Detection and Phylogenetic Analysis of a Novel Tick-borne Virus in *Haemaphysalis Longicornis* Ticks and Sheep From Shandong, China

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Short report

Keywords: Dabieshan tick virus, ticks, sheep, Shandong Province

Posted Date: July 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-680468/v1>

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Version of Record: A version of this preprint was published at Virology Journal on November 27th, 2021.
See the published version at <https://doi.org/10.1186/s12985-021-01704-y>.

Abstract

Dabieshan tick virus (DTV) was first identified in *Haemaphysalis longicornis* from Hubei Province, China in 2015. However, its pathogenic potential to animals and human remains to be further explored. In this study, the presence of DTV in ticks and sheep samples was investigated. A total of 170 engorged ticks were collected from May to September 2019 from Taian and Yantai city, Shandong Province and were pooled individually to screening for DTV. The results of qRT-PCR revealed the positive rate of 13.6% (3/22) in serum of sheep and 8.2% (14/170) in attached ticks, respectively. Phylogenetic analysis demonstrated a close evolutionary relationship among those DTV isolates from animal and ticks, and DTV was relatively conservative in evolution. These findings are the first to demonstrate molecular evidence of DTV in domestic animals. Nonetheless, whether or not could cause disease in sheep and other animals, DTV deserves further investigation.

Background

Ticks are recognized as the second largest group of ectoparasites after mosquito that are exclusively specialized obligate hematophages[1]. They are the main arthropod vectors of pathogens including protozoa, bacteria and viruses with hosts ranging from wildlife to domestic animals and humans worldwide[2]. Recently, much attention has been paid to emerging diseases caused by tick-borne viruses (TBVs) because of their significant impact on domestic animals and human health[3]. A variety of viruses from different genera are included in TBVs, among which the genus *Phlebovirus* are important components, usually named tickborne phleboviruses (TBPVs). The TBPVs contain a large group of virus members, including several important emerging viruses that are pathogenic to human beings, such as severe fever with thrombocytopenia syndrome virus (SFTSV) and Heartland virus (HRTV) [4, 5].

Dabieshan tick virus (DTV) is also one of the emerging TBPVs and was first identified in *Haemaphysalis longicornis* from Hubei Province in 2015[6]. It is reported that DTV is an intermediate species with very close evolutionary relationships to several TBVs, such as Yongjia tick virus 1 and Uukuniemi virus[7, 8]. Previous studies have found that DTV was widely distributed in ticks from Shandong Province and Zhoushan area, and there was no significant difference in sequence between those isolates from Shandong and Hubei Province, indicating that DTV was relatively conservative in evolution. In addition, the carrying rate of DTV in ticks from Zhoushan area was much higher than Shandong Province. However, less is known about the arthropod vector ecology, the host range and the animal pathogenicity of DTV.

In this study, we performed sequence analyses of DTV in ticks and sheep sera sampled from DTV-endemic areas of Shandong Province, to further understand the circulation of DTV in China. Through sequence gathering, evolution matrix construction, and evolutionarily related analysis, we come to understand the genomic characterization as well as the evolutionary relationship among different virus strains. Our findings suggested domestic animals were likely to play an important role in the spread of DTV as replication or preservation hosts.

Methods

Ticks collection and RNA extraction

Sample collection took place in Taian (116°91'E, 36°19'N) and Yantai (120°03'E, 37°15'N), Shandong Province, China (Fig. 1). Sheep was the most common domesticated animal species in these regions and adult sheep raised in free range were selected in this study. Approximately 3 mL of blood samples were collected from sheep jugular veins and sera were obtained after blood clotting. According to the actual situation, 5–15 engorged ticks were collected from the body surface of each sheep with tweezers. After identified morphologically and 2 weeks at room temperature, individual tick specimen was homogenized in 500 µL of chilled DMEM and centrifuged at 4°C and 10,000 g for 5 min. Then, the supernatant of tick homogenates and sera were prepared for RNA extraction using TIANamp RNA extraction kit (Tiangen, China) following the manufacturer's instructions.

PCR for detection of pathogens in ticks

The detection of DTV was performed by qRT-PCR targetting the S segment, with primer sets DTV-F and DTV-R (TGC TCC TCT CCG CAC ACC T and TGG CAA GTA GAG GAA ACT GGT GA, respectively) and probes (FAM-TCC CTC CAG CCA TCA CCA CCT CC-BHQ1) described before [7]. All qRT-PCR-positive samples were further confirmed by nested PCR using primer sets Out-F, R and In-F, R (GGC AGC ACT TTC ACG GAT G, CCC CTG TCA TGT CTA ATC AAT GG and GCA AGC AGA GCC TCA AGA AGC, GCC AGA TTG CGA TCC AAG TAT G, respectively). Amplified products were visualized by SYBR® Safe (Thermo, Waltham, USA) after 1% agarose gel electrophoresis.

Bioinformatics analysis

The sequences obtained were subjected to edit, alignment using SeqMan and MegAlign of DNASTAR software package (Lasergene). MEGA5.1 software was used for phylogenetic analysis by constructing the neighbor-joining evolutionary tree with 1,000 replicates, compared with previous published viral sequences of DTV strains.

Ethical clearance

All of the animal experiments were carried out in strict accordance with the guidelines of the Animal Committee of Weifang Medical University. The study was reviewed and approved by the Ethic Committee of Weifang Medical University. The collection of animal blood was permitted by Daolang District and Laizhou County Forestry Bureau.

Results And Discussion

Tick collection

In this study, we collected 170 engorged ticks from 22 sheep in Taian and Yantai regions of Shandong Province from May through September 2019, of which 89 ticks from 12 sheep in Taian city, and 81 ticks

from 10 sheep in Yantai city (Table 1). All ticks collected from sheep were engorged *Haemaphysalis longicornis* and store in a cool and ventilated place to digest the blood in body. Then, each tick and serum sample was grouped into individual pool in preparation for viral RNA detection assays.

Table 1
Detection of DTV viral RNA in sheep and ticks collected from Shandong, China.

	Sheep			Attached ticks		
	Sample ID	qRT-PCR	Nested-PCR	NO.	qRT-PCR	Nested-PCR
Taian	TADL-001	-	/	12	-	/
	TADL-002	-	/	9	-	/
	TADL-003	+	+	5	+(4 of 5)	+(2 of 4)
	TADL-004	-	/	3	-	/
	TADL-005	-	/	5	-	/
	TADL-006	-	/	7	-	/
	TADL-007	-	/	7	-	/
	TADL-008	-	/	6	-	/
	TADL-009	-	/	9	+(2 of 9)	+(1 of 2)
	TADL-010	-	/	15	+(2 of 15)	+(1 of 2)
	TADL-011	+	-	3	+(1 of 3)	-
	TADL-012	-	/	8	-	/
total		2	1	89	9	4
Yantai	LZSS-001	-	/	11	-	/
	LZSS-002	-	/	14	-	/
	LZSS-003	-	/	7	+(1 of 7)	+(1 of 1)
	LZSS-004	-	/	8	-	/
	LZSS-005	-	/	8	-	/
	LZSS-006	+	+	4	+(3 of 4)	+(1 of 3)
	LZSS-007	-	/	7	-	-
	LZSS-008	-	/	9	+(1 of 9)	-
	LZSS-009	-	/	7	-	/
	LZSS-010	-	/	6	-	/
total		1	1	81	5	2
"/" indicates that no experimental operation has been carried out.						

Detection of DTV RNA in ticks and sheep

According to the result of qRT-PCR, DTV RNA were detected in 14 tick pools (out of 170, 8.2%), three sera pools (out of 22, 13.6%) in this study, including nine tick pools (out of 89, 10.1%), two sera pools (out of 12, 16.7%) from Taian, and five tick pools (out of 81, 6.2%), one sera pool (out of 10, 10%) from Yantai, respectively (Table 1). This result indicated a high prevalence of DTV in engorged ticks and domestic animals, compared to that of unfed ticks (0.67%) in the same region[7]. It seemed probable that domestic animals could enhance virus replication or play a positive role in virus spread, which also verified the role of domestic animals in the life cycle of DTV. Besides, we also found that the DTV RNA was detected in sheep from different areas, and the prevalences were high, maintaining at about 10%. It perhaps because the sheep in this study were grazed on pastures or hills during the day and kept in household backyards at night, leading to a heavy infestation with ticks carrying DTV.

In addition to transmission by ingesting the blood of infected hosts, arboviruses have been proved to spread from infected ticks to other ticks that infect the same host by co-feeding, even in seropositive hosts[9, 10]. Under experimental conditions, transmission of SFTSV from infected to uninfected *Haemaphysalis longicornis* occurred by co-feeding in BALB/c mice and the author speculated that this transmission pathway is of great significance to the survival of TBEV in nature[11]. In the present study, 22 groups of corresponding RNA samples were obtained from ticks and sheep serum samples and, DTV was detected simultaneously in sheep serum and corresponding ticks among three groups, which suggested that the potential viremia might promote the flow and transmission of virus in ticks and animal hosts. Nevertheless, not all ticks from sheep with viremia were detected carrying DTV, perhaps because of the insufficient blood intake of some ticks, resulting in too few copies of the virus to reach the detection threshold of qRT-PCR. Otherwise, the viral RNA of DTV was positive in some ticks of four groups of samples, but negative in corresponding sheep blood and the discordant results suggest that ticks carrying DTV failed to cause high level of viremia in host animals owing to some factors, such as high level of serum antibody and insufficient blood exchange.

Infectious virus, a competent insert vector, and susceptible reverse hosts are the three basic elements for the stable existence of virus cycle[12]. It has been proved that threshold viremia levels of $10^{2.0-4.7}$ 50% lethal dose/mL is sufficient for some TBVs infections, like Colorado tick fever virus, Russian spring-summer encephalitis virus, and louping ill virus[13–15]. In our study, the DTV RNA in the domestic animal were detected, although the copy numbers of viral RNA were low (approximately 6.0×10^4 copies/mL, 95% CI = $5.6-7 \times 10^4$ copies/mL). Successful amplification of the partial S segment of DTV confirmed the occurrence of DTV viremia in this kind of domestic animal. However, only 2 isolates were obtained from all serum samples, which indicated that most infected sheep might have either a short period of viremia, a low level of viremia, or that few infectious virions might be present under the neutralization pressure of high level of serum antibodies.

Phylogenetic analysis

According to the latest taxonomic information on viruses from the International Committee on Taxonomy of Viruses (ICTV) in 2019, DTV is classified as a new member of Uukuvirus genus, Phenuiviridae[16]. Recent studies have demonstrated that DTV was closely related with Yongjia tick virus, Uukuniemi virus and Okutama tick virus in phylogeny and its pathogenicity to animals and human remains unclear due to the lack of further research on this novel virus[7, 8]. However, DTV deserves more attention as an emerging threat to public health. In this study, the partial S segment of DTV was amplified and sequenced from 8 of 17 qRT-PCR-positive samples and all the sequences obtained from sheep and ticks have a 95.1–99.8% nucleotide identity with each other. Pairwise distances analysis demonstrated a close evolutionary relationship among those DTV isolates from animal and ticks in these epidemic regions. Interestingly, the isolate called 19YTLZ-13-P from tick shared nearly the same identity (98.2%) with 19YTLZ-17-P from attached sheep (LZSS-006). Meanwhile, similar results occurred in another group of specimens from Taian, that is, the isolates 19TADL-12-P and 19TADL-16-P from ticks grouped more closely with 19TADL-8-P from attached sheep (TADL-003). These suggested a potential link of DTV infections between sheep and ticks, and DTV might circulate in the endemic areas among ticks and many kinds of domestic animals, such as cattle, pigs, dogs, etc. Besides, these eight S sequences of DTV strains obtained in this study were closely related to that found in Hubei Province, indicating that the sequences of DTV from different regions had high similarity and DTV was relatively conservative in evolution, which was also consistent with our previous research results.

Conclusions

Our data provided epidemiological evidences for DTV infection in ticks and sheep in endemic areas and suggested that *Haemaphysalis longicornis* might serve as a vector for the transmission of DTV to sheep and even other animals, since this tick had been also found on cattle and dog. Whether or not DTV causes disease in sheep and other animals remains to be elucidated.

Abbreviations

DTV: Dabieshan tick virus

TBVs: tick-borne viruses

TBPVs: tickborne phleboviruses

SFTSV: severe fever with thrombocytopenia syndrome virus

HRTV: Heartland virus

ICTV: International Committee on Taxonomy of Viruses

Declarations

Acknowledgements

We thank Professor Zongdong Liu of Lazizhou Center for Disease Control and Prevention, Shandong Province, China.

Authors' contributions

LJS, RHC, YJW, YG, SQW; curated, analyzed and interpreted the data. LL; Statistical analysis. HYS and GYN; drafting and critical revision of manuscript. All the authors checked and approved the final draft of the paper.

Funding

This work was supported by the Natural Science Foundation of Shandong Province (Grant No. ZR2020MH300 and ZR2019BH035) and National Major Project for Infectious Disease Control and Prevention (2018ZX10731-101). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The research was approved by the Ethic Committee of Weifang Medical University. Not applicable for consent to participate.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Location of Shandong Province in China (left) and the location of Taian city, Yantai city, within the province where tick samples were collected, in 2019.

Figure 2

Phylogenetic analysis of DTV S-segment sequences amplified from sheep and ticks samples. A phylogenetic tree based on S-segment sequences (548bp) by the NJ method using MEGA 5.1 is shown. DTV-D3-Wuhan indicates the tick-derived sequence amplified from Hubei Province in 2015. DTV-2018-Shandong indicates the tick-derived sequence amplified from Shandong Province in 2018.