Tang *et al. Zoonoses* (2023) 3:35
DOI 10.15212/ZOONOSES-2023-0023

ORIGINAL ARTICLE

# Monitoring the Process and Characterizing Symptoms of Suckling Mouse Inoculation Promote Isolating Viruses from Ticks

**Shuang Tang[1,#], Sijing Hu[1,#], Jian Xiao[1], Yanfang Zhang[1], Zhengyuan Su[1], Qiaoli Wu[1], Juan Yang[1], Xijia Liu[1], Zhong Zhang[1], Qiong Zhu[1], Junming Shi[1,a], Abilimiti Moming[1], Yali Deng[1], Zhaojun Fan[1], Rong Guo[2], Surong Sun[3], Yujiang Zhang[2], Shu Shen[1,4,*] and Fei Deng[1,*]**

## Abstract

**Objective:** Suckling mouse inoculation is an important method that has been used for years to isolate viruses from ticks; however, this method has usually been briefly described in the literature on a case-by-case basis upon successful isolation rather than providing extensive details.

**Methods:** This study describes the procedure from preparation of tick homogenates to identification of virus isolation using the suckling mouse inoculation method. The transient and persistent features were characterized and the incidence of manifestations that developed in the suckling mice, especially in mice from which viruses were isolated, is reported.

**Results:** We identified 22 symptoms that developed in mice, including 13 transient symptoms that recovered by the end of the observation period and 7 persistent symptoms that the mice suffered from throughout the observation period. Persistent symptoms (lateral positioning and dead) and transient symptoms (malaise, emaciation, and difficulty turning over) were the main symptoms based on the high overall incidence. Moreover, we showed that mice from which viruses were isolated had a concentrated period and advanced days of disease onset.

**Conclusion:** This study provides detailed information necessary for better use of suckling mouse inoculation to isolate viruses from ticks, which may benefit optimization of this method to identify, discover, and acquire tick-borne viruses.

**Key words:** tick-borne viruses, virus isolation, suckling mouse inoculation, ticks, isolating viruses from ticks

[#]These authors contributed equally to this work.
***Corresponding authors:**
shenshu@wh.iov.cn (SS); df@wh.iov.cn (FD)

[1]Key Laboratory of Special Pathogens and Biosafety and National Virus Resource Center, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China
[2]Center for Disease Control and Prevention of Xinjiang Uygur Autonomous Region, Urumqi, China
[3]Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi, China
[4]Hubei Jiangxia Laboratory, 430200 Wuhan, China
[a]Current address: Department of Pathogen Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Received: June 8 2023
Revised: July 26 2023
Accepted: August 9 2023
Published Online: August 30 2023

## INTRODUCTION

Ticks are the second largest group of parasitic vectors (after mosquitoes) for carrying and transmitting pathogens, including viruses, bacteria, and parasites. Isolation of viruses from ticks is important to better our understanding of tick-borne virus (TBV) spillover potentials and to facilitate the collection of TBV resources [1]. Cell incubation and blind passaging are the most common methods used in the laboratory to isolate viruses; these methods are easy to perform and have short cycles. A variety of viral pathogens have been obtained by

incubating and subculturing human and/or mammalian samples with appropriate cell lines. Pathogenic TBVs can also be isolated using this method by incubating tick homogenates with human or mammalian cell lines. The African green monkey kidney cells (Vero) and the derivative cell line (Vero E6) have been most commonly used to incubate with tick homogenates, from which viruses associated with human disease have been isolated, such as Tacaribe virus (*Arenaviridae, Mammarenavirus*) [2], phlebovirus (*Bunyaviridae, Phlebovirus*) [3], Zahedan rhabdovirus (*Rhabdoviridae*, *Rhabdovirus*) [4], and Jingmen tick virus (unclassified) [5]. In addition, the human lung cancer cell line, A549, and the hamster kidney cell line, BHK-21, have been used to isolate tick-borne encephalitis virus (*Flaviviridae, Flavivirus*) from *Dermacentor reticulatus* [6] and deer tick virus (*Flaviviridae*, *Flavivirus*) from *Ixodes scapularis* [7]. Because most human or animal cells are intolerant to heterologous substances, incubation of tick homogenates with these cell lines tends to have a deleterious effect on cells, affecting cell growth and morphology or even leading to cell death [8]. A previous study reported the use of Vero cells to isolate Guertu virus (GTV; *Phenuiviridae, Bandavirus*) from *Dermacentor nuttalli*. Compared to use of *D. nuttalli* homogenates at a low dilution (1:4), incubating *D. nuttalli* homogenates at a high dilution (1:40) with Vero cells was associated with more efficient virus infection and proliferation, resulting in fewer passages of cell subculture to isolate GTV [9]. This finding indicated that the tick homogenates had an inhibitory effect on viral infection in mammalian cells. When the homogenates were highly diluted, the inhibitory effect weakened, and promoted virus infection and proliferation as a result.

Suckling mice inoculation with tick homogenates for virus isolation has been utilized much longer than cell lines. In 1964 Hughes et al. [10,11] first reported isolating Hughes virus (*Nairoviridae, Orthonairovirus*) by inoculating suckling hamsters with homogenates prepared from ticks (*Ornithodoros capensis*), which is nearly 40 years earlier than the first report of isolating virus from ticks using cell lines [12]. In 1974 Butenko et al. [13] inoculated homogenates of Ixodid ticks into suckling mice and isolated Barur virus (*Rhabdoviridae*, *Ledantevirus*). The next year, Kemp et al. [14] isolated 15 strains of Nyamanini virus (*Nyamiviridae*, *Nyavirus*) and 16 strains of Quaranfil virus (*Orthomyxoviridae*, *Quaranjavirus*) from ticks [*Argas* (*Persicargas*) *arboreus*] by suckling mice inoculation [14]. Subsequently, a number of novel viruses, including Saumarez Reef virus (*Flaviviridae, Flavivirus*) [15], Dhori virus (*Orthomyxoviridae, Thogotovirus*) [16], Palma virus (*Marnaviridae, Sogarnavirus*) [17], Midway virus (*Nyamiviridae, Nyavirus*) [18], Mono Lake virus (*Reoviridae, Orbivirus*) [19], Sindbis virus (*Togaviridae, Alphavirus*) [20], and Kadam virus (*Flaviviridae, Flavivirus*) [20], were isolated by inoculating the tick homogenates into suckling mice. These reports were from different laboratories in Australia, the United States, Portugal, Japan, and Saudi Arabia, suggesting a wide use of this method to obtain viruses from ticks. Between 1967 and 1993, researchers from the Virus Laboratory (Faculty of Medicine of Brest, Brest, France), exerted considerable effort to obtain viruses from ticks. Suckling mice inoculation was repeatedly performed with Ixodid tick homogenates and isolated Essaouira virus (unclassified), Kala Iris virus (unclassified), Soldado virus (*Nairoviridae, Orthonairovirus*), Eyach virus (*Spinareoviridae, Coltivirus*), and Meaban virus (*Flaviridae, Flavivirus*) [21-24]. In addition, attempts to isolate viruses from *Ixodes ricinus* and *Ixodes ventalloi* ticks using mammalian cell lines failed [25], which further demonstrated the effectiveness of suckling mice inoculation to isolate viruses from ticks compared to cell lines.

After a variety of TBVs were identified and isolated from ticks in different countries, the association with human and animal diseases was further unveiled. It was also shown that some viruses isolated from ticks, including Dhori virus (*Orthomyxoviridae, Thogotovirus*), Palma virus (*Marnaviridae, Sogarnavirus*), and Eyach virus (*Spinareoviridae, Coltivirus*), are the causative agents of infectious diseases, which cause fevers, neurologic abnormalities, encephalitis, and even death in humans and animals [26]. Nyamanini virus (*Nyamiviridae, Nyavirus*), Midway virus (*Nyamiviridae, Nyavirus)*, and Meaban virus (*Flaviviridae, Flavivirus*) infected birds or other animal hosts [27,28]. There are many other viruses isolated from ticks, the spillover potential, virulence, and pathogenicity of which have yet to be clarified. Isolating viruses from ticks is necessary to understand the risk of cross-species transmission of TBVs and also facilitates epidemiologic surveys of virus distribution. The resulting data would provide fundamental information to interrupt TBV transmission and infection, and to prevent and control the emerging infectious diseases caused by novel TBVs.

Suckling mice are very sensitive to pathogens due to an immature immune system and thus would exhibit different manifestations of illness onset after inoculation with tick homogenates. The ill mice may demonstrate an established infection with pathogens, including viruses. The manifestations and time of illness onset after inoculation are important signals to determine the appropriate timing to collect tissues from diseased mice for subsequent pathogen detection and passaging. Previous studies reported successful virus isolation from tick homogenates [13,15-17,29]; however, manifestations in suckling mice are described separately. Thus far, a comprehensive understanding involving suckling mice to isolate viruses from ticks, analyses of disease onset, and manifestations related to virus isolation are lacking. In the current study tick samples were collected and suckling mice were inoculated. Brains of diseased mice were harvested from the first generation of diseased mice inoculated with tick homogenates, then used for the second generation of inoculations, which may be followed by a third generation of inoculations. The entire procedure from preparing tick homogenates to obtaining virus isolates, including the development of symptoms, time of illness onset, and the incidence and persistence of different symptoms in each generation

and different groups of suckling mice were characterized, summarized, and discussed. The data systematically depicted the entire process of isolating viruses from ticks by inoculating suckling mice, which would promote the use of this method for virus isolation and facilitate optimization of the method for improving its effectiveness to obtain TBVs.

## METHODS

### Tick collection and homogenate preparation

A total number of 25,620 ticks were collected in northern Xinjiang in 2016 and 2017, and were classified as *D. nuttalli* and *Hy. asiaticum* based on morphology. These ticks were grouped according to species and geographic distribution. Alternatively, one tick group contained 50-100 ticks of the same tick species that were randomly selected from the same sampling site. Ticks were thrice-washed with phosphate buffered saline (PBS [pH 7.4]), and homogenates were prepared in 2 mL of pre-cooled PBS using a tissue cell-destroyer (D1000; Novastar, Wuhan, China). The homogenates were centrifuged at 4000 rpm for 10 s, stopped for 10 s, and repeated for 2 cycles at 4°C. The clarified supernatant containing 100 U/mL of penicillin and 100 μg/mL of streptomycin was used for suckling mice inoculation or storage at −80°C until further use [30].

### Suckling mice inoculation and passaging

Pregnant Kunming mice were obtained from the Experimental Animal Research Center of Hubei Provincial Center for Disease Control and Prevention and reared until giving birth to offspring in a specific pathogen-free environment. When the pinkies were born and fed by the doe for 1-2 days, the pinkies were inoculated with clarified tick homogenates as the first generation (F1). Generally, all suckling mice (6-12 mice for one birth) given birth by one mother were inoculated as one experimental group with homogenates prepared from one group of ticks. Each mouse was inoculated simultaneously via the intracranial (10 μL) and intraperitoneal routes (30 μL), as previously described [30]. A group of suckling mice inoculated with PBS was the negative control for each inoculation.

Within 14 days after inoculation, the suckling mice were monitored by daily inspection at least 3 times in the morning, afternoon, and evening. A mouse appearing with any symptoms was immediately dissected, the brain was harvested, and immersed in 1 mL of pre-cooled PBS at 4°C. Homogenates were prepared by a high-performance tissue cell-destroyer (D1000; Novastar). The clarified supernatant was used for virus detection or the next round of suckling mice inoculation. When performing the subsequent passages (F2-F3), inoculation was performed via the same routes and volumes of homogenates as the F1 generation inoculation. Mice that survived or did not show any symptoms were euthanized at the end of the observation period.

### Data recording and analysis

During the 14-day observation period, the following information was recorded every day, including the day of disease onset, types of symptoms, and outcomes. The coding number of each diseased mouse from which brains were harvested was also recorded to ensure virus isolation could be traced back to mouse and tick groups. To analyze the correlation between illness onset and virus isolation, suckling mice that died or were eaten by the does within 48 h after inoculation were not included in the statistical analysis, as recommended in a previous study [31]. Transient and persistent symptoms were distinguished based on the daily records of different manifestations. The transient symptoms appeared in mice in the beginning, but only lasted for several days and disappeared from any of the diseased mice within the 14-day observation period. The persistent symptoms appeared after inoculation and never disappeared from the mice until the last day of observation. The brains of diseased mice collected from the F1-F3 generations were selected for metagenomic sequencing, as described in previous studies [9,30]. Successful isolation of different viruses was indicated by further isolating the viruses from the brain homogenates using cell lines, as described in previous studies [32-34], and by obtaining full genome sequences of viruses in the brain tissues. The full-genome sequences of viruses isolated from ticks using this method were deposited in GenBank under the following accession numbers: KY354080-KY354082; MG659722-MG659727; MH688511; MT815989-MT815994; MT248418-MT248421; MH688532; MH688535; MH688538-MH688539; and MH688513-MH688517.

### Ethics statement

The animal experiments were conducted in an animal biosafety level 2 (ABSL-2) laboratory, and would be conducted in ABSL-3 or BSL-3 laboratory for further isolation once a highly pathogenic virus was identified, according to the Directory of Pathogenic Microorganisms Transmitted among Humans, which was issued by the Chinese Ministry of Health (http://www.nhc.gov.cn/qjjys/s7948/202308/b6b51d792d394fbea175e4c8094dc87e.shtml). Animal experiments were approved by the Ethics Committee of Wuhan Institute of Virology (Chinese Academy of Sciences) under approval number WIVA33201702.

## RESULTS

### Differential symptoms indicated that infection was established in suckling mice after inoculation

A total of 575 groups of suckling mice were inoculated, including 364 groups with tick homogenates in the F1 generation, 154 groups in F2 inoculated with diseased mouse brain homogenates prepared from F1, and 57 groups in F3 inoculated with diseased mouse brain homogenates prepared from F2 (Table 1, Fig 1). Within 14 days after inoculation, 478 groups had illness onset and

**TABLE 1** | Summary of the morbidity characteristics in suckling mice after inoculation in the first (F1), second (F2), and third (F3) generations.

| Parameters | | Total (N=575) | | Passages on suckling mice (groups, %) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | F1 (N=364) | | F2 (N=154) | | F3 (N=57) | |
| Disease onset[#] | Asymptomatic | 97 | 16.87% | 72 | 19.78% | 17 | 11.04% | 8 | 14.04% |
| | Symptomatic | 478 | 83.13% | 292 | 80.21% | 137 | 88.96% | 49 | 85.96% |
| | Transient symptom | 180 | 37.66% | 113 | 38.70% | 45 | 32.85% | 22 | 44.90% |
| | Persistent symptom | 298 | 62.37% | 179 | 61.30% | 92 | 67.15% | 27 | 55.10% |
| Groups in which virus isolation was detected* | Total | 42 | 25.00% | 23 | 27.71% | 9 | 14.52% | 10 | 43.48% |
| | Transient symptom | 9 | 21.43% | 7 | 30.43% | 2 | 22.22% | 0 | 0.00% |
| | Persistent symptom | 33 | 78.57% | 16 | 69.57% | 7 | 77.78% | 10 | 100.00% |

[#]The occurrence of suckling mice eaten by mothers within 48 h after inoculation was not included.
*Percentages were calculated based on the mouse groups from which virus isolation was confirmed by metagenomics sequencing analyses and the total groups which were used for sequencing. In the F1, F2, and F3 generations, brain tissues from 83, 62, and 23 groups, respectively, were prepared for metagenomics sequencing and used for data analyses.

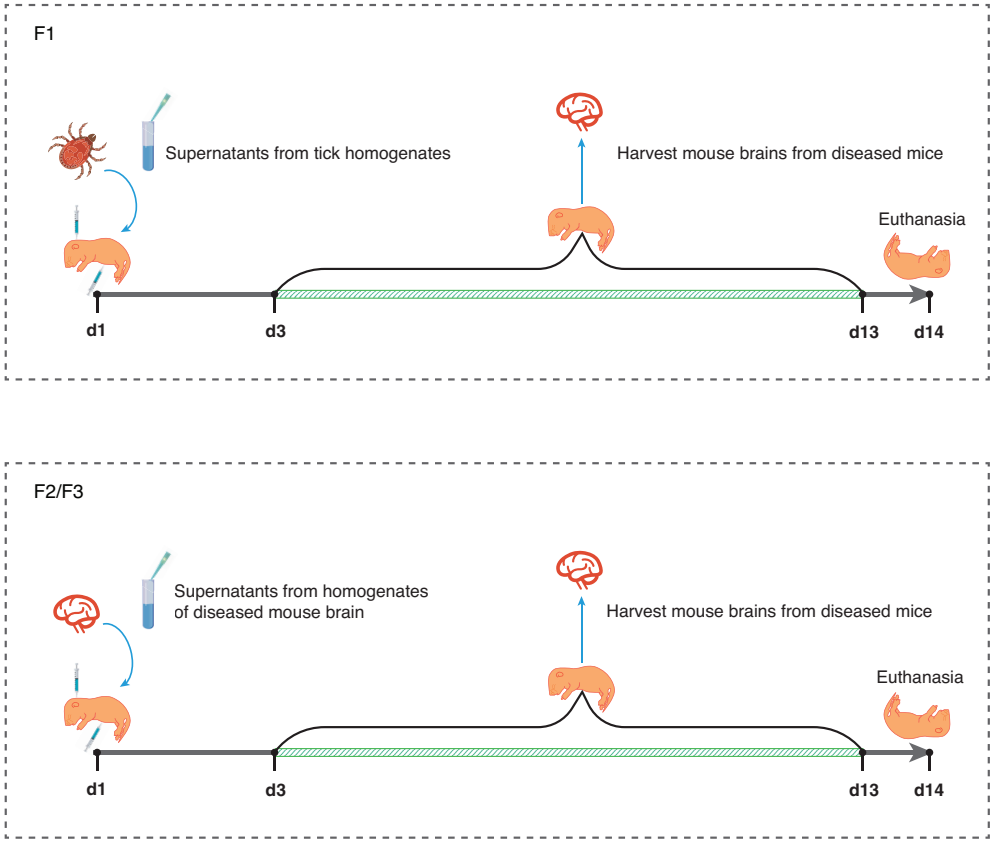


**FIGURE 1** | Schematic diagram of the process of suckling mice inoculation of the first (F1), second (F2), and third (F3) generations. For the inoculation process of the F1 generation, supernatants from tick homogenates were prepared and inoculated intracranially and intraperitoneally into the suckling mice. Mice were observed daily for 14 days after inoculation. During days 3-13, once the mice having disease onset was noted, they were dissected and their brain tissues were harvested. Brain tissues from the F1 inoculation were prepared into homogenates and subsequently inoculated into suckling mice as the second or third passages. The inoculation and observation were performed in the same way as in F1. By the end of the observation period, mice that survived or did not exhibit any symptoms were euthanized.

developed different symptoms (83.13%), suggesting that the groups were infected or injured, whereas the other 97 groups that did not exhibit symptoms were considered asymptomatic or not infected. The diseased mouse groups included 292 groups in F1 (80.22%), 137 groups in F2 (88.96%), and 49 groups in F3 (85.96%). There was a slightly increased incidence of disease onset in F2 and F3 compared to F1.

Mice that had illness onset in the beginning and subsequently recovered were considered to have a transient infection and exhibit transient symptoms. Of all mice, 37.66% (180/478) of the symptomatic mouse groups had mice presenting with transient symptoms, including 38.70% (113/292) in F1, 32.85% (45/137) in F2, and 44.90% (22/49) in F3 (Table 1). Most of the mouse groups (>50%) among the 3 generations developed persistent symptoms (Table 1).

The virus-related sequences from the brain tissues of 168 diseased mice in different groups were analyzed, including 83 groups from the F1 generation, 62 from the F2 generation, and 23 from the F3 generation. Homogenates were prepared and aliquoted into separate pools, then used for metagenomic sequencing. The resulting data of each pool represented the results of virus detection from one mouse group. The sequencing results confirmed that a total of 42 pools (25.0% [42/168]) had high abundance of viral genomic sequences, each with a complete sequence relating to one specific virus, suggesting that viruses were isolated from ticks and maintained in diseased mouse brains. Twenty-three groups (27.71%) were confirmed to have virus isolation among the 83 analyzed groups in the F1 generation, 9 groups (14.52%) in the F2 generation, and 10 groups (43.48%) in the F3 generation. Among the groups in which virus isolation was confirmed, 30.43% (7/23) of the groups in the F1 generation had transient symptoms, while this percentage decreased to 22.22% (2/9) in the F2 generation, and no groups in the F3 generation. In contrast, the proportion of groups that exhibited persistent symptoms was 69.57% (16/23) in the F1 generation, which increased to 77.78% (7/9) in the F2 generation. All of the sequenced groups in the F3 generation had persistent symptoms. The increase in persistent symptoms from the F1 to F3 generations may be attributed to the establishment of persistent infections caused by pathogen(s) after passages rather than physical damage.

## Suckling mice exhibited differential manifestations within 14 days post-inoculation

The ratios of the number of diseased mice among the total number of mice each day were calculated to represent the daily incidence. The accumulated incidence was also determined for three generations. As shown in Fig 2A, illness onset in the F1 generation was observed on days 3-13 after inoculation. The daily incidence increased from day 3, reached a peak of 22% on day 6, then decreased until day 13, suggesting that days 3-6 were the outbreak period in which the number of diseased mice rapidly increased. The daily incidence on days 3-9 were >5%, which was considered the concentrated outbreak period of disease. Mice in the F2 generation generally had disease onset during days 3-11, with the outbreak period on days 3-5, peaking at approximately 28% on day 5 and declining rapidly thereafter. The concentrated outbreak period (daily incidence rate >5%) was on days 4-8. Mice in the F3 generation had disease onset during days 3-13, with days 3-6 considered the outbreak period, during which there was a sharp increase in the daily incidence. The incidence peaked at approximately 27% on days 5-6, followed by a decline, a small rebound on day 9, and a continuous slow decline on days 9-13. The concentrated outbreaks were mainly on days 4-7. Overall, all F1-F3 mice had disease onset on days 3-13, and the peak daily incidence increased from F1 to F3. The concentrated outbreaks of the daily incidence >5% in all generations concentrated on days 4-7.

The proportion of each symptom, as indicated by the number of mice presenting with symptoms per day over the total number of symptomatic mice were recorded and statistically analyzed over time (Fig 2B). In total, 22 different symptoms were observed from the diseased mice, including thin body habitus, loss of balance, abdominal enlargement, a startle response, roach back, stereotypic circling, paddling, hepatosplenomegaly, malaise, straying, diarrhea, sluggish behavior, bloody ascites, poor appetite, unkempt hair, head and neck huddling, euphoria, difficulty turning over, lateral positioning, claudication, dying, and dead (Fig 1B). Of the 22 symptoms, 5 symptoms, including lateral positioning, malaise, thin body habitus, dead, and difficulty turning over, had an incidence ≥10% of the overall symptomatic mice, and therefore considered the most predominant manifestations in diseased mice. Except for straying and head and neck huddling, which were observed in a very small number of mice (n=2), the other symptoms appeared in > 5 mice; 15 symptoms (lateral positioning, malaise, thin body habitus, deceased, difficulty turning over, abdominal enlargement, loss of balance, claudication, bloody ascites, sluggish behavior, hepatosplenomegaly, roach back, a startle response, euphoria, and poor appetite) appeared early since day 3. Stereotypic circling appeared slightly late on days 4-6, and unkempt hair and straying were first observed on day 7 (Fig 2B). Days 3-10 were a relatively intense period for developing various symptoms, while the percentage of each symptom over the total were at a low level on days 11-14. The 14-day monitoring period after inoculation covered the intense period of illness onset, which facilitated observation of various symptoms in the suckling mice.

Not all of the 22 symptoms were observed in each generation. We noted 20 symptoms in the F1 generation, 21 in the F2 generation, and 17 in the F3 generation (S1 Fig). Indeed, lateral positioning, thin body habitus, malaise, deceased, and difficulty turning over were considered the dominant symptoms because each had an overall proportion > 9% of diseased mice in each generation (data not shown). Moreover, the onset period of these five symptoms in the F1-F3 generations was more concentrated than the other symptoms. While the appearance of a thin body habitus, malaise, deceased, and difficulty turning over were concentrated during days 4-8, lateral positioning was observed in suckling mice to be slightly delayed to days 4-8 in the F2 generation and days 4-9 in the F3 generation (S1 Fig).

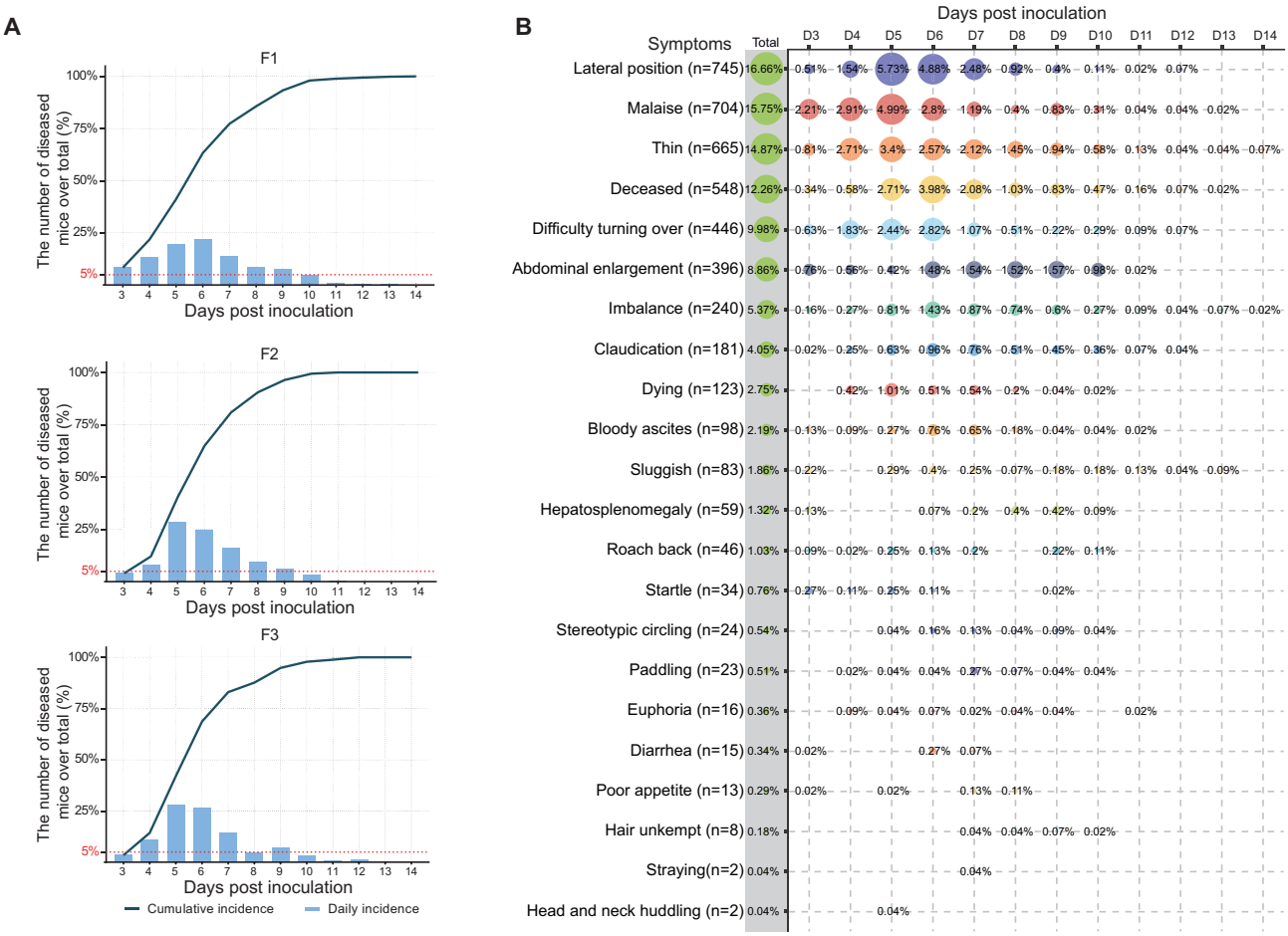


**FIGURE 2** | Overall course of disease onset and incidence of different symptoms over time in three generations of suckling mice. (A) The daily and cumulative incidences for disease onset among suckling mice of the F1, F2, and F3 generations. Daily incidence is shown in bars, and the curves show the cumulative incidence. Daily incidence is expressed by the number of symptomatic mice on a particular day over the total number of diseased mice in 14 days, and the cumulative incidence was determined by the total number of symptomatic mice up to the particular day over the total number of diseased mice in 14 days. A high incidence > 5% is indicated by a red dash line. (B) The total and daily incidences of 22 symptoms in suckling mice. The total incidence is expressed as the number of mice (n) having a symptom over the total number of diseased mice. The daily incidence of each symptom was shown by the number of mice having the symptom on each day over the total number of symptomatic mice within 14 days.

## Characterization of onset time and incidence of transient and persistent symptoms in suckling mice

Because the transient and persistent symptoms had different characteristics, we analyzed the proportions of mice having transient or persistent manifestations during the 14-day observation period. Two symptoms (straying, and head and neck huddling) were excluded from the analysis because only two mice exhibited these two symptoms (Fig 3A). For each analysis, the proportion of mice having a transient manifestation was expressed as the number of mice showing the symptom at the beginning with recovery at a later stage over the overall number of mice with the same symptom. For the mice with persistent symptoms, the proportion was calculated by the number of mice having a symptom throughout the 14 days over the number of total mice with the same symptom. Thirteen symptoms has transient properties because > 5% of mice had these symptoms at the beginning and recovered later (Fig 3A). Diarrhea was the symptom with the most pronounced transient feature; 46.67% of mice had diarrhea early and recovered later, and 53.33% of mice exhibited continuous diarrhea during the observation period. The proportion of mice with a specific symptom that exhibited transience was as follows in descending order: diarrhea (46.67%) > sluggish behavior (32.53%) > a startle response (32.35%) > malaise (31.82%) > bloody ascites (28.57%) > hepatosplenomegaly (25.42%) > unkempt hair (25.00%) > thin body habitus (23.31%) > loss of balance (14.17%) > abdominal enlargement (13.64%) > difficulty turning over (12.56%) > poor appetite (7.69%) > claudication (6.08%). The incidence of these transient symptoms (> 5% as for each symptom) decreased gradually from the F1 to F3 generations. Eleven symptoms were considered to have transient properties in the F1 generation, which was reduced to 10 symptoms in the F2 generation and 8 symptoms in the F3 generation. Diarrhea, a startle response, malaise, and a thin body habitus

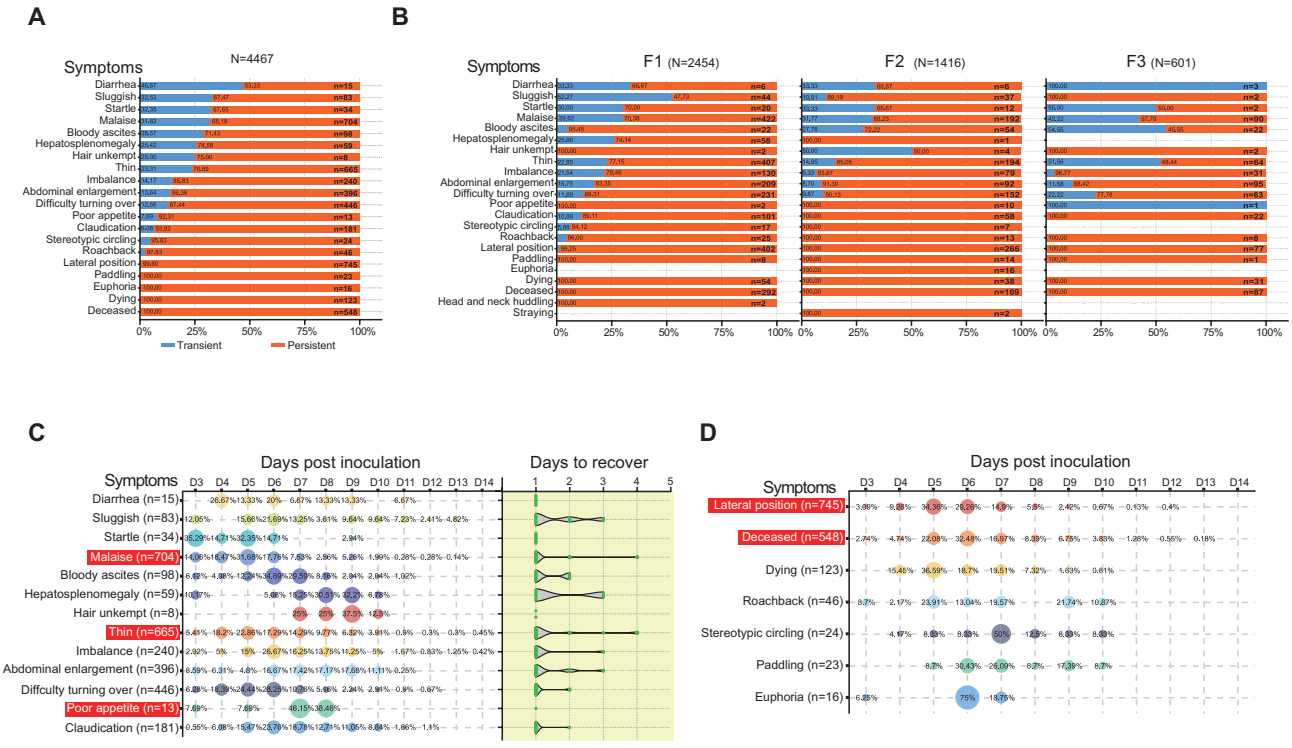


**FIGURE 3** | Characteristics of transient and persistent symptoms in three generations of mice. (A) The proportion of all mice exhibiting symptoms with a transient or persistent nature after inoculation. For each symptom, the total number of mice (n) having the symptom was set as 100%. The transient property of each symptom was shown by the number of mice having the symptom in the beginning and recovered late over the total mice with this symptom (transient). The remaining mice having a particular symptom throughout the entire observation period over all mice with this symptom represent the proportion of mice having the persistent symptom (persistent). (B) Proportions of different symptoms in each generation with transient and persistent features. (C) Normalized daily incidence of 13 transient symptoms among all symptomatic mice (left) and the duration periods for mice to recover from each transient symptom (right). The daily incidence of each transient symptom was normalized by accounting for the number of mice presenting the transient symptom each day over the mice with this transient symptom over 14 days. The duration period shows how many days the mice need to have the symptom onset from the first day to the last until recovery. (D) Normalized daily incidence of seven persistent symptoms in the symptomatic mice. Daily incidence of each persistent symptom was normalized by accounting the number of mice having the persistent symptom each day over the mice with that persistent symptom over 14 days. "n" indicates the total number of mice with each transient or persistent symptom. The five major symptoms are shaded in a red background in (C) and (D).

maintained a transient nature in all generations, and mice with these transient symptoms accounted for >20% of the total number of mice with the same symptoms (Fig 3B). These symptoms were considered the predominant mild manifestations of inoculated mice.

The daily incidence of the 13 symptoms were analyzed and expressed as the number of mice having one symptom per day over the total number of mice having the same symptom (Fig 3C [left]). Results showed that Transient symptoms were present during days 3-13. There were 11 transient symptoms (malaise, thin body habitus, difficulty turning over, abdominal enlargement, loss of balance, claudication, bloody ascites, sluggish behavior, hepatosplenomegaly, a startle response, and poor appetite) observed since day 3, and most of the symptoms appeared intensively on days 3-9. A startle response appeared since day 3 with a high incidence and subsequently disappeared after day 7 in spite of a nominal rise on day 9. Hepatosplenomegaly, unkempt hair, and poor appetite appeared late on day 7 and had a concentrated outbreak thereafter until day 10. The duration of transient symptoms (the days from the first observation of a transient symptom in mice to remission of the symptom) was counted (Fig 3C [right]). Most of these mice (83.47% [414/496]) exhibited transient symptoms that lasted only 1 day and rapidly remitted, while several mice (16.53% [82/496]) showed transient symptoms that lasted up to 4 days. The transient symptoms, including a startle response, diarrhea, poor appetite, and unkempt hair lasted for only 1 day, while most symptoms (sluggish behavior, bloody ascites, hepatosplenomegaly, loss of balance, abdominal enlargement, and difficulty turning over) lasted for 2-3 days, and only malaise and a thin body habitus lasted for 4 days (Fig 3C right).

Almost all of the diseased mice (>95%) exhibited seven persistent symptoms throughout the observation period, including deceased, dying, paddling, euphoria, lateral positioning, roach back, and stereotypic circling (Fig 3A). These symptoms were the main pathogenic features of the disease in mice after inoculation. Compared to transient symptoms, the persistent symptoms appeared in a

relatively short period of time after illness onset. The mice developed persistent symptoms within 7 days with the peak daily incidence on day 5 or 6, except for stereotypic circling, which had a peak incidence on day 7 (Fig 3D).

Of the 5 major symptoms exhibited by mice with an overall daily incidence ≥ 10% (Fig 2B), malaise, a thin body habitus, and difficulty turning over were symptoms having a transient nature, and lateral positioning and dead were persistent symptoms (Fig 3C and D). Compared with other symptoms, the relatively long duration of developing the five symptoms further suggested that the symptoms could be regarded as the main symptoms, which should attract more attention than other symptoms during the observation period.

### The diseased mice from which viruses were isolated had a concentrated period and advanced days of disease onset

Crimean-Congo hemorrhagic fever virus (CCHFV), Yanggou tick virus, Thogotovirus, Tamdy virus (TAMV), and Karshi virus (KSIV) were isolated from ticks, as confirmed by next-generation sequencing (NGS), which obtained the full-length genome sequences. Further, the virus-containing mouse brain homogenates were incubated with susceptible cell lines, and these viruses from cell culture were obtained (unpublished data). Of these viruses, three strains of CCHFV, four strains of TAMV, and one strain of KSIV were characterized for etiologic properties, pathogenicity, and prevalence in our previous studies [30,32,35,36]. Symptoms and the timeline of disease onset of the mouse groups, which were confirmed for CCHFV, TAMV, and KSIV isolation, are summarized in Table 2 and Fig 4. Generally, only a few of these mice showed transient symptoms (malaise) in the F1 generation, from which CCHFV and TAMV were isolated in the subsequent generations, while none of the mice had transient symptoms for KSIV (Table 2). Lateral positioning and malaise were the major persistent symptoms from the mice with CCHFV infections through the three generations, while the major symptoms for TAMV were thin body habitus and a loss of balance for two generations. Mice with KSIV infections developed variable persistent symptoms in the three generations (Table 2). The daily and cumulative incidences of the mouse groups confirmed with CCHFV, KSIV, and TAMV isolations were also characterized (Fig 4). From F1 to F3 (or F2 as for TAMV), the periods during which mice having disease onset were shortened to be more concentrated within 5 days, suggesting a stable infection has been established after passages. Moreover, the incidence curves showed advanced days of illness onset along with subsequent passages in mice with CCHFV, KSIV, or TAMV isolation (Fig 4).

## DISCUSSION

Suckling mouse inoculation is an effective method that has long been used to isolate viruses from ticks. Previous studies have focused on the successful isolation of viruses from ticks using this method, but have not systematically characterized the entire process and the discernible events occurring in the inoculated mice. Successive passages by preparing homogenates of brains from diseased mice in the

**TABLE 2** | Summary of symptoms observed in mouse groups of different generations which were confirmed by virus isolation.

| Events | Virus isolated from ticks (GenBank accession number) | Generations (number of mice) | Symptoms* | | References |
|---|---|---|---|---|---|
| | | | Transient (%) | Persistent (%) | |
| 1 | Crimean-Congo hemorrhagic fever virus (KY354080-KY354082 and MG659722-MG659727) | F1 (n=18) | Malaise (5.56%) | Lateral positioning (50.00%) > difficulty turning over (38.89%) > malaise (33.33%) > dead (22.22%) > thin body habitus (5.56%) and sluggish behavior (5.56%) | [30,35] |
| | | F2 (n=10) | - | Malaise (90.00%) > lateral positioning (70.00%) > dead (30.00%) | |
| | | F3 (n=12) | - | Lateral positioning (100.00%) > malaise (8.33%) | |
| 2 | Karshi virus (MH688511) | F1 (n=10) | - | Dead (10.00%), claudication (10.00%), difficulty turning over (10.00%), paddling (10.00%), sluggish (10.00%), a startle response (10.00%), and malaise (10.00%) | [32] |
| | | F2 (n=15) | - | Lateral positioning (100.00%) > sluggish behavior (46.67%) > roach back (6.67%) | |
| | | F3 (n=19) | - | Thin body habitus (57.89%) > loss of balance (42.11%) > dead (31.58%) > abdominal enlargement (5.26%) | |
| 3 | Tamdy virus (MT815989-MT815994) | F1 (n=36) | Malaise (22.22%) | Thin body habitus (25.00%) > malaise (16.67%) > loss of balance (11.11%) > dead (2.78%) and roach back (2.78%) | [36] |
| | | F2 (n=10) | - | Loss of balance (100.00%) > thin body habitus (90.00%) | |

-: Transient symptoms were not noted in mice.
*The percentage of mice having each symptom among the total number of mice in every generation is presented.

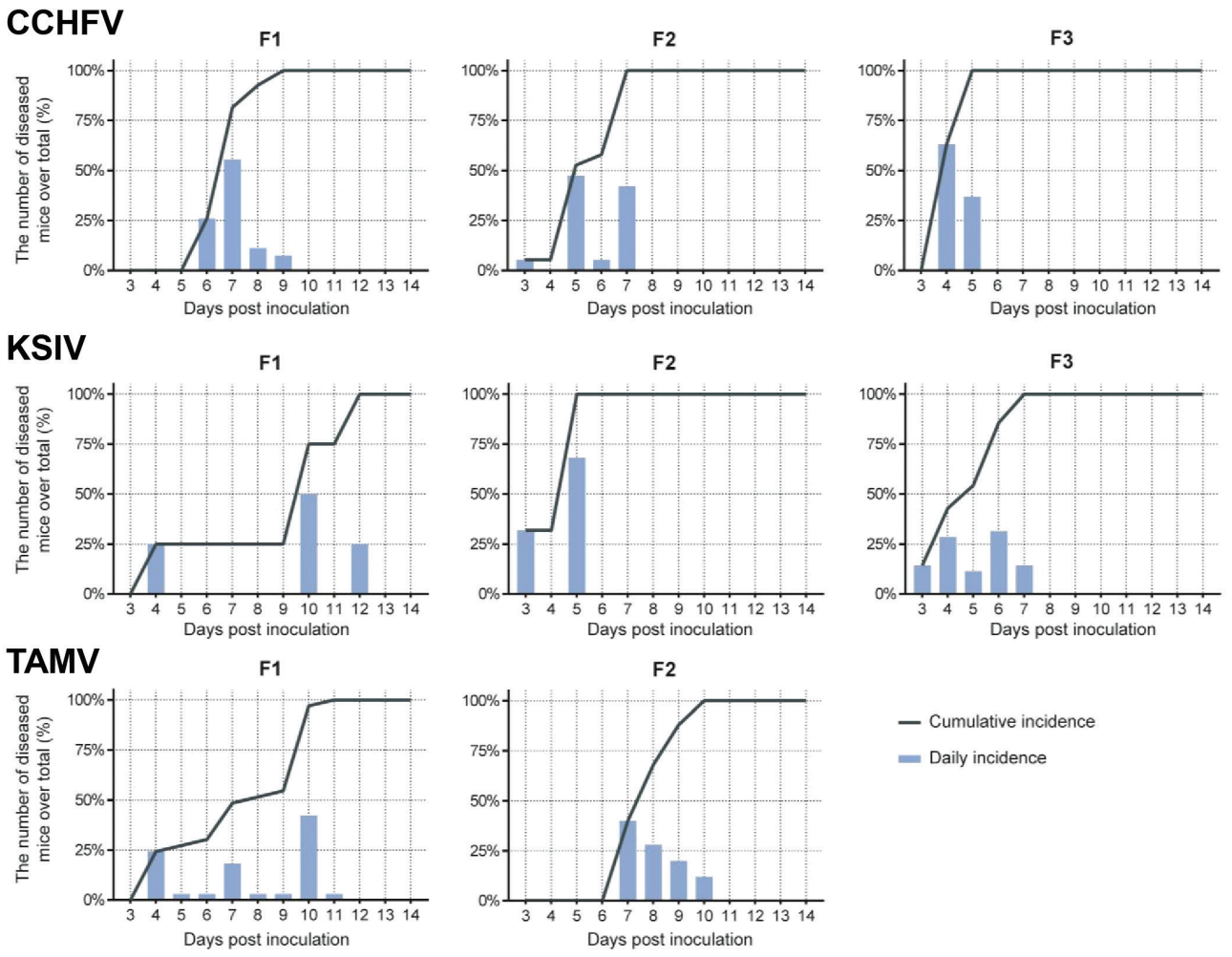


**FIGURE 4** | The daily and cumulative incidences in three generations (F1, F2, and F3) of mice, which were confirmed with virus isolation after NGS and cell culture. Confirmed results about isolating CCHFV (A), KSIV (B), and TAMV (C) were described in previous studies [30,32,35,36, respectively]. F3 data are not available for TAMV because passages on the third generation were not performed.

F1 generation and continuously inoculating into the F2 and F3 generations would help reduce the negative effects of tick-derived materials on mice, which promotes establishing pathogen infection in the subsequent generations. Therefore, the illness onset in suckling mice could be associated with pathogenic infection and indicates successful isolation. Recognizing the appropriate time points when mice are having an acute infection is important for obtaining mouse tissues with high viral loads. Therefore, understanding the process and timing of illness onset and disease progress in mice may facilitate better virus isolation.

Harvesting the brain tissues from diseased mice containing a virus isolate and having the sample incubated with cells is an effective way to obtain virus cell culture from ticks. By using this method, new strains of CCHFV were isolated from *Hyalomma asiaticum* ticks, which promoted an understanding of CCHFV evolution and distribution in recent years [30, 35]. TAMV (*Nairoviridae, Orthonairovirus*) was also isolated from *Hy. asiaticum* ticks using this method, which was a pathogen associated with febrile human diseases in 2007 [36]. The tick-borne flavivirus, KSIV (*Flaviviridae, Flavivirus*), was isolated from *Hy. asiaticum* ticks, which was associated with encephalitis in mice and widespread in northwestern China [32]. These studies suggested that suckling mouse inoculation is an effective method to isolate viruses from ticks, which could help to discover potential pathogens before the emergence of virus-related disease and provide clues to identify causative pathogens of previous outbreaks.

The current study characterized the disease course, identified different symptoms, and analyzed the incidence of different symptoms in suckling mice inoculated with tick homogenates and subsequent two rounds of passages. Greater than 80% of mice developed symptomatic disease after inoculation, which was manifested by 22 different symptoms with a concentrated onset period of 4-7 days. A previous study reported a variety of symptoms observed in mice inoculated with homogenates of *Haemaphysalis parva*, *H. punctata*, and *Hy. marginatum*, in which the symptoms of poor appetite, malaise, and claudication were also found in the present study [37]. This finding suggests some manifestations in common with the disease caused

by tick inoculation in mice. We further showed that lateral positioning, a thin body habitus, malaise, dead, and difficulty turning over were the predominant symptoms, accounting for ≥ 10% of the overall number of symptomatic mice. These symptoms persisted throughout the three generations, suggesting that the symptoms are the predominant manifestations of disease in mice infected with tick-derived pathogens. Moreover, these symptoms were also the predominant symptoms exhibited by the group of mice from which virus was obtained, sequenced, and validated (Table 2). Therefore, focusing on the timing of the development of these symptoms may help to select the right time to harvest diseased mouse tissues for passage and testing, and may help to "pre-identify" the group with persistent symptoms for efficient virus isolation.

Transient symptoms may indicate a transient infection occurring in mice, while persistent symptoms may indicate ongoing pathologic damage. Increasing the frequency of observing the mice after inoculation during the concentrated onset period, differentiating and identifying transient symptoms, and harvesting diseased mouse tissues before disease recovery are crucial to identifying as much of pathogens as possible. Of these 22 symptoms, 13 (diarrhea, sluggish behavior, a startle response, malaise, bloody ascites, hepatosplenomegaly, unkempt hair, a thin body habitus, loss of balance, abdominal enlargement, difficulty turning over, poor appetite, and claudication) were of a transient nature. Seven symptoms (death, dying, paddling, euphoria, lateral positioning, roach back, and stereotypic circling) were of a persistent nature. The groups of mice exhibiting persistent symptoms increased in the subsequent generations, suggesting that some pathogen(s) have established a stable infection in the mouse population. Therefore, it is therefore suggested that increased attention be paid to the groups of mice that develop persistent symptoms and that the brain tissue of these diseased mice be collected and tested as a priority. It is also recommended to increase the observation frequency and to record all types of symptoms in mice after inoculation to harvest brain tissue from mice with priority symptoms in a timely manner.

In the current study brain tissues from suckling mice with lateral positioning and/or dying were selected for the next generation of inoculation, according to previous reports [31]. This finding may affect the data on the percentage of different symptoms in the F2 and F3 generations; however, the results provided a detailed reflection of the population morbidity in mice after inoculation. The mice confirmed with CCHFV, TAMV, and KSIV isolation showed different symptoms in the three generations, probably due to the different virologic properties and pathogenicity. These mice had a more concentrated period of advanced days of illness onset in the F2 and F3 generations, suggesting that stable virus infection was established. This was also evidenced by the reduced persistent symptoms and lack of transient symptoms appearing in the F2 and F3 generations. In addition, infection with different TBVs may have different tissue tropisms, i.e., tick-borne encephalitis virus is more likely to invade nervous tissue and severe fever with thrombocytopenia syndrome virus is more sensitive to immune-related tissues, such as the spleen [38]. The different tissue tropisms and lesions caused by viruses may result in developing different symptoms. In the current study only brain tissue from diseased mice was collected for subsequent inoculation and testing, which may be a possible reason why this method allows the isolation of viruses that are likely to be neurotropic or sensitive to brain tissue [39]. In fact, some of the diseased mice were shown to have hepatomegaly and splenomegaly when being dissected. We tried to have suckling mice inoculated with homogenates prepared from those tissues of a small number of diseased mice and observed different manifestations among these mice, from which a high abundance of viruses was further detected (unpublished data). Therefore, we suggest that this traditional method of isolating viruses by harvesting brains from diseased mice could be optimized according to analyses of the etiology, pathogenicity, and tissue tropism of different viruses. Further in-depth analysis of the pathogenic process and symptoms in groups of mice from which different viruses have been obtained can be carried out to promote understanding and selection of the right time to harvest tissues for virus isolation. Other potential target organs for virus infection that brain tissue can be selected for subsequent inoculation and detection, which can help to obtain a wider range of TBV species.

The current study summarized the disease progress and manifestations among suckling mice after inoculation with tick homogenates and subsequent passages with brain tissues from diseased mice. The results revealed 22 different symptoms among the mice, suggested 5 major symptoms, and analyzed the daily and accumulative incidence. These symptoms are worth noting to decide the right time to collect tissue samples for subsequent passages and identifying isolated viruses. The findings would promote understanding of the entire procedure and methodology of suckling mice inoculation and benefit further optimization of this method. Monitoring the entire process and evaluating the manifestations of this suckling mouse inoculation method, along with analyses of various tick-borne pathogens, may also further our understanding of the spillover ability of various tick-borne pathogens and the correlations between these pathogens and related diseases in the future.

**ACKNOWLEDGEMENTS**

This work was supported by the National Key R&D Program of China (2019YFC1200700, 2021YFC2300900, and 2013YF113500), the Key Deployment Projects of the Chinese Academy of Sciences (KJZD-SW-L11), the Biological Resources Program of the Chinese Academy of Sciences (KFJ-BRP-017-74 and KFJ-BRP-017-06), the European Virus Archive Global (EVA-GLOBAL) Project (871029), and the Hubei Provincial Science and Technology Innovation Platform Special Project (2021DFE014).

## CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## REFERENCES


1. Shi J, Hu Z, Deng F, Shen S. Tick-borne viruses. Virol Sin. 2018;33(1):21-43.
2. Sayler KA, Barbet AF, Chamberlain C, Clapp WL, Alleman R, Loeb JC, et al. Isolation of Tacaribe virus, a Caribbean arenavirus, from host-seeking Amblyomma americanum ticks in Florida. PLoS One. 2014;9(12):e115769.
3. Wang J, Selleck P, Yu M, Ha W, Rootes C, Gales R, et al. Novel phlebovirus with zoonotic potential isolated from ticks, Australia. Emerg Infect Dis. 2014;20(6):1040-1043.
4. Dilcher M, Faye O, Faye O, Weber F, Koch A, Sadegh C, et al. Zahedan rhabdovirus, a novel virus detected in ticks from Iran. Virol J. 2015;12:183.
5. Dincer E, Hacioglu S, Kar S, Emanet N, Brinkmann A, Nitsche A, et al. Survey and characterization of Jingmen Tick virus variants. Viruses. 2019;11(11):1071.
6. Chitimia-Dobler L, Lemhöfer G, Król N, Bestehorn M, Dobler G, Pfeffer M. Repeated isolation of tick-borne encephalitis virus from adult Dermacentor reticulatus ticks in an endemic area in Germany. Parasit Vectors. 2019;12(1):90.
7. Dupuis AP, Peters RJ, Prusinski MA, Falco RC, Ostfeld RS, Kramer LD. Isolation of deer tick virus (Powassan virus, lineage II) from Ixodes scapularis and detection of antibody in vertebrate hosts sampled in the Hudson Valley, New York State. Viro J. 2015;12:183.
8. Kholodilov I, Belova O, Burenkova L, Korotkov Y, Romanova L, Morozova L, et al. Ixodid ticks and tick-borne encephalitis virus prevalence in the South Asian part of Russia (Republic of Tuva). Ticks Tick Borne Dis. 2019;10(5):959-969.
9. Shen S, Duan X, Wang B, Zhu L, Zhang Y, Zhang J, et al. A novel tick-borne phlebovirus, closely related to severe fever with thrombocytopenia syndrome virus and Heartland virus, is a potential pathogen. Emerg Microbes Infect. 2018;7(1):95.
10. Philip CB. Hughes virus, a new arboviral agent from marine bird ticks. J Parasitol. 1965;51:252.
11. Hughes LE, Clifford CM, Thomas LA, Denmark HA, Philip CB. Isolation and characterization of a virus from soft ticks (Ornithodoros capensis group) collected on Bush Key, Dry Tortugas, Florida. Am J Trop Med Hyg. 1964;13:118-122.
12. Attoui H, Stirling JM, Munderloh UG, Billoir F, Brookes SM, Burroughs JN, et al. Complete sequence characterization of the genome of the St Croix River virus, a new orbivirus isolated from cells of Ixodes scapularis. J Gen Virol. 2001;82(Pt 4):795-804.
13. Butenko AM, Gromashevsky VL, L'vov DK, Popov VF. First isolations of Barur virus (Rhabdoviridae) from ticks (Acari: Ixodidae) in Africa. J Med Entomol. 1981;18(3):232-234.
14. Kemp GE, Lee VH, Moore DL. Moore. Isolation of Nyamanini and Quaranfil viruses from Argas (Persicargas) arboreus ticks in Nigeria. J Med Entomol. 1975;12(5):535-537.
15. St George TD, Standfast HA, Doherty RL, Carley JG, Fillipich C, Brandsma J. The isolation of Saumarez Reef virus, a new flavivirus, from bird ticks Ornithodoros capensis and Ixodes eudyptidis in Australia. Aust J Exp Biol Med Sci. 1977;55(5):493-509.
16. Filipe AR, Casals J. Isolation of Dhori virus from Hyalomma marginatum ticks in Portugal. Intervirology. 1979;11(2):124-127.
17. Filipe AR, Alves MJ, Karabatsos N, de Matos AP, Núncio MS, Bacellar F. Palma virus, a new bunyaviridae isolated from ticks in Portugal. Intervirology. 1994;37(6):348-351.
18. Takahashi M, Yunker CE, Clifford CM, Nakano W, Fujino N, Tanifuji K. Isolation and characterization of Midway Virus: a new tick-borne virus related to Nyamanini. J Med Virol. 1982;10(3):181-193.
19. Calisher CH, Schwan TG, Lazuick JS, Eads RB, Francy DB. Isolation of Mono Lake virus (family Reoviridae, genus Orbivirus, Kemerovo serogroup) from Argas cooleyi (Acari: Argasidae) collected in Colorado. J Med Entomol. 1988;25(5):388-390.
20. Al-Khalifa MS, Diab FM, Khalil GM. Man-threatening viruses isolated from ticks in Saudi Arabia. Saudi Med J. 2007;28(12):1864-1867.
21. Chastel C, Launay H, Roguès G, Beaucournu JC. Isolation in France of Soldado virus (arbovirus, Hughes serogroup) from Ornithodoros (A.) maritimus Vermeil and Marguet 1967. C R Seances Acad Sci D. 1979;288(5):559-561.
22. Chastel C, Main AJ, Guiguen C, le Lay G, Quillien MC, Monnat JY, et al. The isolation of Meaban virus, a new Flavivirus from the seabird tick Ornithodoros (Alectorobius) maritimus in France. Arch Virol. 1985;83(3-4):129-140.
23. Chastel C, Guiguen C, Le Lay G, Le Goff F. First isolation of Soldado virus in southern France. Acta Virol. 1988;32(2):191.
24. Chastel C, Main AJ, Bailly-Choumara H, Le Goff F, Le Lay G. Essaouira and Kala iris: two new orbiviruses of the Kemerovo serogroup, Chenuda complex, isolated from Ornithodoros (Alectorobius) maritimus ticks in Morocco. Acta Virol. 1993;37(6):484-492.
25. Chastel C, Main AJ, Couatarmanac'h A, Le Lay G, Knudson DL, Quillien MC, et al. Isolation of Eyach virus (Reoviridae, Colorado tick fever group) from Ixodes ricinus and I. ventalloi ticks in France. Arch Virol. 1984;82(3-4):161-171.
26. Moutailler S, Popovici I, Devillers E, Vayssier-Taussat M, Eloit M. Diversity of viruses in Ixodes ricinus, and characterization of a neurotropic strain of Eyach virus. New Microbes New Infect. 2016;11:71-81.
27. Kuhn JH, Bekal S, Caì Y, Clawson AN, Domier LL, Herrel M, et al. Nyamiviridae: proposal for a new family in the order Mononegavirales. Arch Virol. 2013;158(10):2209-2216.
28. Wilhelmsson P, Jaenson TGT, Olsen B, Waldenström J, Lindgren PE. Migratory birds as disseminators of ticks and the tick-borne pathogens Borrelia bacteria and tick-borne encephalitis (TBE) virus: a seasonal study at Ottenby Bird Observatory in South-eastern Sweden. Parasit Vectors. 2020;13(1):607.
29. Henderson BE, Tukei PM, McCrae AW, Ssenkubuge Y, Mugo WN. Virus isolations from Ixodid ticks in Uganda. II. Kadam virus--a new member of arbovirus group B isolated from Rhipicephalus pravus Dontiz. East Afr Med J. 1970;47(5):273-276.
30. Zhang Y, Shen S, Fang Y, Liu J, Su Z, Liang J, et al. Isolation, characterization, and phylogenetic analysis of two new Crimean-Congo hemorrhagic fever virus strains from the northern region of Xinjiang Province, China. Virol Sin. 2018;33(1):74-86.
31. Feng Z. The methodology of laboratory detection of Crimean Congo hemorrhagic fever virus. Endem Dis Bull. 2004;19:101-115.
32. Bai Y, Zhang Y, Su Z, Tang S, Wang J, Wu Q, et al. Discovery of Tick-Borne Karshi virus implies misinterpretation of the Tick-Borne encephalitis virus seroprevalence in Northwest China. Front Microbiol. 2022;13:872067.
33. Fujita R, Ejiri H, Lim CK, Noda S, Yamauchi T, Watanabe M, et al. Isolation and characterization of Tarumizu tick virus: a new coltivirus from Haemaphysalis flava ticks in Japan. Virus Res. 2017;242:131-140.
34. Lwande OW, Lutomiah J, Obanda V, Gakuya F, Mutisya J, Mulwa F, et al. Isolation of tick and mosquito-borne arboviruses from ticks sampled from livestock and wild animal hosts in Ijara District, Kenya. Vector Borne Zoonotic Dis. 2013;13(9):637-642.
35. Guo R, Shen S, Zhang Y, Shi J, Su Z, Liu D, et al. A new strain of Crimean-Congo hemorrhagic fever virus isolated from Xinjiang, China. Virol Sin. 2017;32(1):80-88.
36. Moming A, Shen S, Fang Y, Zhang J, Zhang Y, Tang S, et al. Evidence of human exposure to Tamdy virus, Northwest China. Emerg Infect Dis. 2021;27(12):3166-3170.

37. Yurchenko OO, Dubina DO, Vynograd NO, Gonzalez JP. Partial characterization of tick-borne encephalitis virus isolates from ticks of Southern Ukraine. Vector Borne Zoonotic Dis. 2017;17(8):550-557.
38. Jin C, Liang M, Ning J, Gu W, Jiang H, Wu W, et al. Pathogenesis of emerging severe fever with thrombocytopenia syndrome virus in C57/BL6 mouse model. Proc Natl Acad Sci U S A. 2012;109(25):10053-10058.
39. Ruzek D, Avšič Županc T, Borde J, Chrdle A, Eyer L, Karganova G, et al. Tick-borne encephalitis in Europe and Russia: review of pathogenesis, clinical features, therapy, and vaccines. Antiviral Res. 2019;164:23-51.