2004, there was a first report of BTV-16 in Croatia (LISTEŠ et al. 2009.); namely apart from few cases reported in Greece between 1999 and 2000, BTV-16 has never been reported in the Balkan Peninsula before (LISTEŠ et al., 2009.). In 2010, 10 Oryx antelopes were imported to Croatia from the Sultanate of Oman when BTV-1 and BTV-16 were detected in 4 animals housed in the quarantine facility on Veliki Brijun Island (BOSNIĆ et al., 2015.). It was proven that the antelopes had already been infected when transported from the Sultanate of Oman (BOSNIĆ et al., 2015.). While BTV-16 had been previously detected in Dubrovnik-Neretva County (LISTEŠ et al. 2009.), BTV-1 was identified for the first time (BOSNIĆ et al., 2015.).

In October 2014, authorized veterinarians notified suspicion on BT, again in Dubrovnik-Neretva County. The suspicion was based on clinical signs in 42 sheep on 13 different suspicion properties. Between October 2014 and April 2015, 80 outbreaks of 253 cases in 5 counties (Vukovar-Srijem, Zadar, Šibenik-Knin, Split-Dalmatia and Dubrovnik-Neretva Counties) have been reported. In September 2015, BTV-4 reoccurred in Split-Dalmatia County. 1 outbreak of 1 case in sheep was confirmed (NIEDBALSKI, 2016.).



Legend:

- Serotype 4
- Serotype 1

Figure 10. Positive BTV cases in Croatia, 2015.
(source: http://www.veterinarstvo.hr/UserDocsImages/Laboratoriji/Program%20BPJ%20Rev2.pdf)

In September 2016, there was an outbreak of BTV-4 in Metković, Dubrovnik-Neretva County counting. The outbreak was confirmed with c-ELISA and real-time PCR positive testing.

Figure 11. Positive BTV-4 cases in Croatia, 2016.
(source: http://www.veterinarstvo.hr/UserDocsImages/Laboratoriji/Program%20BPJ%20Rev2.pdf)

2.6. BLUETONGUE DETECTION

2.6.1. Serolocal methods

2.6.1.1. Competitive enzyme-linked immunosorbent assay (c-ELISA)

ELISA is a commonly used analytical biochemistry assay which uses a solid-phase enzyme immunoassay to detect the presence of a ligand (a protein) in a liquid sample using antibodies directed against the protein to be measured. C-ELISA (also known as inhibition ELISA) is the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody. First, the primary antibody is incubated with the sample antigen and the resulting antibody–antigen complexes are added to wells that have been coated with the same antigen. After an incubation period, any unbound antibody is washed off. The more antigen in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be a smaller amount of primary antibody available to bind to the antigen coated on the well, resulting in a signal reduction.

2.6.1.2. Serum neutralization test and serotyping by virus neutralization

Virus neutralization (VN) serology is able to identify serotype-specific neutralizing antibodies as well as determine their titer. It is a specialized type of immunoassay because it does not detect all antigen–antibody reactions but antibody that can block virus replication. This is important because related groups of viruses may share common antigens, but only a fraction of these antigens are targets of neutralizing antibody. A virus serotype is usually based on virus neutralization.

Neutralization tests are, therefore, type specific for the currently recognized BTV serotypes that have been isolated in culture and can be used to serotype a virus isolate or can be modified to determine the specificity of antibody in sera (OIE, 2018.). There is a variety of tissue culture-based methods available to detect the presence of neutralizing anti-BTV antibodies. Cell lines commonly used are BHK, Vero and L929. One of the methods is microtitre neutralization. Approximately 100 TCID50 (50% tissue culture infective dose) of the virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of standard antiserum serially diluted in tissue culture medium (OIE, 2018.). Approximately 104 cells are added per well in a volume of 100 µl, and after incubation for 4–6 days, the test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show 75–100% CPE. The unidentified virus is considered to be serologically identical to a standard BTV serotype if both are neutralized in the test to a similar extent.

2.6.1.3. Immunological serogrouping of viruses

The success of virus isolation techniques is evaluated by testing for the presence of BTV in the cell culture supernatants, embryo tissues or inoculated animal blood. *Orbivirus* isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins which are conserved within each serogroup (VP7) (OIE, 2018.).

Immunofluorescence, a technique based on the use of specific antibodies which have been chemically conjugated to fluorescent dyes is commonly used. Monolayers of BHK or Vero cells on chamber slides (glass cover-slips) are infected with either tissue culture-adapted virus or virus in ECE lysates. After 24–48 hours at 37°C, or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-BTV antiserum or BTV-specific monoclonal antibodies (MAbs) and standard immunofluorescent procedures. Antigen capture enzyme-linked immunosorbent assay (ELISA) is another used technique in which virus derived proteins are captured by antibody adsorbed to an ELISA plate and bound materials detected using a second antibody. The capture antibody may be polyclonal or a serogroup-specific MAb.

2.6.2. Molecular methods

2.6.2.1. PCR method

There are three major steps involved in a PCR which are repeated for 30 or 40 cycles, done on an automated cycler which rapidly heats and cools the test tubes and which contains the reaction mixture. Each step takes place at a different temperature:

1. Denaturation: At 94°C when the double-stranded DNA melts and opens into two pieces of single-stranded DNA.
2. Annealing: At medium temperatures, around 54°C, primers (short, artificial DNA strands of about 18 to 25 nucleotides that match the beginning and end of the DNA fragment to be amplified) pair up (anneal) with the single-stranded "template" (the sequence of DNA to be copied). On the small length of double-stranded DNA (the joined primer and template), the polymerase attaches and starts copying the template.
3. Extension: At 72°C DNA building blocks complementary to the template are coupled to the primer, making a double stranded DNA molecule.

With one cycle, a single segment of double-stranded DNA (dsDNA) template is amplified into two separate pieces of double-stranded DNA. These two pieces are then available for amplification in the next cycle. As the cycles are repeated, more and more copies are generated and the number of copies of the template is increased exponentially.

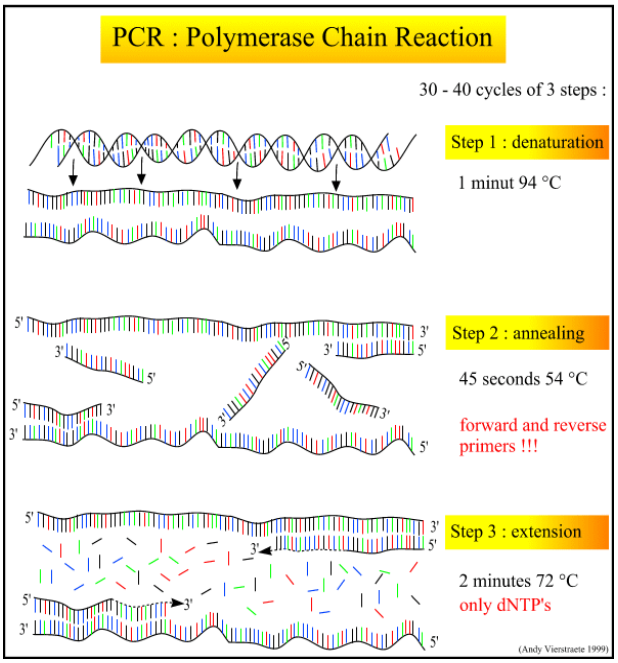


Figure 12. Principle of the PCR.
(source: https://users.ugent.be/~avierstr/principles/pcr.html)

2.6.2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR is a highly sensitive technique for the detection and quantitation of RNA. The technique consists of two parts the synthesis of cDNA (complementary DNA) from RNA by reverse transcription (RT) and the amplification of a specific cDNA by the polymerase chain reaction (PCR). An additional step allows the detection and amplification of RNA. The RNA is reverse transcribed into complementary DNA (cDNA), using enzyme reverse transcriptase (DNA-dependent DNA polymerase activity) into cDNA.

Real-time RT-PCR method provides sensitive and rapid detection of BTV in a one-step procedure. It requires a thermo cycler with an optical system to capture fluorescence and a computer with software capable of capturing the data and performing the final analysis of the reaction. The programs available from diverse manufactures exhibit differences regarding sample capacity, method of excitation and total sensitivity (ALVES VALONES et al., 2009.). The presented method is capable of detecting all known BTV serotypes and strains currently circulating (HOFMANN et al., 2008.) and the assay targets BTV segment 10 (NS3). Kits for the one-step real-time PCR are available commercially (HOFMANN et al., 2008.; OIE, 2018.).

2.6.2.3. Real-Time quantitative RT-PCR (qPCR)

Real-Time qPCR enables detection, measurement and characterization of products generated during each cycle of PCR process (HEID et al., 1996.). Commonly, in RT-qPCR RNA transcripts are quantified by reverse transcribing them into cDNA first, as described above and then qPCR is subsequently carried out. As in standard PCR, DNA is amplified by 3 repeating steps: denaturation, annealing and elongation. However in qPCR, fluorescent labeling enables the collection of data as PCR progresses. This technique has many benefits due to a range of methods and chemistries available (HEID et al., 1996.).

2.7. BLUETONGUE IDENTIFICATION AND CHARACTERIZATION

2.7.1. In-vitro and in-vivo cultures

The most sensitive method of virus isolation procedure for BTV is the inoculation of embryonated chicken eggs (ECE). Primary inoculation of cell cultures like the KC cell line (a cell-line derived from *C. variipennis* midges), also had been confirmed as very sensitive (MCHOLLAND et MECHAM, 2003.; OIE, 2018.). Inoculation of sheep also may be a useful approach. Attempts to isolate virus in cultured cells in vitro may be more convenient, but the success rate is frequently much lower than that achieved with in-vivo systems (GARD et al, 1988.). Samples for virus

isolation include unclotted blood from suspected viraemic animals, blood clots after separation of serum, spleen, lymph nodes or midges (OIE, 2018.).

2.7.2. Isolation in embryonated chicken eggs

Blood has to be collected from suspected viremic animals into an anticoagulant (EDTA, heparin or sodium citrate). The blood cells are washed three times with sterile phosphate buffered saline (PBS). Washed cells are re-suspended in PBS or isotonic sodium chloride and either stored at 4°C or used immediately for attempted virus isolation (OIE, 2018.). Tissue and midge suspension can be also prepared and stored or immediately used (OIE, 2018.).

If samples are frozen, they should be collected in buffered lactose peptone or 10% dimethyl sulphoxide and stored at –70°C or colder (OIE, 2018.). All *Orbiviruses*, including BTV remain durable for several months in whole blood in anticoagulant stored at 4°C.

Organs and tissues samples should be kept and transported at 4°C to a laboratory where they are homogenized in PBS or isotonic saline (1:10), centrifuged (at 1500 revolutions per minute for 10 minutes) and filtered (OIE, 2018.). Virus in the supernatant may be identified either directly as described or after further amplification in cell culture (OIE, 2018.).

2.7.3. Isolation in cell culture

Virus isolation may be performed in BTV susceptible cell cultures such as mouse L, baby hamster kidney (BHK-21), African green monkey kidney (Vero) or *Aedes albopictus* clone C6/36 (AA) (OIE, 2018.). The efficiency of isolation is often significantly lower following inoculation of cultured cells with diagnostic samples compared with that achieved in ECE. Highest recovery rates are achieved by primary isolation of virus in ECE followed by passage in AA cells or mammal cells for further replication of virus. Successful virus isolation has also been reported using primary isolation in cells derived from *Culicoides sonorensis* free of BTV and *Culicoides* viruses and designated as KC (MCHOLLAND et MECHAM, 2003.; WECHSLER et al., 1989.). In case of passage in AA or KC cells, additional passages in mammalian cell lines such as BHK-21 or Vero are usually performed (OIE, 2018). A cytopathic effect (CPE) is not necessarily observed in AA or KC cells but appears in mammalian cells. Cell monolayers are monitored for the appearance of CPE for 5 days at 37°C in 5% CO2 with humidity. If CPE is not evident, a second passage is performed in the mammalian cell culture. Isolated BTV can be detected after each ECE or cell culture passage by antigen detection or polymerase chain reaction (PCR) techniques (OIE, 2018.).

2.7.4. Isolation in sheep

This procedure for isolation of BTV is not frequently used but is useful where laboratory facilities are not available. Sheep are inoculated with washed cells from 10 ml to 500 ml of blood, or 10–50 ml tissue suspension (OIE, 2018.). Inoculation is administered subcutaneously in 10–20 ml aliquots. Large volumes may aid in the virus isolation attempts and should be administered intravenously. The infected sheep are held for 28 days and checked daily for pyrexia and weekly for antibody response using c-ELISA (OIE, 2018.). Sheep blood collected at 7–14 days post-inoculation usually contains the isolated virus, which can be stored viable at 4°C or –70°C, detected and characterized (OIE, 2018.) using mentioned methods.

2.7.5. Next generation sequencing (NGS) and bioinformatic analysis

Next Generation Sequencing (NGS) is a powerful platform that has enabled the sequencing of thousands to millions of DNA molecules simultaneously. NGS technology allows sequencing short fragments of DNA across the whole genome, producing single end or paired end reads of 50–700 base-pairs (TORRI et al., 2012.). The resulting raw DNA-Seq read data must then be analyzed following two computational macro-processes:

1. Mapping and assembling, quality control, quality score re-calibration, realignment in “difficult” regions of the genome;

2. Advanced steps focused on variant calling (SNPs, insertions-deletions (Indels) and CNVs) and annotation (TORRI et al., 2012.).

The application of NGS technology and its various methodological variants now makes it possible to detect different types of microorganisms present within a microbial sample simultaneously, using a culture-independent approach and in a single sequencing run (GOODWIN et al., 2016.).

Illumina dye sequencing is used to determine the series of base pairs in DNA, also known as DNA sequencing. The method is based on reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands.

Illumina sequencing technology works in three basic steps: amplify, sequence, and data analyze. The process begins with purified DNA. The DNA gets chopped up into smaller pieces and given adapters, indices, and other kinds of molecular modifications that act as reference points during amplification, sequencing and analysis. The modified DNA is loaded onto a specialized chip where amplification and sequencing will take place. Along the bottom of the chip are hundreds of thousands of oligonucleotides (short, synthetic pieces of DNA) which are anchored to the chip and able to grab DNA fragments that have complementary sequences. Once the fragments have attached, a phase called cluster generation begins. The goal is to create thousands of copies of each fragment of DNA. Next, primers and modified nucleotides enter the chip. These nucleotides

have reversible 3' blockers that force the polymerase to add on only one nucleotide at a time as well as fluorescent tags. After each round of synthesis, a camera takes a picture of the chip. A computer determines what base was added by the wavelength of the fluorescent tag and records it for every spot on the chip.

After each round, non-incorporated molecules are washed away. A chemical deblocking step is then used in the removal of the 3’ terminal blocking group and the dye in a single step. The process continues until the full DNA molecule is sequenced (MEYER et KIRCHER, 2010.).

With this technology, thousands of places throughout the genome are sequenced at once via massive parallel sequencing. During data analysis and alignment, the newly identified sequence reads are aligned to a reference genome.

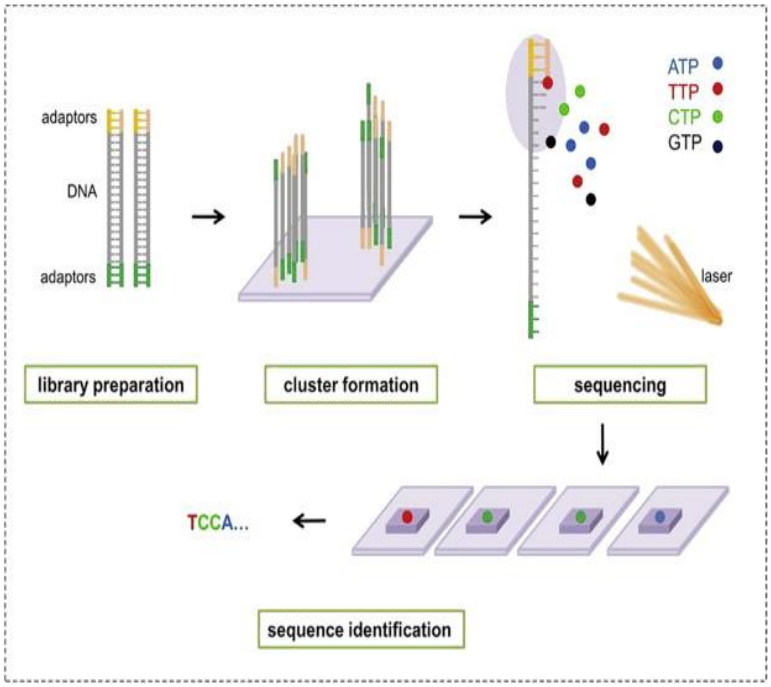


Figure 13. Illumina sequencing.
(source: https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/illumina-dye-sequencing)

2.8. BLUETONGUE SEROTYPE 3 IN THE MEDITERRANEAN BASIN

In November 2016, a novel BTV was identified in a symptomatic sheep located in the north-eastern part of Tunisia (SGHAIER et al., 2017.). Institute de la Recherche Vétérinaire in Tunisia in collaboration with the OIE Reference Laboratory for BT of the Istituto Zooprofilattico Spermentale dell’Abruzzo e del Molise (IZSAM) of Teramo, Italy characterized the etiological agent as a novel

Western bluetongue virus, BTV-3 strain. This was the first time that BTV-3 strain was reported in Tunisia. New strain was named BTV-3-TUN2016 (LORUSSO et al., 2017.) and the outbreak was confirmed to the OIE. Next recorded events included a widespread presence of a BTV-3 and belonging antibodies in sheep through whole Tunisian country (LORUSSO et al., 2017.).

Approximately one year after occurrence in Tunisia, in November 2017 an adult female of a crossbred sheep belonging to a flock of near 400 animals positioned in the Western part of the island of Sicily, showed clinical signs including fever, nasal discharge, oedema of the head and depression. Veterinary services of a local health unit were alerted due to symptoms consistent with BT infection. They visited the entire herd and obtained EDTA blood and serum samples from the symptomatic sheep. Samples were sent to the Istituto Zooprofilattico Sperimentale of Sicily for BT diagnosis.

A real time reverse transcriptase-polymerase chain reaction (RT-PCR) molecular assay which detects Seg-10 of BTV genome (RT-qPCRNS3) (HOFMANN et al., 2008.) was performed (SGHAIER et al., 2017.). Beside RT-PCR, c-Elisa for detection of BTV specific antibodies was also performed using the serum samples. Whole blood and serum samples resulted positive for BTV RNA and antibodies. Following to the Italian legislation, BT positive samples were sent to the BT National Reference Laboratory at the National Reference Centre for Foreign Diseases of Animals of the IZSAM for the confirmation of BTV infection and characterization of the BTV-3 strain.

Moreover, an additional Western BTV-3 strain named BTV-3 TUN2016/Zarzis was identified in the Southeastern regions of Tunisia, nearby the border with Libya (LORUSSO et al., 2017.).

2.9. ASSESSMENT OF NOVEL PROTOCOLS FOR DIAGNOSING BTV-3

2.9.1. Development of a specific BTV-3w quantitative real time PCR, RT-QPCRBTV-3

Primers were designed to amplify a specific 104 bp fragment of Seg-2 sequence of BTV-3w (BTV-3TUN2016, KY432370) and then verified by the Primer Express 3.0.1 software test tool (Applied Biosystems) (LORUSSO et al., 2018.). Primer BTV-3w forward sequence was 5’-AAATTTAATGAAGATAGATATCGTGAGATGATC-3’ (position 1393–1425), and primer BTV-3w reverse sequence was 5’-TTACCTTCTTCCTCAAGGATYTTATACATT-3’ (position 1496–1467). Probe and primers were synthesized by Eurofins Genomics (Ebersberg, Germany). BTV-3w TaqMan probe (CAGTCGGTAATTGATGATGGGTGGGACC) was dual-labelled with 6-carboxyfluorescein (FAM) at the 5’end and with tetramethylrhodamine (TAMRA) at the 3’ end (position 1426–1453) (LORUSSO et al., 2018.). The thermal profile consisted of a single cycle of reverse transcription at 50 °C for 15 min followed by a denaturation step at 95 °C for 2 min for reverse transcriptase inactivation and DNA polymerase activation. The amplification of cDNA was performed by 45 cycles including denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s.

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2.9.2. Virological and serological analysis

Insect pools, blood and serum samples collected in Tunisia were sent to the IZSAM for confirmation of the presence of BTV RNA and antibodies. Additional genotyping, serotyping, isolation and sequencing analyses were performed. The same molecular methods have been used for BTV RNA detection including RT-qPCRNS3 and BTVEuropean typing. Samples which turned negative by BTVEuropean typing were further tested by additional molecular assays including those specific for novel BTV serotypes. RT-qPCRBTV-3 was used to screen RNAs purified from blood samples tested positive by the RT-qPCRNS3 but negative by BTVEuropean typing. Virus isolation (SAVINI et al., 2004.) was performed only for RT-qPCRNS3-positive samples by passing sonicated blood once onto confluent monolayers of KC (WECHSLER et al., 1989.) cells for ten days at 28°C followed by two blind passages on confluent monolayers of Vero cells at 37°C, 5% CO2. Infection of embryonated chicken eggs (OIE) was performed to isolate BTV-Y TUN2017 from the infected blood samples. Confirmation of BTV isolation either from cells or eggs was given by RTqPCRNS3. SN (SAVINI et al., 2004.) for serotypes 1 to 27 was also performed for c-ELISA positive serum samples. By using a specific protocol, the novel BTV-3 strain has been detected and confirmed (LORUSSO et al., 2017.).

2.9.3. Seg-2 RT-PCR and sequencing

One-Step RT-PCR (Qiagen) and primers BTS2F (5'-TCAGGTATCCATCAGGCTCTC-3') and BTS2R (5'-GCTTCTCCTCTCGTTTTTCATA-3') were employed to determine a portion (650 bp) of Seg-2 from a total number of 23 BTV-3w positive blood samples. cDNA was synthesized at 50 °C for 30 min with denaturation at 95 °C for 15 min. The amplification reaction was carried out for 40 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 2 min.

Amplicons were used for direct sequencing in both directions using the aforementioned primers. The partial Seg-2 sequences of six BTV-3w isolates were also included in the analysis. Primers BTYS2F and BTYS2R designed on Seg-2 of BTV-Y TUN2017, were employed. Amplicons were used for direct sequencing in both directions.

2.9.4. Circulation of BTV-3 strains

In 2018, one year after the first official notification of BTV-3 in Sicily, following guidelines of the national surveillance plan for BTV serum samples were collected from nine sentinel animals including goats and sheep located in a farm in the municipality of Teulada, province of Sud Sardegna. The animals have been previously tested serologically negative for BTV in April, May, June, July and August of the same year. Serum samples were tested by c-ELISA (LORUSSO et al., 2016.) at the local IZS. One sample was positive for the presence of BT antibodies. In order to

detect viral RNA, EDTA blood samples were collected from all animals and purified nucleic acids were tested by real-time RT-qPCR (detection of the Seg-10 of all known existing BTV serotypes).

A total of 4 out of 10 blood samples tested positive by qPCRNS3. Positive samples were sent to the IZSAM for confirmation by qPCRNS3 and genotyping. Genotyping was performed by means of the LSI VetMAX European BTV Typing Kit which detects BTV serotypes (BTV-1, -2, -4, -6, -8, -9, -11 and -16) which have been circulating in previous years in Europe and in the Mediterranean basin. However, samples were negative by qPCRtyping. Based on the epidemiological scenario previously described (LORUSSO et al., 2017.; LORUSSO et al., 2018.) samples were tested by a real-time RT-qPCR specific for detection of the Seg-2 of BTV-3w. All samples were positive for the presence of BTV-3w RNA. Therefore, samples were used for NGS by metagenomic approach in order to obtain information on viral genome sequence and constellation, using the Illumina NextSeq 500 protocol. EDTA blood samples were also processed for virus isolation.

Shortly after the first BTV-3 confirmation in stated animals, veterinary services of Sardinia region reported clinical signs such as depression, fever and nasal discharge, suggesting BTV infection in several animals from four different farms in the same municipality as the sentinel animals (CAPPAI et al., 2019.). Moreover, four sheep died because of the infection and the rest of animals belonging to the same farms also showed clinical signs. In October 2018, the Italian Ministry of Health notified the World Organization for Animal Health (OIE) the BTV-3w outbreak.

Of all the potential routes of induction to explain Italy's first outbreak of BTV-3, the focus was on aerial introduction of infected *Culicoides* spp. as the most likely. During transmission period, legal movements of animal, semen and embryos from Tunisia to Sicily were not possible, since Tunisia have not been considerate as a BT-free region. Illegal movements may have occurred but they are less likely. It is possible that infected midges were transported by airplane or ship; still, no direct flights occurred between Trapani and Tunisia during the study period (AGUILAR-VEGA et al., 2018.). Since the outbreak in Sicily involved BTV-3-naïve animals and did not conduct towards to multiple outbreaks, the hypothesis is that a small number of BTV-3-infected midges arrived at one particular area on the western Sicilian coast and that this small amount of midges was sufficient to transmit BTV to a susceptible host in late September or early October of 2018.

2.9.5. Philogeny of BTV-3 circulating strains

One BTV-3 isolate obtained from the blood sample of a sheep originating from Zarzis has been sequenced by NGS. The genome sequence (BTV-3 TUN2016/Zarzis) has been deposited with the genbank database. The percentage of nt identity of genome segments of BTV-3 TUN2016/Zarzis with homologous segments of BTV-3 TUN2016 (SGHAIER et al., 2017.) ranges from 80.3% (Seg-10) to 98.0 (Seg-6) suggesting the circulation of two different and unrelated BTV-3w strains. In all phylogenetic analyses, BTV-3 TUN2016 and BTV-3 TUN2016/Zarzis genome segments fall in the same clade except for Seg-10 (LORUSSSO et al., 2018.). Homologous sequences of this segment show indeed 80% of nt sequence identity; Seg-10 of BTV-3 TUN2016 has 99% nt identity to that of

BTV-2 SUD1983/05 (KP822001) whereas Seg-10 of BTV-3 TUN2016/Zarzis has 98% nt identity to that of BTV-16 NIG1982/10 (KC853058), representative of the western strains of BTV-16 (CAPPAI et al., 2019.). After quality control and trimming performed using an in house script, a total number of 328.774, 744.026, 185.768 and 221.558 reads remained with mean phred scores ranging from 31 to 33. Reads were used for de novo assembly by spades v.3.12. The assembly of sample 2018TE20821/2 did not show any contings belonging to BTV. Only partial sequence of the Seg-4 was detected in sample 20820/1 and 20821/2. On the other hand, the sequencing run of sample 20820/4 produced a total number of 550 contigs longer than 300 nucleotides. Of these, 16 belonged to BTV. This assembly was refined by mapping the reads and contigs against the closest reference genome BTV-3 TUN2016. As expected, sequence data obtained from the RNA purified from the isolate (BTV-3 SAR2018) were of higher quality and importantly contained the complete coding genome information for 10/10 segments of the virus. BTV-3 SAR2018 was demonstrated to be almost identical (99% of nucleotide identity in Seg-1, -3, -4, -5, -6, 9 and -10; 100% in Seg-2, -7 and -8) across all segments to BTV-3 TUN2016 identified in Tunisia for the first time in November 2016. Likewise, also partial Seg-2 sequences had 100% of nucleotide identity with that of BTV-3 TUN2016. The close relationship between BTV-3 TUN2016 and BTV-3 SAR2018 is also evident in the Seg-2 phylogenetic analysis (Figure 12). BTV-3 SAR2018 clearly clusters with BTV-3 TUN2016 and it is more distantly related (94.1% of nucleotide identity) to BTV-3 TUN2016/Zarzis. In turn, BTV-3 TUN2016/Zarzis is more closely related to a BTV-3w strain detected in Egypt in 2016 and recent BTV-3w strains isolated in Israel. BTV-3 strain from Sicily was demonstrated to be also identical to BTV-3 TUN2016.

Seg-2 sequence of BTV-3 SAR2018 and western BTV-3 strains were aligned and configured for highest accuracy. After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT; Guindon & Gascuel, 2003; Guindon et al., 2010). The HKY85 substitution model was selected assuming an estimated proportion of invariant sites (of 0.465) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 2.751). Reliability for internal branch was assessed using the aLRT test (SHLike). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3; Chevenet, Brun, Banuls, Jacq, & Chisten, 2006).

BTV-3 SAR2018 was demonstrated to be almost identical (99% of nucleotide identity in Seg-1, -3, -4, -5, -6, 9 and -10; 100% in Seg-2, -7 and -8) across all segments to BTV-3 TUN2016 identified in Tunisia for the first time in November 2016. Likewise, also partial Seg-2 sequences had 100% of nucleotide identity with that of BTV-3 TUN2016. The close relationship between BTV-3 TUN2016 and BTV-3 SAR2018 is also evident in the Seg-2 phylogenetic analysis (Figure 3). BTV-3 SAR2018 clearly clusters with BTV-3 TUN2016 and it is more distantly related (94.1% of nucleotide identity) to BTV-3 TUN2016/Zarzis, an additional BTV-3w strain discovered in 2016 in the eastern part of Tunisia nearby the border with Libya (LORUSSO et al., 2018).

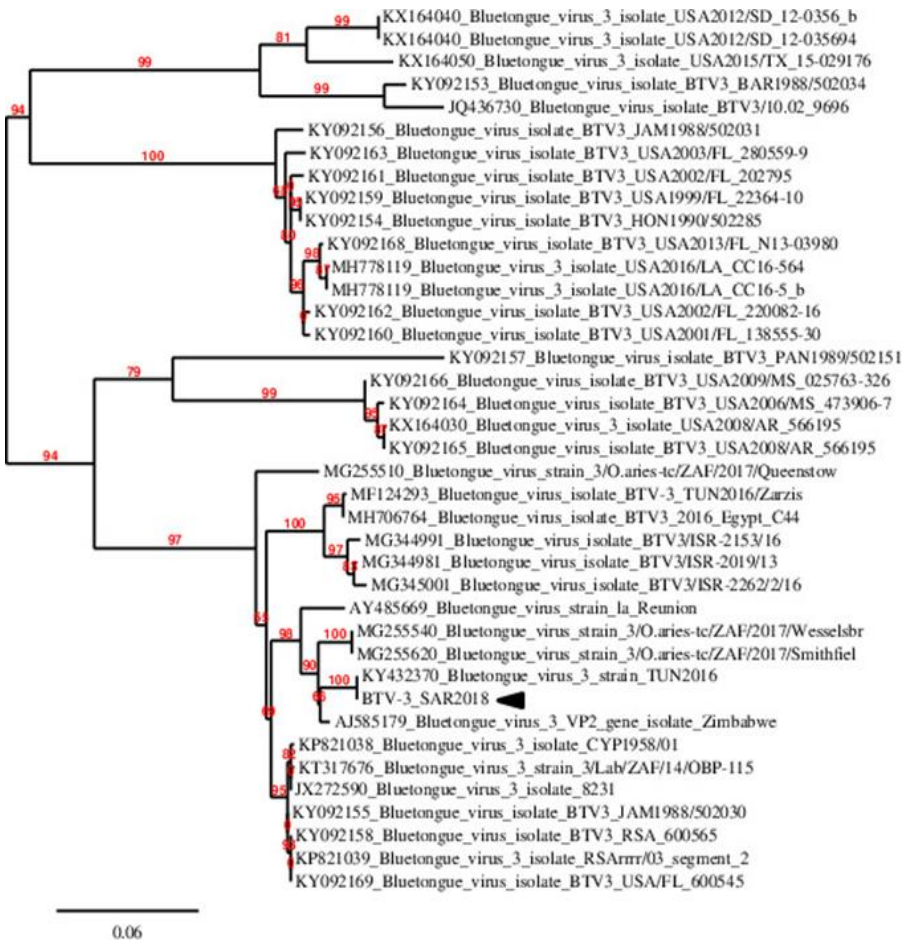

FIGURE 14. Seg-2 phylogenetic tree of western strains of BTV-3.

(source: https://www.researchgate.net/publication/331348508_Western_Bluetongue_virus_serotype_3_in_Sardinia_diagnosis_and_characterization)

3. CONCLUSION

1. The hypothesis which explains the first outbreak of BTV-3 in Italy is that a small number of BTV-3-infected midges arrived at one particular area on the western Sicilian coast and that this small amount of midges was sufficient to transmit BTV to a susceptible host in late September or early October of 2018 in the Sicily.

2. It was crucial to develop a specific and precise RT-qPCR for the detection of BTV-3, which was used to detect BTV-3 circulation in Tunisia and to discover promptly this serotype both in Sicily and Sardinia.

3. NGS is becoming a central and essential diagnostic technique which provides identification and characterization of a BTV.

4. Sentinel animals are a key factor of the BTV surveillance plan since sampling sentinel animals allows quick detection of the virus by seroconversion before other susceptible animals are infected.

5. Finally, it is critical that European authorities collaborate in organizing common surveillance programs to detect early novel BTV strains.

6. It is mandatory to develop specific vaccines and to organize coordinated vaccination campaigns between all BTV at-risk countries.

4. SUMMARY

CHARACTERIZATION OF EMERGING BLUETONGUE VIRUS SEROTYPE 3 STRAINS IN THE MEDITERRANEAN BASIN BY NEXT GENERATION SEQUENCING

Bluetongue (BT) is a vector-borne viral OIE-listed disease of wild and domestic ruminants caused by bluetongue virus (BTV). BT has severe economic repercussions for the livestock industry due to direct losses caused by the infection but also due to indirect losses as a result of restrictions on animal trade. Since 1998, Southern Europe and the Mediterranean basin have experienced multiple incursions of different strains belonging to different serotypes and topotypes of BTV. In 2016 a BT outbreak occurred in Tunisia when the etiological agent was characterized as a novel western BTV serotype 3 strain. One year after the notification in Tunisia, BTV-3 outbreaks were evidenced in Sicily and Sardinia.

Considering the history of the epidemiology of BTV in the Mediterranean basin and the absence of specific vaccines, a novel BTV-3 certainly represents a risk for the European livestock industry. Therefore, in this thesis several aspects were analyzed including the development of a specific and accurate real time RT-PCR for the detection of BTV-3 RNA, the analysis of BTV-3 spread by means of virological and serological methods within the particular emphasis on a genome characterization of BTV-3 using a next generation sequencing (NGS), a novel molecular diagnostic technique.

Key words: Bluetongue, Mediterranean basin, BTV serotype 3, NGS

5. SAŽETAK

TIPIZACIJA EMERGENTNOG VIRUSA BOLESTI PLAVOG JEZIKA SEROTIPA 3 NA PODRUČJU MEDITERANA KORIŠTENJEM METODE SEKVENCIRANJA SLJEDEĆE GENERACIJE (NGS)

Bolest plavog jezika (BPJ) je vektorska bolest divljih i domaćih preživača koja se prenosi virusom bolesti plavoga jezika i koja se nalazi na listi bolesti Svjetske organizacije za zdravlje životinja (OIE). BPJ ima ozbiljne ekonomske posljedice za stočarsku industriju zbog izravnih gubitaka uzrokovanih infekcijom, ali i zbog neizravnih gubitaka kao posljedica ograničenja u trgovini životinjama. Od 1998. godine na području južne Europe i Mediterana došlo je do višestrukih izbijanja bolesti uzrokovanih različitim serotipovima i topotipovima virusa BPJ. 2016. godine došlo je do epidemije BPJ u Tunisu, kada je etiološki agens okarakteriziran kao novi soj virusa bolesti plavog jezika - serotip 3. Godinu dana nakon izbijanja u Tunisu, epidemije virusa bolesti plavog jezika serotipa 3 zabilježene su na Siciliji i Sardiniji.

Obzirom na epidemiološku situaciju pojave virusa BPJ na Mediteranu i na odsutnost specifičnih cjepiva za novi serotip bolesti, navedeni serotip 3 virusa BPJ zasigurno predstavlja rizik za europsku stočarsku industriju. Stoga je u ovoj tezi analizirano nekoliko aspekata, uključujući razvoj specifičnog i preciznog RT-PCR-a u stvarnom vremenu za detekciju RNA serotipa 3 virusa BPJ te širenje serotipa 3 virusa BPJ pomoću viroloških i seroloških metoda s naglaskom na tipizaciju genoma serotipa 3 virusa BPJ korištenjem najnovije tehnike molekularne dijagnostike-sekvenciranja sljedeće generacije (NGS).

Ključne riječi: Bolest plavog jezika, Mediteran, serotip 3 virusa BPJ, NGS

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7. BIOGRAPHY

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