Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France

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During the compulsory vaccination programme against bluetongue virus serotype 1 (BTV-1) in Corsica (France) in 2014, a BTV strain belonging to a previously uncharacterized serotype (BTV-27) was isolated from asymptomatic goats. The present study describes the detection and molecular characterization of two additional distinct BTV-27 variants found in goats in Corsica in 2014 and 2015. The full coding genome of these two novel BTV-27 variants show high homology (90–93 % nucleotide/93–95 % amino acid) with the originally described BTV-27 isolate from Corsican goats in 2014. These three variants constitute the novel serotype BTV-27 ('BTV-27/FRA2014/v01 to v03'). Phylogenetic analyses with the 26 other established BTV serotypes revealed the closest relationship to BTV-25 (SWI2008/01) (80 % nucleotide/86 % amino acid) and to BTV-26 (KUW2010/02) (73–74 % nucleotide/80–81 % amino acid). However, highest sequence homologies between individual segments of BTV-27/FRA2014/v01–v03 with BTV-25 and BTV-26 vary. All three variants share the same segment 2 nucleotide with BTV-25. Neutralization assays of anti-BTV27/FRA2014/v01–v03 sera with a reassortant virus containing the outer capsid proteins of BTV-25 (BTV1\text{VP2/VP5 BTV25}) further confirmed that BTV-27 represents a distinct BTV serotype. Relationships between the variants and with BTV-25 and BTV-26, hypotheses about their origin, reassortment events and evolution are discussed.

INTRODUCTION

A novel 27th bluetongue virus serotype (BTV-27) was recently discovered in asymptomatic goats during a monitoring and mandatory vaccination programme against BTV-1 in Corsica, France, in 2014 (Zientara et al., 2014). To date, 27 BTV serotypes have been determined worldwide. The detection of two putative novel serotypes in a Capripox vaccine in the Middle East (BTV-28) and in an alpaca in South Africa (BTV-29) has recently been suggested, but the criteria that determine a novel serotype by percentage divergence in sequence identity in comparison with other BTV serotypes remain to be clarified (Wright, 2014; Zientara et al., 2014; Maan et al., 2015b).

In general, BTV is notifiable to the World Organisation for Animal Health (OIE), since outbreaks commonly implicate trade and movement restrictions that may cause severe economic losses for affected regions. Furthermore, BTV-infected sheep often show severe clinical signs, while cattle, goats and camels are usually asymptomatic, although some clinical cases in cattle have been observed during the North European outbreak of BTV-8 (Backx et al., 2007; Dal Pozzo et al., 2009a, b; Schulz et al., 2012; Caporale et al., 2014).

BTV-27 is most closely related to BTV-25 and BTV-26 (Jenckel et al., 2015). BTV-25 and 26 were recently detected in asymptomatic goats in Switzerland (Chaignat et al., 2009) and in sheep in Kuwait (Maan et al., 2011a),
respectively. Experimental infection with BTV-25 and 26 did not cause any clinical signs in goats and only a mild clinical disease in sheep (Chaignat et al., 2009; Batten et al., 2014). However, BTV-25 and 26 possess unique characteristics in contrast to the more established BTV serotypes 1 to 24. For example, BTV-26 appears to be transmitted horizontally, rather than by Culicoides, as demonstrated in experimentally infected goats (Batten et al., 2014) and BTV-25 could not be isolated in cell culture (Chaignat et al., 2009). Since the three novel BTV-25, 26 and 27 serotypes have not caused economic losses, restriction measures have not been implemented in the affected regions so far.

BTV comprises 10 dsRNA segments (Seg-1 to Seg-10) that encode for seven viral structural proteins (VP1–VP7) and five non-structural proteins (NS1–NS4 and NS3a), and are enclosed by three concentric capsid layers. The outer capsid (VP2 and VP5), the outer-core capsid (VP7) and the sub-core capsid (VP1, VP3, VP4 and VP6). NS proteins are expressed within the infected cell (Schwartz-Cornil et al., 2008; Ratinier et al., 2011; Maan et al., 2015b).

For BTV-27, a novel serotype was suggested, since Seg-2 sequence identity between BTV-27 and BTV-25 was low (73 % nucleotide/75 % amino acid) and anti-BTV-25 serum could not neutralize BTV-27. Furthermore, unlike BTV-25, BTV-27 grows in cell culture (Zientara et al., 2014).

In the present study, we report the identification, isolation and characterization of two novel variants of BTV-27 discovered in Corsica, France, in 2014.

RESULTS

Detection of three variants of BTV-27 in goats in Corsica

In the field study of BTV-27 infection in Corsican goat herds in 2014 and 2015, approximately 80 goats from 11 different herds were found BTV-27-RNA-positive using conventional BTV-27-specific reverse-transcription (RT)-PCR (Zientara et al., 2014). From 35 of these BTV-27-RNA-positive goats (Fig. 1), amplification products of partial Seg-2 sequences were obtained. Alignment of these amplicons revealed three different clusters of BTV-27 Seg-2 with about 8 % nucleotide divergence between each other (data not shown). We termed these three variants of BTV-27 as BTV-27v01, v02 and v03.

In 2014, Seg-2 sequences from 18 BTV-27v01, one BTV-27v02 and 10 BTV-27v03 were obtained from 29 different goats in seven herds (Fig. 1). In 2015, Seg-2 sequences from five BTV-27v01 and one BTV-27v03 were detected in samples from six goats located in four Corsican herds. These six goats were BTV-27 negative or not tested in 2014. Two of the four herds were already affected by BTV-27v01 in 2014: BTV-27v01 was again detected in the first herd, while BTV-27v03 was initially detected in the second. On the two other farms in Eastern Corsica, BTV-27v01 was identified for the first time (Fig. 1).

Virus isolation results

Two to four passages were required before a cytopathic effect (CPE) could be observed and virus isolation was successfully achieved for less than 10 % of the BTV-27 RT-PCR-positive blood samples inoculated on BSR cells. Finally, eight BTV-27 isolates were obtained. The first three strains were the variants BTV-27v01, v02 and v03, collected...
from three different goats between January and May 2014 (Fig. 1). The five other isolates belonged to the two variants BTV-27v01 and v03.

**Virus neutralization test results**

The three anti-BTV-27v01–v03 sera did not neutralize a reassortant with the outer capsid viral proteins VP2 and VP5 of BTV-25 and the other proteins of BTV-1 (BTV1VP2/VP5 BTV25), whereas they neutralized BTV-27v01, v02 and v03, respectively (data not shown).

**Full-genome sequencing of BTV-27 variants**

Phylogenetic and sequence analyses of the complete coding genomes of the BTV serotypes 1 to 26 with the three BTV-27 variants (Fig. 2) revealed the highest nucleotide (nt) and amino acid (aa) sequence homology to BTV-25 (80 % nt/86 % aa), and a slightly lower homology to BTV-26 (73–74 % nt/80–81 % aa) (Table 1).

**Segment-specific relationships of BTV-27 variants with other BTV serotypes.** Analysing the sequences of the 10 segments separately, Seg-1, 2, 3, 4, 8 and 9 of all three BTV-27 variants show highest sequence homology (72–86 % nt/66–95 % aa) to the homologous segments of BTV-25. Considerable differences in relationships between BTV-27v01–v03 with BTV-25 and BTV-26 were found for the remaining segments/protein-encoding genes: Seg-5/NS1 of BTV-27v01–v03, Seg-6/VP5 of BTV-27v02 and v03, and Seg-10/NS3 of BTV-27v01 and v03 are most closely related to the corresponding genomic segments/protein genes of BTV-26. In contrast, Seg-6/VP5 of BTV-27v01 and Seg-10/NS3 of BTV-27v02 have a higher identity to BTV-25 (Table 1, Fig. S1, available in the online Supplementary Material). Remarkably, Seg-7 nt sequences of BTV-27v01–v03 share a higher identity with BTV-25 (84–85 % nt) than with BTV-26 (82 % nt), while the VP7 aa sequences of the three variants are more homologous to BTV-26 (97 % aa) than to BTV-25 (95–96 % aa).

**BTV-27 inter-variant relationships.** BTV-27v01 and v03 are more closely related to each other (93.2 % nt/94.8 % aa) than to v02 (v02–v03 identity was 92.9 % nt/94.5 % aa while v01–v02 identity was 90.0 % nt/92.7 % aa) (Table S1, available in the online Supplementary Material). BTV-27v01 and v02 inherit one more segment closer related to BTV-25 than v03, but nt and aa sequence identities of BTV-27v01–v03 with BTV-25 are similar (Table 1).

Within the BTV-27 variants, differences between segments/protein-encoding genes were generally small (<8 % nt/<10 % aa), except for Seg-6, 9 and 10 (Table S1). Relationships between BTV-27v02 and v03 of Seg-6/VP5, as well as between v01 and v03 of Seg-9/VP6 and Seg-10/NS3 show a high identity (93–97 % nt/95–98 % aa). In contrast, considerably decreased sequence homologies of ≤90 % (69–88 % nt/69–90 % aa) were found between variants (i) BTV-27v01, respectively, v02 and v03 (70 % nt/76 % aa) for Seg-6/VP5, (ii) BTV-27v01 and v02, as well as between v02 and v03 for Seg-9/VP6 (73 % nt/69 % aa) and Seg-10/NS3 (80–81 % nt/88–90 % aa) (Table S1). Seg-7 sequences of BTV-27v01 and v03 show the highest aa homology (100.0 % aa), and a slightly smaller aa homology (99.4 % aa) between v01 and v02, as well as between v02 and v03, while nt homologies are similar for the three variants (97.2–97.3 %).

**Identification of a BTV-27 serotype.** Seg-2/VP2 sequence identities of BTV-27v01–v03 are similar between the three variants (92–93 % nt/90–91 % aa) and are more closely related to BTV-25 (73–74 % nt/75–76 % aa homology) than to BTV-26 (63 % nt/60 % aa homology). Different BTV isolates included within a single BTV serotype share >68.4 % nt/72.6 % aa homology, while they are discriminated as distinct serotypes of a maximum identity of ≤71.5 % nt/77.8 % aa according to Maan et al. (2011a). For BTV-27v01, a novel BTV serotype was therefore suggested, previously due to the low sequence identity with BTV-25 (Zientara et al., 2014). The close relationship between Seg-2/VP2 of BTV-27v01–v03 strongly suggests that all variants belong to the same serotype.

**Identification of nucleotypes.** BTV nucleotypes have only been determined for BTV Seg-2 and 6 (Maan et al., 2010, 2011b). Seg-2 of BTV-27v02 and v03 share the same nucleotype with BTV-25 (nucleotype 'K') (Fig. 3), as previously described for BTV-27v01 (Zientara et al., 2014), since Seg-2 homology of BTV-27v01–v03 (73–74 % nt/75–76 % aa) with BTV-25 exceeds the minimum sequence identity (≥66.9 % nt/69.4 % aa) required for inclusion into a Seg-2 nucleotype (Maan et al., 2011a; Zientara et al., 2014).

For Seg-6, inclusion of BTV-27v01 in the same eighth Seg-6 nucleotype (nucleotype 'H') with BTV-25 (77 % nt/86.7 % aa homology) has already been described previously (Maan et al., 2015b). A minimum of <76 % nt/85.9 % aa sequence identity is required for inclusion in a Seg-6 nucleotype (Maan et al., 2010, 2011b). In contrast, BTV-27v02 and v03 are more closely related to BTV-26 (72 % nt/83 % aa homology) than to BTV-25 (70 % nt/76–77 % aa homology). According to the current definition of Maan et al. (2010, 2011b), these two variants present a novel tenth nucleotype (nucleotype 'J') of Seg-6 (Fig. 4).

**Identification of topotypes.** In general, most BTV isolates have been assigned to the major ‘eastern’ and ‘western’ topotypes and to further subgroups that reflect the geographic origin (Maan et al., 2011b). According to the criteria defined by Maan et al. (2010), the BTV-27 variants represent novel topotypes for Seg-1 to 6, share a common topotype with BTV-25 and BTV-26 for Seg-7, 9 and 10, and form one topotype with BTV-25 for Seg-8 (data not shown). However, in a recent approach Seg-1, 5, 7, 9 and 10 of BTV-27v01 were included in the respective common topotypes with BTV-25 and BTV-26, while Seg-3, 4 and 8
**Fig. 2.** Phylogenetic relationships based on complete coding genomes of BTV serotypes 1 to 26 with the three BTV-27 variants BTV-27/FRA2014/v01 to v03. The phylogenetic tree of nucleotide sequences was constructed with i3-TREE (Nguyen et al., 2015) by using 1000 bootstrap replicates and the ‘best-fitted model’. GenBank accession numbers of the respective segments of each serotype are given in Fig. S1, available in the online Supplementary Material. Countries of origin are given in brackets, if not given in the isolate description (AUS, Australia; DE, Germany; FRA, France; IND, India; IT, Italy; KUW, Kuwait; NET, the Netherlands; SWI, Switzerland; ZAF, South Africa). Bar, 0.3 nucleotide substitutions per site.
Table 1. Comparison of dsRNA (cDNA copy) genome segment and protein identities of the three variants of BTV serotype 27 (BTV-27/FRA2014/v01–v03) with the most closely related BTV-25 (SWI2008/01) and BTV-26 (KUW2010/02), as well as the GenBank accession numbers of the segment 1 to 10 sequences. Greyed-out numbers represent nucleotide and/or amino acid sequences with a higher identity to BTV-26 than to BTV-25. Comparison of identities between (i) the three BTV-27 variants, (ii) BTV-25 and BTV-26 and (iii) complete coding genomes of BTV-25, -26 and -27 are given in Table S1, available in the online Supplementary Material.

<table>
<thead>
<tr>
<th>Genome segment/protein nomenclature (*)</th>
<th>GenBank accession numbers of BTV-27v01/v02/v03</th>
<th>Highest percentage nt/aa identity with BTV-25†</th>
<th>Highest percentage nt/aa identity with BTV-26‡</th>
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</thead>
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<tr>
<td>Seg-1/VP1 (Pol)</td>
<td>LN713671/ KU760987/ KU760997</td>
<td>85.9/92.3</td>
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<td>Seg-2/VP2 (OCP1)</td>
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<td>73.2/75.0</td>
<td>63.3/59.9</td>
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<td>Seg-3/VP3 (T2)</td>
<td>LN713672/ KU760989/ KU760999</td>
<td>84.3/95.0</td>
<td>76.5/89.7</td>
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<tr>
<td>Seg-4/VP4 (CaP)</td>
<td>LN713673/ KU760990/ KU761000</td>
<td>79.2/90.5</td>
<td>73.6/81.7</td>
</tr>
<tr>
<td>Seg-5/NS1 (TuP)</td>
<td>LN713674/ KU760991/ KU761001</td>
<td>74.0/78.1</td>
<td>79.6/88.2</td>
</tr>
<tr>
<td>Seg-6/VP5 (OCP2)</td>
<td>LN713675/ KU760992/ KU761002</td>
<td>77.4/86.7</td>
<td>79.6/88.2</td>
</tr>
<tr>
<td>Seg-7/VP7 (T13)</td>
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<td>84.2/95.7</td>
<td>79.6/88.2</td>
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<tr>
<td>Seg-8/NS2 (VIP)</td>
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<td>82.6/87.0</td>
<td>79.6/88.2</td>
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<tr>
<td>Seg-9/VP6 (Hel); NS4</td>
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<td>72.4/66.8</td>
<td>70.4/68.6</td>
</tr>
<tr>
<td>Seg-10/NS3; NS3a</td>
<td>LN713679/ KU760996/ KU761006</td>
<td>79.1/86.9</td>
<td>71.1/64.9</td>
</tr>
<tr>
<td>Seg-1 to 10</td>
<td>79.8/86.4</td>
<td>79.8/86.4</td>
<td>73.5/80.5</td>
</tr>
</tbody>
</table>

nt, Nucleotide sequence; aa, amino acid sequence (in bold); VP, viral protein; NS, non-structural protein.

*Protein structure/function: Pol, RNA polymerase; OCP, outer capsid protein; T2, internal protein with T=2 symmetry; CaP, capping enzyme; TuP, tubule protein; T13, internal protein with T=13 symmetry; VIP, viral inclusion body matrix protein; Hel, helicase enzyme.
†GenBank accession no. Seg-1, 3, 4: GQ982522–GQ982524; Seg-2, 5–10: EU839840–EU839846 (see also Table S1).
‡GenBank accession no. Seg-1, 4–6, 8–10: JN255156–JN255162; Seg-2, 3, 7: HM590642–HM590644 (see also Table S1).
of BTV-27v01 were merged into the respective topotypes of BTV-25 (Maan et al., 2011b, 2015b). Due to the close relationship between the BTV-27 variants, the latter division of segments within topotypes may be adapted for BTV-27v02 and v03 (Fig. S1).

For Seg-2 of BTV-27v01–v03, the nt sequence identity to BTV-25 (73–74%) is slightly smaller than the minimum required for inclusion within a major topotype (>78.2) and the maximum required to distinguish between ‘major’ western and eastern topotypes (<75.2%) according to Maan et al. (2010, 2011b). The homology of BTV-27v01–v03 Seg-2 (63%) with the second closely related BTV-26 (which was recently defined as a novel major topotype) is considerably smaller (Table 1). Therefore, we suggest to also include Seg-2 of BTV-27v01–v03 within the western topotype of BTV-25 (Fig. S1).

Sequence identities of Seg-6 show that BTV-27v01 is more closely related to BTV-25 (77% with BTV-25; 69% with BTV-26), while BTV-27v02–v03 are more closely related to BTV-26 (72% with BTV-26; 70% with BTV-25). Different topotypes of Seg-6 are defined to share <79.5% and the same topotypes, >79.8% nt identity (Maan et al., 2010). Accordingly, BTV-27v01 and BTV-27v02–v03 form novel individual Seg-6 topotypes. However, the borderline sequence relationship of Seg-6 of BTV-27v01 with the minimum identity required for inclusion within the same topotype suggests its inclusion within the topotype of BTV-25. In contrast, we suggest that BTV-27v02 and v03 form a novel common topotype (Fig. S1), since the nt identity is...
lower than the nt identity between other BTV serotypes that belong to clades branching from different evolutionary branching points, for example BTV-1 (GenBank accession no. JX861493) and BTV-8 (GenBank accession no. AM498056) (74.1 % nt homology).

**DISCUSSION**

The field study of novel BTV strains in goats in Corsica (France) in 2014, revealed that a minimum of three variants of BTV-27 are circulating in asymptomatic goats. BTV-27v01 seems to be the most frequent in the field (Fig. 1), since this variant was found in the majority (n=23) of the 35 sequenced goat samples. BTV-27v03 was detected in a few (n=11) goats in three herds that were also affected by BTV-27v01. In contrast, BTV-27v02 seems to be rare, because this variant was only detected and isolated from a single goat. BTV-27v01 was the only variant detected in all investigated parts of the island including Eastern Corsica, while BTV-27v02 and v03 were only detected in the western part. Comparing the full coding genomes of the three variants, homologies are similar between BTV-27v01 and v03 and between v02 and v03 (93 % nt/95 % aa), while v01 and v02 are more distantly related to each other (90.0 % nt/92.7 % aa). This is in accordance with the results of the field study (Fig. 1), since BTV-27v03 was only found in herds also affected by BTV-27v01, and BTV-27v02 was found in proximity to two farms where both v01 and v03 occurred together in the herds. The origin and reason for this spatial dissemination of these BTV-27 variants are unknown (Zientara et al., 2014). Similar to BTV-27v01 (Zientara et al., 2014), the complete coding genomes of BTV-27v02 and v03 showed the highest sequence homology to BTV-25 (80 % nt/86 % aa) (Table 1, Fig. 2) detected in goats in Switzerland in 2008 (Hofmann et al., 2008) and a slightly lower homology to BTV-26 (73–74 % nt/80–81 % aa) detected in sheep in Kuwait in 2010 (Maan et al., 2011a).

Neutralizing antibodies and cellular immune responses against the outer-capid protein VP2 (Seg-2) mediate protection against infection with a homologous BTV serotype (Schwartz-Cornil et al., 2008; Maan et al., 2015b). Therefore, identification of the BTV serotype determines the choice of a suitable vaccine strain (Nomikou et al., 2015). A novel BTV serotype was suggested previously for BTV-27v01 (Zientara et al., 2014). The results of the present study further indicate that BTV-27, including all three variants, belongs to a novel serotype: (i) Seg-2/VP2 sequence identities of BTV-27v01–v03 are most closely related to BTV-25 (73–74 % nt/75 % aa), (ii) sequence homology is high between the three variants (92–93 % nt/90–91 % aa), (iii) neutralization test of anti-BTV-27v01–v03 goat sera did not neutralize a BTV-1 reassortant with the outer-capid viral proteins VP2 and VP5 of BTV-25 (BTV1/VP2/VP5 BTV25). In addition, in vitro characteristics of BTV-27v01–v03 are distinct from BTV-25 since all BTV-27 variants can be propagated in cell culture. Cross-neutralization assays between these three BTV-27 variants conducted with sera collected from naturally infected goats in the field showed inconclusive results. However, some of these goats have been vaccinated against BTV-1 (and potentially
against BTV-2 or BTV-4) and the time of BTV-27 infection and infection status of these BTV-27-RT-qPCR-positive animals are unknown. Some of the goats found infected in 2014 could have been previously infected with one or more BTV-27 variants. Indeed, cross-neutralization assays between these three BTV-27 variants have to be carried out with sera collected from animals experimentally infected with any one of three BTV-27 variants.

Sequence identities between individual segments of BTV27v01–v03, and in comparison with the respective segments of BTV-25 and BTV-26, varied considerably (Tables 1 and S1). Accordingly, the following epidemiological evolution may be suggested: BTV-27v01 originates from a common ancestor of BTV-25 and BTV-26, or a BTV-25 that reassorted Seg-5 and Seg-7 with a BTV-26. BTV-27v03 evolved from v01 or a common ancestor, followed by reassortment of Seg-6 of a BTV-26 or a common ancestor of BTV-26. BTV-27v02 evolved from v03 or a common ancestor and was subsequently reassorted with Seg-10 and Seg-9 from a BTV-25. A reasonable explanation for the origin of reassorted segments from serotypes other than BTV-27 (Table 1) may be that BTV-infected animals acquire a protective immune response against infection with a homologous serotype, but they may still be infected with a heterologous BTV serotype (Schwartz-Cornil et al., 2008; Eschbaumer et al., 2010).

The majority of segments of BTV-27v01–v03 share the same topotype (reflecting geographic origin) with the western BTV-25 (Seg-2, 3, 4 and 8) or a common topotype with BTV-25 and the eastern BTV-26 (Seg-1, 5, 7, 9 and 10). Interestingly, Seg-6 of BTV-27v01 shares the same topotype with BTV-25, while Seg-6 of BTV-27v02 and v03 forms a novel, distinct topotype (Fig. S1). Similarly, Seg-6 of BTV-27v01 shares the same nucleotype ‘H’ with BTV-25, while BTV-27v02 and v03 are more closely related to BTV-26 and form a novel distinct tenth nucleotype ‘J’ (Fig. 4) according to Maan et al. (2010, 2011b). In contrast to Seg-6, Seg-2 of all BTV-27 variants belongs to the same nucleotype as BTV-25 (Fig. 3).

The highly variable VP2 (Seg-2) is the major determinant of a BTV serotype, while the second outer-capsid VP5 (Seg-6) is more conserved and co-determines the BTV serotype (Schwartz-Cornil et al., 2008; Maan et al., 2015b). In general, a weak association between Seg-2 and 6 of various BTV serotypes occurs in vivo, due to the extensive functional, but not highly specific interaction (Nomikou et al., 2015). Accordingly, independent reassortment of Seg-2 and 6 observed in the present study may occur, and has recently been described for an Indian BTV-2 (Maan et al., 2015a). Reassortment events are a common feature in BTV evolution (Shaw et al., 2013; Boyle et al., 2014; Nomikou et al., 2015) and may explain the diversity of sequence identities between BTV-27v01–v03 (Tables 1 and S1). In a comprehensive study of BTV genomes from different isolates of the 27 currently recognized BTV serotypes that have circulated within or outside Europe, Seg-2, 6, 7 and 10 were found permissive in reassortment with different genomic backgrounds. In contrast, a core set of Seg-1, 3, 4, 5, 8 and 9 was shown to commonly remain together during reassortment events in vivo (Nomikou et al., 2015). Co-evolution of specific segments was suggested as facilitating optimal function between the respective protein genes (Boyle et al., 2014; Nomikou et al., 2015), for example, the viral replication complex consisting of Seg-1, 4 and 9 (individual functions given in Table 1) (Nomikou et al., 2015). In the present study, Seg-1, 3, 4, 8 and 9 were all found most closely related to BTV-25, but not Seg-5. Seg-5 of BTV-27v01–v03 shares a higher homology to Seg-5 of BTV-26. The reassortment events could have occurred due to positive (diversifying) selection, facilitating a higher fitness for survival (Boyle et al., 2014; Maan et al., 2015b). A correlation between a higher virulence with an enhanced transmission and dissemination of a western Seg-5/NS1 among eastern BTV serotypes was found for BTV outbreaks in India (Maan et al., 2015b). However, the novel serotypes BTV-25, 26 and 27 seem to be apathogenic in goats (Chaignat et al., 2009; Batten et al., 2014; Zientara et al., 2014). In regions where BTV is endemic, clinical signs are rarely observed in susceptible hosts and reassortment between co-circulating or newly introduced BTV serotypes and strains is a common event in regions where BTV is endemic, for example Australia and North America. However, the introduction of novel BTV strains or Culicoides-vector spp. to regions where BTV is endemic or where susceptible hosts are naïve to BTV infection may result in emergence of bluetongue disease (MacLachlan et al., 2009; Boyle et al., 2012, 2014; Caporale et al., 2014). Adaptation to local hosts and vectors may result in positive (diversifying) selection with more virulent quasispecies, followed by negative (stabilizing) selection due to strong evolutionary constraints imposed by replication in alternating hosts (Boyle et al., 2012, 2014; Caporale et al., 2014). Interestingly, in BTV-27v01–v03, no reassorted segments have been detected from a pathogenic BTV-1 strain (Saegerman et al., 2008) that circulated in Corsica in 2013 and 2014 (Zientara et al., 2014). Indeed, recent reverse-genetic studies suggest that some combinations of genomic segments might not reassort with more distantly related BTV strains, at least using current methods (Nunes et al., 2014; Nomikou et al., 2015).

The inter-variant relationships of the full coding genomes of BTV-27v01–v03 (Table S1) are relatively large in comparison with, for example, BTV-1 strains isolated in recent years in France and Italy (segments 1–10 of the BTV-1 isolates FRA2007/18, FRA2008/21 and SAD2013 share ≥99.3 % homology; http://www.ncbi.nlm.nih.gov) after its incursion into Southern Europe in 2006 (Purse et al., 2008; Wilson & Mellor, 2009). In a similar way, the BTV-8 recently isolated in France in 2015 shows only 19 substitutions identified in 18 444 coding nucleotides, with only a consequence of four amino acid changes (in Seg-1, 3, 8 and 9), when compared with BTV-8 strains isolated in France in 2008 (Bréard et al., 2016). Furthermore, all three variants show a similar sequence identity with BTV-26 for Seg-5/
NS1 and VP7 (Table 1). Therefore, a possible explanation for the diversity of the BTV-27 variants may be that reassortment might have occurred with viruses already present on the island or at a more distant place from where the ancestors of BTV-25, 26 and 27 originate. Interestingly, for the highly conserved Seg-7 that encodes for the outer-core capsid viral protein VP7, the nt sequences of BTV-27v01–v03 are more closely related to BTV-25 (84 % nt/95–96 % aa). In contrast, the VP7 aa sequences of BTV-27v01–v03 show the highest identity to BTV-26 (82 % nt/97 % aa) (Table 1). Distances between the three BTV-27 variants are very similar (97 % nt/99.4–100.0 % aa), while sequence identities between BTV-25 and BTV-26 are lower for the nt sequence (81 % nt) and higher for the aa sequence (98 % aa) (Table S1). These characteristics further support the hypothesis that the three serotypes BTV-25, 26 and 27 diverged from a common ancestor and that BTV-27 subsequently experienced a strong conservation pressure, masking regional variations, evident by the large variations in nt sequences of Seg-7 (Maan et al., 2011b). VP7 can mediate attachment and penetration of insect vector cells. The strong conservation pressure could have been driven by an adaptation to regional Culicoides-vector midges together with/or due to an isolated evolution of BTV-27 on the island. On the other hand, BTV-27 has never been detected in Corsica before 2014, despite comprehensive BTV field studies in previous years (Zientara et al., 2014). The BTV-27 variants have possibly been introduced recently to the island either (i) after a single introduction, for example, with infected ruminants or Culicoides vectors emerging on the island with prevailing winds, or (ii) due to several introductions from a more distant place, such as North Africa. Multiple introductions of different BTV strains from North Africa to the European part of the Western Mediterranean Basin have been reported (Mintiens et al., 2008; Wilson & Mellor, 2008). Furthermore, Culicoides imicola populations in the Western Mediterranean Basin (including North Africa and Corsica) belong to a common genetic cluster (Jacquet et al., 2015). Accordingly, a marked adaptation of the BTV-27 variants to Corsican C. imicola midges was assumably not essential (Boyle et al., 2012, 2014; Caporale et al., 2014), which may explain the high homology between the VP7 aa sequences of BTV-27v01–v03.

CONCLUSION

In summary, the results of the phylogenetic and sequence analyses of the full coding genomes of BTV-27v01–v03 with other BTV serotypes, as well as the neutralization test with BTV1VP2/VP3 BTV25 indicate that three variants of BTV-27 have been isolated, and further support that BTV-27 (with all three variants) should be classified as its own serotype.

Buetongue is a notifiable disease to the OIE since outbreaks generally implicate trade and movement restrictions that may cause severe economic losses for affected regions. To date, the apathogenic BTV-25, 26 and 27 serotypes have not caused economic losses and restriction measures have not been implemented in the affected regions. The recent detection of BTV-25, 26 and 27, and of two putative novel BTV serotypes (BTV-28 and 29) within a few years due to molecular detection techniques (Wright, 2014; Zientara et al., 2014; Maan et al., 2015b) suggests that the discovery of additional novel BTV serotypes is very likely in the future. Accordingly, it seems reasonably important to define clear, objective serological and genetic criteria that allow the discrimination between ‘atypical’ apathogenic BTV and ‘traditional’ potentially pathogenic BTV strains and an appropriate assessment of whether restriction zones and trade restrictions should be implemented or not. Although BTV virulence has been associated with particular viral proteins, including VP2, NS4 and NS3, pathogenicity is a complex process, depending on functional and structural interaction of BTV proteins, as well as on various other factors such as vector competence, host, environment and climate (Boyle et al., 2012, 2014; Caporale et al., 2014; Celma et al., 2014; Pages et al., 2014; Janowicz et al., 2015; Ratinier et al., 2016). Future research is needed to investigate (i) the role and competence of Culicoides-vector spp. important for the transmission of traditional BTV serotypes, as well as their impact on pathogenicity of atypical BTVs, and (ii) whether reassortment of apathogenic BTV strains belonging to topotypes of BTV-25, 26 and 27 with pathogenic BTV serotypes is possible, and might lead to virulent strains of those topotypes, or to a spill over to cattle.

METHODS

Field study. In 2014, EDTA-blood was collected from goats (male and female) on different Corsican farms during a mandatory vaccination programme against BTV-1, which was combined with a monitoring programme of animals with clinical signs similar to bluetongue disease and fatalities as described previously (Zientara et al., 2014). In November 2015, 18 to 25 goats were sampled on each of seven Corsican farms where at least one BTV-27 variant was detected in 2014 and on two additional farms that introduced goats previously testing positive for BTV-27-specific RT-PCR (see below).

RT-PCR for BTV-27 variant determination. The BTV-27 variants have been detected in the EDTA-blood of goats without clinical signs using a commercial BTV-group-specific real-time quantitative RT-PCR (RT-qPCR) kit that amplifies a portion of the segment 10 of all 27 BTV serotypes (ADIAVET™ BTV Real time PCR kit; BioMérieux). The different BTV-27 variants were identified by alignment of partial segment 2 sequences obtained with conventional RT-PCR by using primers as described by Zientara and colleagues (2014). The amplified products were directly sequenced (see below).

Virus isolation. As described previously (Zientara et al., 2014), isolation of these BTV-27 variants was successfully achieved only on BSR (a clone of BHK-21) cells. Red blood cells from 200 µl of EDTA-blood were washed twice with PBS. An osmotic choc was performed with 600 µl of sterile water. Finally, 1.4 ml of PBS was added. The BSR cell monolayer was then inoculated with this inoculum for 1 h at room temperature. The inoculum was removed and 6 ml of medium containing 5 % FBS was added onto BSR cells. After 9 to 10 days of incubation at 37 °C and 5 % CO2, supernatants were tested by commercial BTV-group-specific RT-qPCR (see earlier). For BTV RT-qPCR-positive samples, another passage was performed until a cytopathic effect (CPE) was observed or a negative RT-qPCR result was obtained.
Virus neutralization test with BTV\textsubscript{1}P/VP2/VP5 BTV\textsubscript{25}. Three goats, sera positive with serum neutralization test (Sailleau et al., 2000) against the respective BTV-27v01 to v03 were used for virus neutralization test (VNT) against the genetically modified BTV\textsubscript{1}P/VP2/VP5 BTV\textsubscript{25}, a reassortant of BTV serotype 1 (Nunes et al., 2014). VNT was carried out in microtitre plates. Briefly, constant amounts of serum (containing four neutralizing units against the respective BTV-27 variant) were added to a 10-fold dilution series of BTV\textsubscript{1}P/VP2/VP5 BTV\textsubscript{25} and to BT2v01, v02 and v03 as controls. The serum–virus mixtures were incubated for 1 h at 37°C and 5% CO\textsubscript{2}. BSR cells (20 000 cells/well) were then added at the plates were incubated for 6 days at 37°C and 5% CO\textsubscript{2} (Sailleau et al., 2000).

Sequencing. Preparation of the samples, library construction, sequencing and sequence data assembly of the coding regions of the 10 segments for each of BTV-27v02 and BTV-27v03 were conducted as described previously for BTV-27v01 (Jenckel et al., 2015). Phylogenetic trees were constructed with BTV strains, representative for any one of the 27 BTV serotypes (i) separately, for each of the 10 segments (Figs 3, 4 and S1) and (ii) in combination, for all segments together (Fig. 2). BTV strains were selected according to the availability of all 10 segments for each of the 27 serotypes. In addition, phylogenetic trees were calculated for Seg-2 and Seg-6 coding for the outer-capsid proteins VP2 and VP5 with the 27 reference strains of the different BTV serotypes (Figs 3 and 4) (Maan et al., 2004, Singh et al., 2004). Sequences were aligned with MAFFT (version 7.017, Katoh & Fitch, 2002) and phylogenetic trees were constructed with IQ-TREE (version 1.2.2) (Nguyen et al., 2015) using 1000 bootstrap replicates and the 'best-fitted model'.

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