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Risk-based serological survey of bluetongue and the first evidence of bluetongue virus serotype 26 circulation in Tunisia

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Abstract

Background: Bluetongue (BT), a vector-borne disease of wild and domestic ruminants, is responsible for severe economic losses in flocks. To reduce this impact, a surveillance and control plan was implemented in Tunisia. However, the epidemiological situation of BT remains incompletely understood, especially for the circulating serotypes.

Objective: The aim of this survey was to determine the seroprevalence, to identify the circulating serotypes and to identify the associated risk factors for bluetongue virus (BTV) circulation in Tunisia using risk-based sampling (RBS).

Methods: A total of 3314 blood samples were randomly collected from 67 sectors using risk-based sampling and screened by competitive enzyme-linked immunosorbent assays (c-ELISAs). Out of the 1330 positive samples, 200 samples were analysed by serum neutralization test (SNT) to identify circulating BTV serotypes.

Results: Of 3314 sera, 1330 were c-ELISA-positive (40.1%) for antibodies against the BTV structural protein VP7. The result of SNT showed the presence of BTV-1, BTV-2, BTV-3, BTV-4 and, for the first time in Tunisia, BTV-26. The logistic regression model revealed that older animals had nearly two times the odds of being infected with BTV compared to younger animals. Flocks with a history of BT were almost 1.5 times more likely to be at risk for contracting BTV infection. The flock size, housing indoors and intensive production system were significant protective factors.

Conclusions: High seroprevalence of BTV among sheep was highlighted in Tunisia. The neutralization test showed the presence of the following BTV serotypes: BTV-1, BTV-2, BTV-3, BTV-4 and, for the first time in Tunisia, BTV-26. Age, production system and flock size were important variables associated with BTV infection in sheep. This finding is crucial, as it will allow the adjustment of the BT control programme in Tunisia.

KEYWORDS
bluetongue, risk factors, risk-based sampling, serotyping, sheep, Tunisia

1 | INTRODUCTION

Bluetongue (BT) is a vector-borne disease predominantly transmitted by biting midges (*Culicoides* spp.) to domestic ruminants such as sheep, goats and cattle and wild ruminants such as deer, roe deer and Cantabrian chamois (Chaignat et al., 2009; Lorca-Oró et al., 2014). This disease is caused by infection with Bluetongue virus (BTV) of the genus *Orbivirus*, family *Reoviridae*, which contains 10 segments of double-stranded RNA with a high rate of mutation (Alkhamis et al., 2020). Thirty-five serotypes have been described so far. These include the 24 classical BTV serotypes and the other 11 considered atypical (Ries et al., 2020). BT is an infectious noncontagious disease capable of causing severe economic losses due to direct (loss of body weight and condition, drop in milk production and poor subsequent reproductive performance) and indirect economic impacts (restriction on the international trade of livestock) (Gethmann et al., 2020). Currently, BTV is widespread in all continents except Antarctica (Ma et al., 2019). Climate change has resulted in a shift in midge populations, hence a shift in disease/viral emergence (Jacquot et al., 2017). In North Africa, the first reported incursion of BTV in Tunisia was in 1999 (Ben Fredj et al., 2003). A total of 14,775 clinical cases and 1286 deaths in sheep caused by serotype 2 (BTV-2) were reported by Tunisian veterinary services, and since then, the disease has been endemic (Dhaou, 2017; Kilani et al., 2020). In 2000, Algeria reported the presence of BTV-2 in the northeast of the country (Hammami, 2004), and molecular studies comparing genomic segments 2 and 7 of the virus isolated in Algeria to those isolated in Tunisia in May 2000 showed that the two isolates were probably of the same origin (Kardjadj & Luka, 2016). BT was not reported in Morocco before 2004, but an incursion of BTV-10 in 1956 in the southern area of Larache and west of Arbaoua was described based on a serological study (Youssef et al., 2017). In 2004, outbreaks of BTV-4 appeared for the first time in Larache province in the Northwest Atlantic littoral of Morocco (Youssef et al., 2017). Since 1999, many serotypes, including BTV-1, BTV-2, BTV-3 and BTV-4, have been identified in sheep in North African countries with clinical signs such as swollen head with nasal discharge and depression (EFSA, 2007). Serological surveys were conducted to identify circulating serotypes. In Libya, the serological investigation carried out by Mahmoud A et al. during 2015-2016 revealed the presence of BTV-1, BTV-2, BTV-3, BTV-4, BTV-9 and BTV-26 (Mahmoud et al., 2018). A putative novel BTV serotype (BTV-Y TUN2017) related to the BTV strain, contaminating a commercially available sheep pox vaccine, and to BTV-26 was identified in sheep in the centre of Tunisia in 2017 (Lorusso et al., 2018). Although BTV is endemic in Tunisia, there is currently limited information available on the prevalence of this virus in sheep. The circulation of several serotypes throughout North African countries at the same time raises several questions about the role of livestock movements in the introduction and spread of BTV in this region (Gahn et al., 2020; Walton, 2004). In Tunisia, since its first detection in 1999 and up to 2017, BTV has been controlled through the mass vaccination of sheep using a live vaccine for the first period (1999–2016) and an attenuated vaccine for the second period (2016–2017) against BTV-1, BTV-2 and BTV-4. Active and passive surveillance had
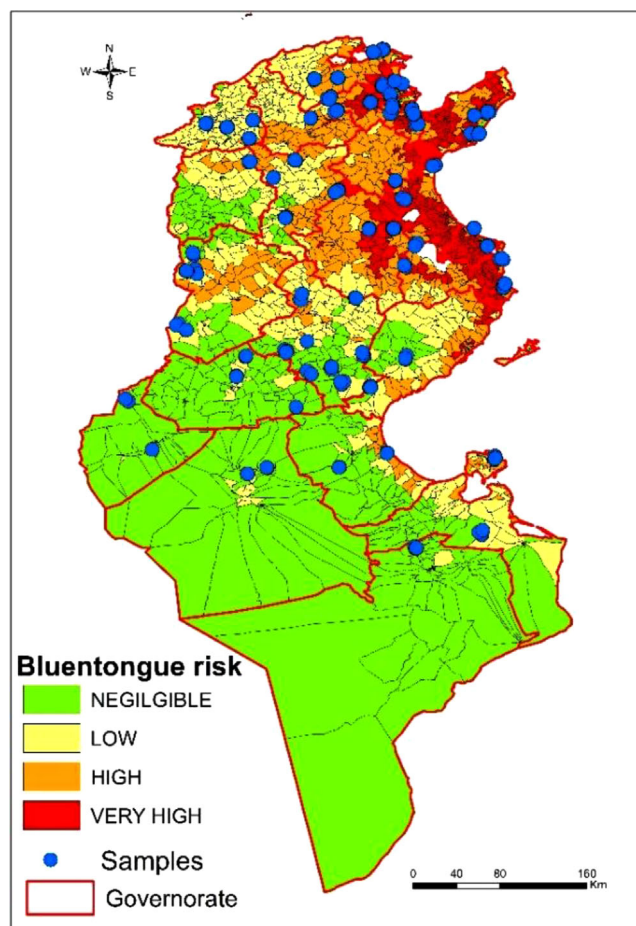

FIGURE 1 Risk map of bluetongue (BT) exposure in Tunisia demonstrating sheep farms within specific governorates (red sections) and sectors (black sections) of four different risk categories (negligible, low, high and very high) in which samples were taken

been implemented throughout the country since the occurrence of the disease. Considering the epidemiological situation, conventional methods such as passive and active surveillance were insufficient to control BTV, especially with uncontrolled livestock movements. The surveillance of BT could be strengthened by implementing risk-based surveillance (RBS) that takes into consideration the main risk factors, such as vector density, animal movement and animal density, to optimize the surveillance for early detection and to target zones where the disease is more likely to be introduced and spread. The aim of this study was to use RBS to determine the seroprevalence, serotypes and associated risk factors of BTV circulation in Tunisia.

2 | MATERIALS AND METHODS

2.1 | Identification of risk areas and sampling

Tunisia is subdivided into 24 governorates (administrative units) that are further divided into 2084 sectors (Figure 1). This study was conducted in 67 sectors randomly selected from the 2084 sectors in

Tunisia, which were classified into four risk classes (negligible, low, high and very high risk areas) for exposure to BTV using a qualitative risk assessment method based on SQRA methodology (Kalthoum et al., 2021; Squarzoni-Diaw et al., 2020).

The primary step involved in the identification of risk areas for exposure to BT in Tunisia was the identification of the potential risk factors for BTV spread. Thus, risk factors used to characterize the different levels of risk of exposure to BTV (negligible, low, high and very high) included the following:

- The probability of the presence of the main vector (*Culicoides imicola*) was determined using the maximum entropy model (MaxEnt version 3.4.1, http://www.cs.princeton.edu/wschapire/MaxEnt/) (Phillips et al., 2006), the climatological factors (temperature and rainfall) and the presence/absence data on the vector retrieved from previous studies on vector trapping in Tunisia (Chaker et al., 2005; Hammami et al., 2008; Slama et al., 2016; Slama et al., 2014; Slama et al., 2015; Sghaier et al., 2017). The probability of the presence of the vector in the generated raster was calculated at the sector level using ArcGIS software version 10.4.
- Sheep population density expressed in units of the number of heads/km^2 at the sector level was calculated using Gridded Livestock of the World animal density data set (Robinson et al., 2014) and the area of sectors, which was performed with ArcGIS software version 10.4.
- National livestock movement data: A cross-sectional survey was conducted by the national Center for Zoosanitary Vigilance (CNVZ) at all livestock markets in Tunisia. Data on the origin/destination, number of animals and traded species of all movements that had occurred during the survey were registered. The degree and betweenness of the recorded flows were calculated using social network analysis at the sector level (Baccar et al., 2018; Martínez-López et al., 2009).

To combine these risks, all quantitative risk factors (sheep population density, probability of the presence of the vector and degree and betweenness of the national livestock movement) were discretized (quantiles) into four qualitative categories of risk according to their distribution per sector (negligible, low, high and very high) (Table 1).

For the qualitative risk factors, only two classes were established: absence and presence and were coded as zero and one, respectively.

To enable categorized risks to be viewed in an easily understandable format, each categorized risk factor was mapped at the sector level. Finally, classified risk factors were combined using the Boolean combination at the level of sector as Squarzoni-Diaw et al., 2020 did for foot and mouth disease (FMD) (Squarzoni-Diaw et al., 2020) (Figure 1).

The following are the expected prevalence rates in the four risk strata (very high, high, low and negligible) (Figure 1):

- A very high risk stratum with an expected prevalence rate p1 of 50%. There are 453 sectors in this stratum.
- A high risk stratum with an expected prevalence rate p2 of 40%. There are 536 sectors in this stratum.

TABLE 1 Combination table of risk factors to estimate the risk of exposure of bluetongue (BT) in Tunisian sectors

Sector risk level	Qualitative combination of risk factors
4: Very high	["very high" sheep density AND "very high" probability of vector] OR ["very high" probability of vector AND "very high SNA degree and betweenness]
3: High	["high" sheep density AND "high" probability of vector] OR ["high" probability of vector AND "high SNA degree and betweenness"]
2: Low	["low" sheep density AND "low" probability of vector] OR ["low" probability of vector AND "low SNA Degree and Betweenness"]
1: Very low	All remaining sectors

A low risk stratum with an expected prevalence rate p3 of 15%. There are 574 sectors in this stratum.

- A negligible stratum with an expected prevalence rate p4 of 10%. There are 521 sectors in this stratum.

A total of 67 randomly sampled sectors were needed. The number of required sectors in each risk category was calculated based on an absolute precision of 2.5% and a risk error of 5%. These 67 sectors were distributed as follows: 15, 17, 18 and 17 sectors from very high, high, low and negligible risk areas, respectively. Fifty animals per sector were randomly sampled. Animals younger than 4 months were excluded from the sampling to avoid the detection of antibodies due to maternal immunity. A total of 3350 samples were needed and 3314 were collected in sheep in 333 flocks during the period of the study. The gap in the number of samples corresponds to the number of samples discarded because of insufficient quantity or broken tubes.
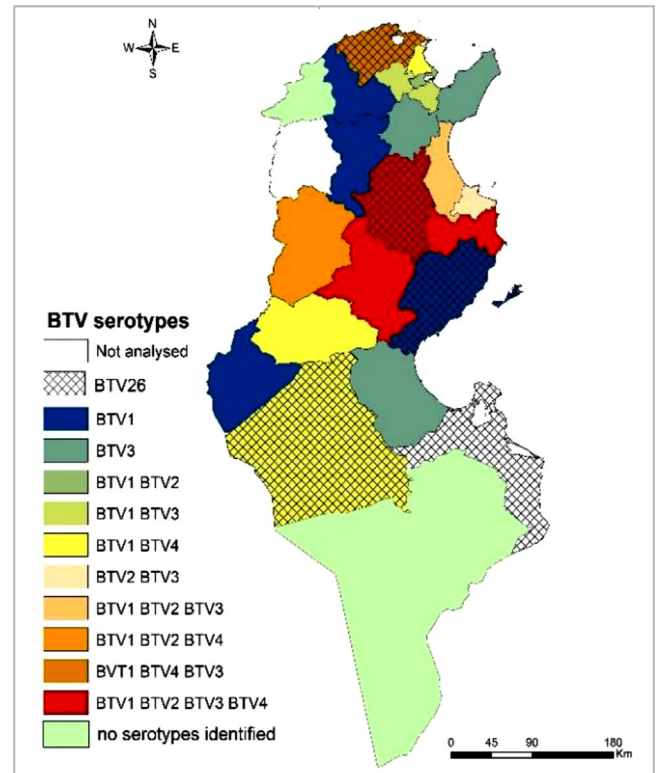
2.2 | Data collection and questionnaire design

The study was conducted between November 2019 and January 2020, as this period coincides with the highest levels of seroconversion in the animals in the study. Data on breeders and animals were collected using a structured questionnaire. First, the questionnaire was pretested in 10 flocks to verify whether the questions were understandable by both investigators and breeders. Data collected included information on breeders and farms (number of animals, geographical coordinates, history of BTV in the farm and in neighbouring farms and vaccination against BT) and on sampled animals (age, sex and breed). Questions on risk factors related to exposure to the BTV both in animals (age, sex, breed, housing) and flocks (flock size, presence of stagnant water, proximity to wetlands, grazing near wetlands, production system, exchange of animals and introduction of new animals) were included. Face-to-face interviews were conducted with breeders to collect data.

TABLE 2 Distribution of the 200 samples selected for the serum neutralization test according to the governorates

Governorate	Samples	Percentage
Ariana	7	3.5
Beja	5	2.5
Ben arous	10	5
Bizerte	11	5.5
Gabes	6	3
Gafsa	7	3.5
Jendouba	2	1
Kairouan	29	14.5
Kasserine	14	7
Kébili	2	1
Mahdia	8	4
Mannouba	8	4
Médenine	3	1.5
Monastir	3	1.5
Nabeul	3	1.5
Sfax	3	1.5
Sidi bouzid	48	24
Siliana	5	2.5
Sousse	4	2
Tataouine	8	4
Tozeur	5	2.5
Tunis	5	2.5
Zaghouan	4	2
Total	200	100

FIGURE 2 Bluetongue virus (BTV) serotypes circulating across 67 sampled sectors ($n = 200$ sheep sera) in Tunisia between November 2019 and January 2020 as identified by serum neutralization test following a positive competitive enzyme-linked immunosorbent assay (c-ELISA) result

2.3 | Blood samples and laboratory analysis

A total of 3314 sera were collected and tested using a commercial competitive enzyme-linked immunosorbent assay (c-ELISA) kit (ID SCREEN® Bluetongue Competition ELISA, IDVet, Grabels, France) according to the manufacturer's instructions. Sera with a percentage inhibition of <35% were considered positive. Samples with a percentage inhibition between 35% and 45% were retested, and those above 45% were classified as negative. Sera with doubtful results were retested. Among the ELISA-positive samples, 200 were selected based on seropositivity and representativeness of the governorates, with the exception of one governorate where samples were not analysed as they showed weak ELISA-positive results. The number of samples ranged between two and 48 (Table 2). The 200 samples were sent to the OIE Reference Laboratory for Bluetongue (IZSAM) Teramo, Italy, to identify serotypes by serum-neutralization test (SNT) assay using the reference serotypes 1, 2, 4, 6, 8, 9, 14, 15, 16 and 26 (Gard & Kirkland, 1993; Savini et al., 2004). Duplicate technical replicates were performed for the SNT.

2.4 | Data analysis

Data entered into Microsoft Access were analysed using R software version 3.4 (R Core Team, 2017). Environmental Systems Research Institute (ESRI) Maps in Figures 1 and 2 were generated with ArcGIS version 10.4 (https://www.esri.com/). The overall seroprevalence of BTV was calculated by dividing the total number of positive samples in c-ELISA by the total number of tested samples. Flock size was divided into three classes based on the quartile classification method (Ross, 2014). Animals were grouped into three classes of age (Class 1: ≤24 months; Class 2: >24 months to ≤48 months; Class 3: >48 months). Associations between the seroprevalence and its potential risk factors were first screened in a univariable analysis using the chi-square test, and the significance level α was set at $p < 0.05$. Then, multivariable analysis using a logistic regression model (LRM) was performed using the significant results of the univariable analysis (significance level α was set at $p < 0.02$). Odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated using R software. BTV seroprevalence was considered the dependent variable and significant risk factors were considered independent variables.

TABLE 3 Sroprevalence of bluetongue (BT) infection in Tunisian sheep according to the category of the risk areas

Risk category	Negative samples	Positive samples	Total	Seroprevalence (%)	95% CI
Very high	437	306	743	41.2	37.6%–44.7%
High	502	346	848	40.8	37.4%–44.1%
Low	529	361	890	40.6	37.3%–43.7%
Negligible	516	317	833	38.1	34.7%–41.3%
Total	1984	1330	3314	40.1	38.4%–41.8%

3 | RESULTS

3.1 | BTV seroprevalence

Out of the 3314 samples, 1330 sera showed antibodies against BTV by c-ELISA. The overall seroprevalence of BTV among sheep surveyed in Tunisia was 40.1% (1330/3314; 95% CI: 38.4%, 41.8%). The seroprevalence was homogeneously ($p = 0.55$) distributed among the four categories of risk areas, and no significant variation was detected (Table 3).

The seroprevalence varied significantly at the governorate level ($p = 0.000$). The lowest seroprevalence (20.5% [41/200]) was recorded in Jendouba (northwest Tunisia). High values of seroprevalence were observed in the central (Kairouan 62.8% [125/199], Sousse 56% [56/100]), southern (Tataouine 73.4% [36/49]) and northern governorates (Kef 58.8% [53/90], Mannouba 48.9% [48/98]) (Table 4).

3.2 | Strain typing and identification

Out of the 200 positive samples in c-ELISA, which were analysed by the SNT test to identify BTV serotypes, 105 samples had neutralizing antibodies, which represented 52.5% of the tested samples. Titres range from 1:10 to more than 1:640 against one or more of the following serotypes: BTV-1, BTV-2, BTV-3, BTV-4 and BTV-26. The highest titres for BTV-1 and BTV-2 were 1:640 and 1:320, respectively, recorded in the governorate of Sidi Bouzid (centre of Tunisia). For BTV-3, the highest titre (1:320) was detected in the governorate of Gabes (south of Tunisia). However, for BTV-4 only low titres (1:10 and 1:20) were recorded in eight governorates from the north, centre and south of Tunisia. BTV-26 was discovered in sheep for the first time in the north (Bizerte governorate), the centre (Sousse and Sfax governorates) and the south (Medenine, Kebili governorates) (Figure 2). Titres ranged between 1:10 and 1:80. The highest titre was registered in the governorate of Kebili (south of Tunisia). BTV-1 was the most prevalent serotype (33.3%), followed by BTV-3 (13.3%), BTV-2 (8.6%), BTV-4 (3.8%) and BTV-26 (1%). We found that 60% (63/105) of samples showed the presence of neutralizing antibodies against only one serotype. The results highlighted double, triple or quadruple infections in 40% of the samples. The combination of BTV-1 with BTV-4 was the most predominant (14 samples), followed by the circulation of BTV-1 with BTV-2 (nine samples). In the central governorates, infection with four serotypes (BTV-1, BTV-2, BTV-3 and BTV-4) or (BTV-1,

BTV-2, BTV-3 and BTV-26) was observed in three samples. BTV-1 is the only serotype identified in the northwest governorates (Siliana and Beja). No serotypes were identified in the governorates of Jendouba and Tataouine.

3.3 | Risk factors associated with the seroprevalence of BTV

Univariate analysis showed that five of the 14 analysed factors (age, housing, production system, flock size and BTV history on the farm) were statistically significantly associated with the seroprevalence of BTV. Older animals (>48 months) were more frequently infected by BTV and appeared more at risk than young animals ($\leq$ 24 months) ($p = 0.0000$). The flock level risk factors housing (indoors/outdoors), production system, flock size and BTV history on the farm were significantly associated with the seroprevalence of BTV ($p < 0.05$) (Table 5). Housing sheep outdoors was found to be associated with a decreased risk of seropositivity for BTV ($p = 0.008$). In addition, smaller flock sizes were found to be a protective factor ($p = 0.02$). For the production systems, the lowest seroprevalence was found to be associated with intensive farming systems ($p = 0.01$). However, breed, proximity to wetlands, presence of stagnant water, grazing near wetlands, introduction of new animals, presence of BTV in neighbouring farms, exchange of animals and presence of cattle in the farm were found to be not significant ($p > 0.05$).

In the final mixed effects logistic regression model, older animals (>48 months) had a nearly two times (OR = 1.6, 95% CI = 1.32–1.93) higher risk of being infected by BTV than younger animals (<24 months) ($p < 0.001$). Flock size was a significant protective factor for small flocks, and a decrease in flock size on the farm decreased the risk of being positive for BTV ($p = 0.02$). Similarly, the intensive production system (OR = 0.68, 95% CI = 0.53–0.87) and housing indoors (OR = 0.72, 95% CI = 0.58–0.88) were identified as protective factors. Flocks with a history of BTV have a two times (OR = 1.5, 95% CI = 1.11–2.02) higher risk of being infected by BTV than noninfected flocks (Table 6).

4 | DISCUSSION

The current surveillance programme for BT in Tunisia is based on a passive component consisting of direct detection of the suspected

TABLE 4 Seroprevalence of bluetongue virus (BTV) infection in sheep based on competitive enzyme-linked immunosorbent assay (c-ELISA) according to the Tunisian governorates

Governorate	Number of tested animals	Positive samples	Seroprevalence (%)	95% CI
Jendouba	200	41	20.5	14.9–26
Tunis	90	22	24.4	15.5–33.3
Monastir	110	28	25.4	17.3–33.5
Tozeur	100	26	26	17.4–34.5
Medenine	100	29	29	20.1–37.3
Kasserine	150	45	30	22.6–37.3
Gabes	100	32	32	22.8–41.1
Siliana	199	69	34.6	28–41.2
Kebili	99	36	36.3	26.8–45.8
Ben Arous	150	55	36.6	28.8–44.3
Mahdia	190	72	37.8	30.9–44.7
Beja	100	38	38	28.4–47.5
Gafsa	150	59	39.3	31.5–47.1
Sidi Bouzid	299	125	41.8	36.2–47.3
Nabeul	196	82	41.8	34.9–48.7
Ariana	200	86	43	36.1–49.8
Bizerte	198	93	46.9	40–53.9
Zaghouan	50	24	48	34.1–61.8
Mannouba	98	48	48.9	39–58.8
Sfax	97	50	51.5	41.6–61.4
Sousse	100	56	56	46.2–65.7
Le Kef	90	53	58.8	48.7–69
Kairouan	199	125	62.8	56–69.5
Tataouine	49	36	73.4	61.1–85.8

clinical cases. However, depending on the climatic changes and the intensity of the uncontrolled animal movement, passive surveillance alone may not be sufficient to monitor the disease due to the occurrence of subclinical cases. It is foreseen that supplementing existing passive surveillance with risk-based targeted sampling will increase the chances of detecting the infection (Mastin et al., 2020; Stärk et al., 2006). Here, we present the results of a risk-based serological surveillance study investigating BTV seroprevalence and the circulation of different BTV serotypes in sheep across 67 sectors of Tunisia. The primary step consisted of identifying risk areas according to the occurrence of BTV. Classification and categorization of risk factors at the sector level were adapted from the methodology recently applied to FMD in Tunisia (Squarzoni-Diaw et al., 2020). Samples were collected from 67 sectors classified into four categories of risk (negligible, low, high and very high). Overall, our results show that the seroprevalence rate of BTV was 40.1% among sheep in Tunisia. This is higher than the seroprevalence rates in sheep reported in Morocco (33.3%–37%), Algeria (Moustafa et al., 2016) (5.7%) and Ethiopia (30.6%) (Abera et al., 2018) but lower than what was found in Libya (50.3%) (Mahmoud et al 2019), Iran (76.4%) (Hasanpour et al., 2008), Pakistan (44.2%)

(Sohail et al., 2018) and Saudi Arabia (Yousef et al., 2012). However, the seroprevalence rates identified in this study should be interpreted with some caution. While it is known that the flocks selected for this survey were not vaccinated against BTV at the time (the vaccination of sheep flocks in Tunisia against BTV was stopped in 2017), their origin and past vaccination history were not always known, so seroprevalence data could reflect vaccination status in animals born before 2017.

The highest values of seroprevalence were recorded in the centre of Tunisia (governorates of Kairouan and Sousse) and the northern governorate (Mannouba), where BT clinical cases are recorded every year. *Culicoides imicola*, the main vector of BTV throughout Africa, was the most abundant species in the central and northern Tunisia (Hammami et al., 2008; Slama et al., 2017; Thameur et al., 2021). The high number of positive samples in the south of Tunisia (governorate of Tataouine), where *Culicoides imicola* was not detected (Hassine et al., 2017), can be explained by animal movement (Mintiens et al., 2008; Sumner et al., 2017; Turner et al., 2012) or the presence of other *Culicoides* species vectors. This could also be related to the uncontrolled introduction of animals from neighbouring countries (Elmahi et al., 2020). Lorusso et al., in a previous study conducted in Tunisia, detected a new strain of

TABLE 5 Univariable (chi-square) analysis of the risk factors associated with bluetongue virus (BTV) seroprevalence in Tunisia

Variable	Croups	Negative Samples	Positive samples	Number of tested animals	p Value
Age	Class 1 (<24 months)	1007	584	1591	0.0000[a]
	Class 2 (> = 24 and < = 48 months)	636	425	1061	
	Class 3 (>48 months)	341	321	661	
Breed	Barbarine	418	310	728	0.18
	Queue fine de l'Ouest	1039	654	1693	
	Noir de Thibar	129	101	230	
	Others	398	265	663	
Housing	Indoor housing	220	189	409	0.008[a]
	Outdoor housing	1764	1141	2905	
Production system	Extensive	662	437	1099	0.01[a]
	Intensive	282	147	429	
	Semi-intensive	1040	746	1786	
Proximity to wetlands	Yes	1354	914	2268	0.8
	No	630	416	1046	
Presence of stagnant water	Yes	465	295	760	0.4
	No	1519	1035	2554	
Flock size	Large	462	357	819	0.02[a]
	Medium	1005	670	1675	
	Small	517	303	820	
Grazing near wetlands	Yes	624	436	1060	0.4
	No	1360	894	2254	
Introduction of new animals	Yes	427	289	716	0.9
	No	1557	1041	2598	
BT history in the farm	Yes	85	91	176	0.001[a]
	No	1899	1239	3128	
Presence of BT in neighbouring farms	Yes	37	33	70	0.2
	No	1737	1117	2854	
Exchange of animals	Yes	369	269	638	0.2
	No	1615	1061	2676	
Presence of cattle in the farm	Yes	570	393	963	0.6
	No	1414	937	2351	

[a]Variables selected and used in the multiple analysis ($p \leq 0.02$).

BTV, named BTV-Y TUN2017, in sheep originating from Libya (Lorusso et al., 2018), which is an atypical strain where the transmission is horizontal. Whether it is animal movement or the presence of the vector, our study demonstrates the widespread seroprevalence of BTV among sheep in Tunisia.

According to the SNT results, out of the 200 samples, 105 were positive for neutralizing antibodies. We did not analyse all c-ELISA-positive samples by SNT for economic reasons. Neutralizing antibodies against BTV-1, BTV-2, BTV-3, BTV-4, BTV-6, BTV-8, BTV-9, BTV-14, BTV-15, BTV-16 and BTV-26 were analysed because these serotypes have been circulating or circulated in the Mediterranean region (Kundlacz et al., 2019; Mahmoud et al., 2019; Nomikou et al., 2009). As expected, the results revealed the presence in Tunisia of four serotypes (BTV-1, BTV-2, BTV-3 and BTV-4) that were sequenced during previous outbreaks. Cocirculation of different BTV serotypes (2, 3 or 4) within the same governorate and animal species was not unexpected since no tetravalent vaccine for the circulating serotypes was used to control the disease. Similarly, uncontrolled animal movements could be the cause of the permanent introduction of any of the four serotypes that circulate in neighbouring countries. It is important to identify the circulating serotypes of BTV to adapt to the control programme, especially when vaccination is the main measure used. Our results revealed multiple infections with BTV serotypes, indicating the high risk of reassortment events that could generate novel virus strains (Guimarães et al., 2017).

TABLE 6 Multivariable analysis of the risk factors associated with bluetongue virus (BTV) seroprevalence in Tunisia

Variable	Groups	Odds ratios	95% CI	p Value
Age	Ref. Class 1			
	Class 2	1.15	0.97–1.35	0.09
	Class 3	1.6	1.32–1.93	<0.001
Production system	Ref. = Extensive			
	Intensive	0.68	0.53–0.87	0.003
	Semi-intensive	0.95	0.81–1.13	0.62
Housing	Outdoors/indoors	0.72	0.58–0.88	0.002
BT history in the farm	Yes/No	1.51	1.11–2.02	0.008
Flock size	Ref. Large			
	Medium	0.81	0.68–0.98	0.02
	Small	0.72	0.59–0.9	0.002

BTV-1 was the most prevalent serotype, with 33.3% of the analysed samples suggesting the persistence of the circulation of this serotype since the age of the positive animals with BTV1 varied between 6 and 24 months.

Interestingly, this study is the first to report anti-BTV-26 antibodies detected in sheep sera in Tunisia. This serotype was detected for the first time in sheep in Kuwait (Maan et al., 2011). The main characteristic of this serotype was the absence of clinical signs and direct transmission between ruminants without the presence of the vector (Batten et al., 2014; Maclachlan et al., 2015). The detection of the serotype BTV-26 for the first time in Tunisia was not surprising since this serotype had recently circulated in Libya (Mahmoud et al., 2019). It was reported that BTV-26 was introduced in Libya through illegal and uncontrolled animal movement (Mahmoud et al., 2019). The presence of BTV-26 in the governorate of Medenine is in favour of its introduction into Tunisia from Libya. In fact, uncontrolled animal movements between Tunisia and Libya could facilitate the introduction of emerging serotypes and other diseases (Bouguedour & Ripani, 2016; Kalthoum et al., 2021). The circulation of BTV-26 in the centre (Gafsa, Kairouan, Sousse and Sfax governorates) and in northern Tunisia (Bizerte governorate) could be explained by its spread via animal movement in livestock markets (Baccar et al., 2018). It was demonstrated that the mobility of animals at the national level occurs throughout the country with a concentration in the centre and the north and where several livestock markets were qualified as a superspreader of the diseases (Baccar et al., 2018). Tunisia is at a permanent risk of introduction of new serotypes of BTV, such as BTV-9 and BTV-16 identified in Libya (Mahmoud et al., 2019) and BTV-8, BTV-14 and BTV-16 detected in Morocco (Drif et al., 2018; Drif et al., 2014). Our results showed that 95 samples were not positive for SNT. In this study, only serotypes BTV-1, BT-V2, BT-V3, BTV-4, BTV-6, BTV-8, BTV-9, BTV-14, BTV-15, BTV-16 and BTV-26 were investigated because these are the main serotypes circulating in the Mediterranean region. Consequently, the 95 samples could be positive for other serotypes that have not been evaluated, such as atypical serotypes found in Tunisia previously (Lorusso et al., 2018) or classical serotypes. Moreover, no serotypes were identified in Tataouine and Jendouba, suggesting that there could be other serotypes circulating in these governorates. Further investigation targeting serotypes other than those known to circulate in the Mediterranean area, especially atypical serotypes, would be recommended.

Fourteen risk factors were investigated with univariable and multivariable analyses using a LRM. Only six factors were associated with the variation in BTV seroprevalence. The current study revealed a statistically significant association between age and the seroprevalence of BTV, which increased with increasing age of the sampled animals, probably reflecting an increased duration of exposure. This result is comparable to those of studies conducted in different areas worldwide (Christie, 2010; Mahmoud & Khafagi, 2014; Mohammadi et al., 2012; Yilma & Mekonnen, 2015). In contrast, no association between age and BTV seroprevalence was found in the study conducted by Mozaffari and Khalili (2012). Here, a significant relationship was identified between BTV seroprevalence, flock size and intensive production systems. Our results suggest that a small flock size and intensive production system are protective factors. This result can be explained by the fact that in intensive systems, farms have better management of disease prevention and control and biosecurity measures (Gong et al., 2021). Similar results were found in studies conducted in Spain assessing risk for BTV-1 (Pascual-Linaza et al., 2014) and in Nepal (Gaire et al., 2014). Furthermore, economic losses due to BT are higher in semi-intensive farms than in other production systems (Kumar & Pandian, 2020).

For the management practices (housing indoors/outdoors), a high seroprevalence rate of BTV was detected in the farms where animals were kept indoors compared to those kept outdoors. Our result is in disagreement with previous studies that demonstrated that indoor animals are expected to be more exposed to infected vectors and BTV infection (Adam et al., 2014). However, the indoor and outdoor behaviour of *Culicoides* is strongly influenced by temperature and climatic conditions (Jess et al., 2018), and the high prevalence found in animals kept indoors could be due to the presence of the vector inside the buildings, as the investigation was carried out in winter.

This result can also be explained by the probability of the presence of vectors in Tunisia other than *Culicoides imicola* that are able to transmit the disease. The endophilic behaviour of many species of *Culicoides* has been reported in many studies worldwide. In Belgium, the number of *Culicoides* trapped indoors was 15–22 times higher than that in outdoor environments (Zimmer et al., 2010). Baldet T et al., in a study conducted in France, described for the first time the indoor activity of several species of *Culicoides*, such as *C. obsoletus*, *C. scoticus* and *C. dewulfi*, known by their outdoor activity (Baldet et al., 2008). In eastern Slovakia, high abundances of *C. punctatus* and *C. newsteadi* were detected indoors, suggesting variation in the behaviour of these species (Sarvašová et al., 2016). Overall, there is a crucial need to conduct further investigation to advance knowledge on the ecology of Tunisian *Culicoides* species to better control the disease.

Additionally, this study highlighted gaps in knowledge in many areas, such as vector specificity (abundance, behaviour in different climatic conditions and competence of other species of *Culicoides*), the circulating serotypes of BTV that influence the control of the disease. Therefore, further investigations are highly recommended to confirm the origin and identify the routes of spread of the introduced serotypes and to improve knowledge on the ecology of the main vector.

5 | CONCLUSION

Our results indicate a high seroprevalence of BTV among sheep and widespread BT disease across Tunisia. Five serotypes were detected in this study, and multiple infections were highlighted. This finding is crucial, as it will allow the adjustment of the BT control program in Tunisia. For the first time, we discovered the circulation of BTV-26 in Tunisia. Age, production system and flock size were important variables associated with BTV infection in sheep. Despite climate change and the circulation of many serotypes in neighbouring countries, the mass vaccination campaign had been stopped since 2017. The control of this endemic disease has become a challenge for veterinary services. Thus, the national BTV surveillance and control strategy needs to be reinforced through the implementation of systematic active surveillance, the development of an early warning system based on eco-climatic variables and the establishment of a database for animal movement.

CONFLICT OF INTEREST
The authors declare no conflic of interest. The funders had no role in the study (sampling, analyses, interpretation of results) or in the decision to publish the results.

AUTHOR CONTRIBUTIONS
Conceptualization: Kalthoum Sana and Seghaier Chedia; Formal analysis: Kalthoum Sana, Sghaier Soufien, Teodori Liana, Spedicato Massimo, Savini Giovanni, Thabet Sarah and Hammami Salah; Investigation: Guesmi Kaouther, Gharbi Raja, Hajlaoui Haikel, Bel Haj Mohamed Bassem, Khalfaoui Wiem, Lachtar Monia, Ben Salem Ameni, Fatnassi Naouel and Dhaouadi Anissa; Data curation: Ben Ali Mehdi; Writing – original draft: Kalthoum Sana, Seghaier Chedia, Ben Hassine Thameur, Savini Giovanni, Ben Ali Mehdi and Hammami Salah; Writing – review and editing: Kalthoum Sana, Seghaier Chedia and Hammami Salah.

DATA AVAILABILITY STATEMENT
no data are available.

ETHICAL DISCLOSURES
The authors declare that the study was conducted according to the national guidelines without causing harm to the animals and respecting their welfare. The authors also declare that the data related to breeders have not been published.

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