Crimean-Congo haemorrhagic fever virus in ticks collected from livestock in Balochistan, Pakistan

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Abstract

Background: Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne zoonotic pathogen. It causes a fatal haemorrhagic disease in humans. Hard ticks, in particular Hyalomma spp., are considered to function as reservoir as well as vector for CCHFV.

Methods: A cross-sectional study was conducted in the province of Balochistan, Pakistan, from September to November 2017. Ticks were collected from cattle, sheep and goats in livestock farms. The ticks were morphologically identified, followed by confirmation with molecular methods (PCR and sequencing). Furthermore, ticks were examined for CCHFV genomes (S segment) by a one-step multiplex real-time RT-qPCR and positive samples were sequenced to determine the CCHFV genotype.

Results: In total, 525 of 529 livestock infesting adult ticks belonged to the genus Hyalomma, and 4 ticks to the genus Rhipicephalus (R. microplus 3×, R. turanicus 1×). In the genus Hyalomma, H. marginatum (28%), H. excavatum (26%), H. dromedarii (22%), H. anatolicum (16%) and H. scupense (8%) ticks were identified. Tick infestations were as follows: sheep 58%, goats 28% and cattle 14%. Four per cent (20/525) of ticks were CCHFV genome-positive, and all genomes clustered in CCHFV genotype Asia 1. Among CCHFV-positive ticks, 75% (15/20) were female and 25% (5/20) male. CCHFV genomes were most frequently detected in H. marginatum (30%, 6/20), followed by H. dromedarii (25%, 5/20), H. excavatum (20%, 4/20), H. anatolicum (20%, 4/20) and H. scupense (5%, 1/20). All CCHFV-positive ticks were found on sheep. The largest number of CCHFV-positive ticks were detected in the district of Kalat (60%, 12/20), followed by the districts of Quetta (30%, 6/20) and Killa Abdullah (10%, 2/20).

Conclusions: This study confirms the circulation of CCHFV in ticks in Balochistan, southwestern Pakistan. It is imperative to take effective tick control measures in this area, especially to control livestock tick infestations to prevent CCHF infections in humans.

Keywords
Crimean-Congo haemorrhagic fever virus, cross-sectional study, livestock, Pakistan, ticks

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1 | INTRODUCTION

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne zoonotic pathogen that can cause fatal haemorrhagic disease in humans (Spengler & Estrada-Peña, 2018). CCHFV belongs to the genus Orthohantavirus in the family Nairoviridae (ICTV, 2018). It has a negative-sense single-stranded RNA with a segmented genome comprised of small (S), medium (M), and large (L) segments (Papa et al., 2018).

Ticks, in particular species of the genus Hyalomma, are the main vectors and reservoir for CCHFV (Gonzalez, Camicas, Cornet, Faye, & Wilson, 1992; Logan, Linthicum, Bailey, Watts, & Moulton, 1989; Spengler & Estrada-Peña, 2018). Within tick populations, CCHFV is transmitted by co-feeding and passed on transovarially and transstadially (Gonzalez et al., 1992; Logan et al., 1989). Once infected, ticks remain positive for CCHFV for their whole lifespan (Gargili et al., 2017).

Many vertebrate species are considered to be subclinically affected amplifying hosts for CCHFV, with only a transient viraemia, whereas humans are developing a clinical manifestation (Gargili et al., 2017). Humans acquire the infection by tick bites, nosocomially from an infected patient or through contact with viraemic animals, their tissues or body fluids (Gargili et al., 2017). In humans, clinical signs include fever, dizziness, severe headache, vomiting, nausea, diarrhoea, cardiovascular and neuropsychiatric changes and haemorrhages (Whitehouse, 2004). The case/ fatality ratio recorded in humans ranges from 5% to 80% depending on the availability of suitable medical and the average severity of the clinical manifestation upon initial diagnosis (Kouhpayeh, 2019; Yilmaz et al., 2009).

The first known human outbreak of Crimean-Congo Haemorrhagic Fever (CCHF) was detected on the Crimean Peninsula in 1944. Today, the virus is endemic in Africa, Asia, south-east Europe, and Middle East (Whitehouse, 2004). CCHFV is phylogenetically divided into seven genotypes (Europe 1–2, Asia 1–2, Africa 1–3), which are also grouped according to the geographical area of origin (Bente et al., 2013).

The CCHFV strains circulating in Pakistan belong to the genotypes Asia 1 and Asia 2 (Bente et al., 2013). CCHFV is endemic in many countries in Asia, especially countries neighbouring Pakistan, that is Afghanistan, Iran, China and India (Chinikar, Ghiasi, Hewson, Moradi, & Haeri, 2010; Mostafavi et al., 2012; Mourya et al., 2015; Sun et al., 2009). In Pakistan, the first known human CCHF case was reported in 1976, followed by multiple reports about cases in the country (Atif, Saqib, Ikram, Sarwar, & Schall, 2017). CCHF cases appeared in almost all parts of the country, and a larger number of cases clustered in the south-western region, that is the province of Balochistan (Abbas, Younus, & Muhammad, 2015; Alam, Khurshid, Sharif, Shaukat, Rana, et al., 2013; Atif et al., 2017). Balochistan is an epicentre for livestock-related activities, especially for breeding small ruminants, which contribute a major share to the country’s annual total meat production. The climate in Balochistan is arid and semi-arid, and thus provides a favourable habitat for ticks (Alam, Khurshid, Sharif, Shaukat, Rana, et al., 2013). There is no previous report of the identification of CCHFV genotypes in ticks in Balochistan. Considering the zoonotic importance of CCHFV infection in humans, we determined the geographic distribution of the CCHFV tick vectors, circulation of CCHFV genotypes among them and the risk arising from infected ticks to the human population.

2 | METHODOLOGY

2.1 | Study area

Pakistan is geographically located in South Asia. It is divided into four administrative units called provinces. Balochistan is the largest province located in the south-western part of the country, spreads around 347,190 km² and comprises 44% of the country’s total land mass. Topographically, Balochistan is divided into four parts: upper highlands in the north-eastern and central part, lower high lands in south-eastern part, plains in southern part and deserts in the western part (Majeed, 2015). The climate is arid and semi-arid with annual precipitation from 50 mm in the western part to over 400 mm in the eastern part of the province. The ranges in Balochistan are used for producing forage for livestock, medicinal plants, wildlife habitat, recreational activities and wood fuel. The husbandry system of livestock in the province can be nomadic, transhumant/semi-nomadic or sedentary (Mirza, Athar, & Qayyum, 2009).

2.2 | Study design

A cross-sectional study was conducted from September to November 2017 in the province of Balochistan, Pakistan. The sample size was calculated for large populations with 50% expected prevalence to avoid potential loss of precision due to a higher or lower true prevalence, at the 95% confidence level and for 10% desired precision, resulting in 96 livestock farms to be sampled (Cannon & Roe, 1982). The details of the administrative units were obtained from the local municipality authority. A multi-stage cluster sampling approach was used to select the livestock farms in each division. Four out of six divisions in Balochistan were randomly selected. In each division, three districts, and in each district, two union councils were selected. In each union council, four livestock farms were randomly selected. In each farm, a minimum of three animals of each existing species (cattle, sheep or goats) was randomly selected irrespective of age, sex and breed. The animals on the farm were not tagged. Therefore, a systematic sampling approach was used, so that each animal at a farm had an equal chance to be selected. Before sampling, the farmer was informed about the purpose of sampling and his consent was obtained.

2.3 | Tick collection

Ticks were collected from cattle, sheep and goats on the livestock farms. Adequate personal measures were adopted by wearing
protective clothing to cover the whole body during the tick collection process to protect the tick collectors from tick-borne infections. The entire bodies of the animals were examined for ticks, in particular ears, neck, chest, scrotum, perineum and base of the tail. The ticks were eventually collected from the hosts with blunt forceps, transferred into appropriately labelled safety lock Eppendorf tubes®. The ticks were stored at −20°C until further processing. They were then transferred to the University of Veterinary and Animal Sciences, Lahore, Pakistan, for morphological and molecular identification securing the cold chain. For further analysis, the ticks were shipped frozen on dry ice to maintain cold chain to the Friedrich Loeffler Institute, Greifswald-Insel Riems, Germany.

2.4 | Tick identification

2.4.1 | Morphological identification

Ticks were identified based on their morphological features under the stereomicroscope using a multiple electronic key (Walker, Matthews, & Preston, 2005) and the re-description of tick species by Apanaskevich and Horak (2005); Apanaskevich, Filippova, and Horak (2010). The ticks were identified up to the species level. After morphological identification, 10 specimens that belonged to different species were selected to confirm the morphological identification using a molecular method.

2.4.2 | Molecular identification

DNA was extracted using Halos et al. (2004) protocol with the following modifications: ticks were crushed separately using liquid nitrogen and 1.5 ml of lysis buffer, followed by the addition of 0.125 µl of 20 mg/ml of proteinase K to each tube. The samples were incubated at 65°C for overnight. The concentrations of the extracted genomic DNA samples were quantified using a NanoDrop ND-100 instrument. The extracted genomic DNA samples were stored at −20°C until further use. The amplification of a partial fragment (750 nt) of the second internal transcribed spacer (ITS2) gene was performed using the primers as described earlier by Rehman et al. (2017). PCR was performed in a total reaction volume of 20 µl, containing 2 µl of each primer, 2 µl template DNA, 10 µl of 2× Green Master mix with Taq polymerase (Wiz Bio Solutions) and 4 µl of DEPC water. The product was amplified in a thermocycler (G-storm) with initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 57 °C for 30 s and 72°C for 50 s. A final extension at 72°C for 5 min was also performed. The product was visualised on ethidium bromide-stained 1.5% agarose gels using a UV illuminator. The amplicons obtained were isolated and purified using a gel purification kit (GeneAll), and the purified products were sent to 1st BASE, Singapore, for sequencing.

2.5 | Molecular analysis of ticks for CCHFV genome

2.5.1 | RNA extraction and real-time RT-qPCR

Each tick was homogenized in a 2-ml safety lock Eppendorf tube® with 500 µl PBS and a steel bead with 5 mm in diameter (Qiagen) by using TissueLyser II (Qiagen) for 3 min at 30 Hz. The homogenate was centrifuged for 10 min at 10,000 rpm. One-hundred-and-forty µl supernatants were added afterwards to 560 µl AVL buffer (Qiagen) in a 1.5-ml safety lock Eppendorf tube®. The tick homogenization procedure adopted in this part (2.5.1) and in the part 2.4.2 was performed according to two different protocols. RNA was extracted using the NucleoMag® VET kit (Macherey-Nagel) on a KingFisher Flex instrument (Thermo Fisher Scientific) according to the manufacturer’s instructions. Ten µl MS2-phage RNA were added to each well as an extraction control. In addition, 100 µl FKS P84 (RIC) was added to one well in each row of the plate as a negative control. The extracted RNA was stored at −80°C until further processing.

To detect the RNA of the CCHFV S segment, a one-step multiplex real-time RT-qPCR developed by Sas et al. (2018) was used with the following modifications: it included primer sets for all known CCHFV genotypes. The AgPath-ID™ One-Step RT-PCR kit (Thermo Fisher Scientific) was used. The total reaction volume was 25 µl, consisting of 20 µl master mix (2 µl RNase free water, 12.5 µl 25× RT-PCR Buffer, 2 µl MS2-phage RNA Primer Probe mix, 2.5 µl genotypes-specific CCHF-Primer-Probe-CoProbe mix and 1 µl 25× µl RT-PCR Enzyme Mix) and 5 µl RNA template. As a positive control, CCHFV synthetic RNA was used as described by Sas et al. (2018). The following cycling protocol was applied: 48°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. A CFX96 Real-Time PCR thermal cycler (Bio-Rad Laboratories) was used for the real-time RT-qPCR.

To confirm the results of the one-step multiplex real-time RT-qPCR for the S segment, a one-step multiplex real-time RT-qPCR was performed to detect CCHFV L-segment RNA, which again included primer sets for all known CCHFV genotypes. The AgPath-ID™ One-Step RT-PCR kit (Thermo Fisher Scientific) was used. The total reaction volume was 15 µl, consisting of 12 µl master mix (1.9 µl RNase free water, 7.5 µl 25X RT-PCR Buffer, 2 µl genotypes-specific CCHF-MS2-phage RNA Primer-Probe-CoProbe mix and 0.6 µl 25X µl RT-PCR Enzyme Mix) and 3 µl RNA template. As a positive control, synthetic CCHFV RNA was used. The following cycling protocol was applied: 48°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. A CFX96 Real-Time PCR thermal cycler (Bio-Rad Laboratories) was used for the real-time RT-qPCR.

2.5.2 | Sequencing

Samples that were positive in the one-step multiplex real-time RT-qPCR were further analysed to determine the
CCHFV genotypes. The RNA template was amplified by using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase kit (Thermo Fisher Scientific) according to manufacturer’s recommendations using a Bio-Rad C1000™ thermal cycler (Bio-Rad Laboratories). All genotype-specific CCHFV primers were used as described by Sas et al. (2018). The presence of the expected cDNA product obtained by RT-PCR was confirmed by electrophoresis in 2% agarose gels. The respective band was excised, cleaned using the Monarch® PCR & DNA Cleanup Kit (BioLabs) and sent to Eurofins Germany for sequencing. For phylogenetic analysis, CCHFV S segment reference strains of different genogroups were obtained from GenBank (www.ncbi.nlm.nih.gov). Multiple alignments of sequences were performed, and a phylogenetic tree was constructed with the maximum likelihood method using the Kimura 2-parameter model (Guindon et al., 2010) in the Geneious 11.1.5 software (Biomatters).

2.6 | Statistical analysis

For statistical analyses, R software (R Core Team, 2013, Vienna, Austria; http://www.R-project.org/) and RStudio (an integrated development environment for R) (RStudio, 2016) were used. ArcGIS (version 10.5.1; Esri, 380 New York Street, Redlands, California, USA) was used to prepare maps. The CCHFV prevalence among ticks was calculated by dividing the number of CCHFV-positive ticks by the total number of ticks analysed. 95% confidence intervals (CI) for proportions were estimated by using the exact 95% Clopper–Pearson interval method with the binom.test function in the binom package in R (Dorai-Raj, 2014).

3 | RESULTS

3.1 | Tick species identification and geographical distribution

Five-hundred-and-twenty-five of 529 ticks (99%, CI: 98%–100%) were identified as *Hyalomma* spp. and four (1%, CI: 0.2%–2%) ticks belonged to the genus *Rhipicephalus*, both genera belonging to the family Ixodidae (hard ticks) (Table 1). In the genus *Hyalomma*, the following species were identified: *H. marginatum* (28%, CI: 24%–32%), *H. excavatum* (26%, CI: 22%–30%), *H. dromedarii* (22%, CI: 19%–26%), *H. anatolicum* (16%, CI: 13%–19%) and *H. scupense* (8%, CI: 6%–11%). In the genus *Rhipicephalus*, three ticks were *R. microplus* and one tick *R. turanicus*. Tick infestation on ruminants was detected in 58% (CI: 54%–62%) of the examined sheep, 28% (CI: 24%–32%) of the goats and 14% (CI: 11%–18%) of cattle. All collected ticks were identified as adults. The geographical distribution of the ticks at the district level is shown in Figure 1.

3.2 | CCHFV prevalence in ticks

Five-hundred-and-twenty-five *Hyalomma* ticks were analysed, out of which 20 (4%, CI: 2%–6%) ticks were positive for CCHFV S segment

| District     | Host species | *Hyalomma* anatolicum | *Hyalomma* excavatum | *Hyalomma* marginatum | *Hyalomma* dromedarii | *Hyalomma* scupense | *Rhipicephalus* microplus | *Rhipicephalus* turanicus |
|--------------|--------------|------------------------|----------------------|-----------------------|-----------------------|----------------------|---------------------------|---------------------------|
| Quetta       | Sheep        | 12                     | 5                    | 1                     | 5                     | 3                    | 1                         | 5                         |
|              | Cattle       | 1                      | 1                    | 1                     |                       |                       |                           |                           |
| Killa        | Sheep        | 1                      | 1                    | 2                     | 2                     | 1                    |                           |                           |
| Abdullah     | Sheep        | 1                      | 1                    | 1                     |                       |                       |                           |                           |
| Pishin       | Cattle       | 5                      | 1                    | 12                    | 4                     | 1                    |                           |                           |
| Kalat        | Sheep        | 6                      | 14                   | 1                     |                       |                       |                           |                           |
| Khuzdar      | Goat         | 1                      | 3                    | 1                     | 22                    | 59                   |                           |                           |
| Lasbela      | Sheep        | 2                      | 2                    | 3                     | 9                     | 33                   | 3                         | 3                         |
|              | Goat         | 1                      | 1                    | 1                     | 1                     | 12                   | 2                         | 5                         |
| Sibi         | Cattle       | 4                      | 12                   | 12                    | 17                    | 1                    | 2                         | 2                         |
| Ziarat       | Sheep        | 1                      | 1                    | 6                     |                       | 4                    |                           |                           |
| Zhob         | Sheep        | 15                     |                      | 4                     | 30                    | 4                    | 11                        |                           |
| Loralai      | Sheep        | 1                      | 8                    | 6                     | 6                     | 1                    | 1                         | 4                         |
|              | Goat         | 1                      | 1                    | 4                     | 12                    |                       |                           |                           |
| Sherani      | Sheep        | 3                      | 3                    | 1                     |                       |                       |                           |                           |
| Total        |              | 61                     | 21                   | 96                    | 39                    | 92                   | 57                        | 101                       | 16                        | 36                        | 6                         | 3                         | 1                         |
genome. All sequenced amplicons of the positive ticks clustered in the genotype Asia 1 (Figure 2). The Balochistan-42-2017-Pakistan sequence showed the closest proximity (99% nucleotide identity) with a CCHFV strain from Oman (DQ211645), followed by CCHFV strains from Iran (KJ566219, 97% nucleotide identity) and Pakistan (U88414, 97% nucleotide identity). Among the CCHFV-positive ticks, 75% (15 out of 20) were female and 25% (5 out of 20) were male. CCHFV genomes were detected most frequently in *H. marginatum* (30%, 6 out of 20), followed by *H. dromedarii* (25%, 5 out of 20), *H. excavatum* (20%, 4 out of 20), *H. anatolicum* (20%, 4 out of 20) and *H. scupense* (5%, 1 out of 20) (Figure 3). All positive ticks were found on sheep. The highest number of ticks was CCHFV-positive in the district of Kalat (60%, 12 out of 20), followed by the districts of Quetta (30%, 6 out of 20) and Killa Abdullah (10%, 2 out of 20) (Figure 4). In Kalat, CCHFV genome was detected in *H. marginatum* (5 out of 12), *H. dromedarii* (4 out of 12), *H. anatolicum* (2 out of 12) and *H. excavatum* (1 out of 12). In Quetta, *H. excavatum* (3 out of 6), *H. anatolicum* (2 out of 6) and *H. scupense* (1 out of 6) were positive for the CCHFV genome. In addition, in Killa Abdullah district, one *H. marginatum* and one *H. dromedarii* were positive for CCHFV genomes.

## DISCUSSION

Crimean-Congo haemorrhagic fever virus is endemic in Pakistan and causes a large number of human infections often with lethal outcomes. Livestock animals serve as feeding source and thus foster the increase in local tick populations. Moreover, they serve as amplification hosts for the virus. Both factors can lead to an increased exposure of the rural human population to this dangerous tick-borne disease by contact with blood or tissues from infected animals or tick bites, for example during activities in the field.

In this study, ticks collected from livestock (sheep, goats and cattle) in Balochistan, Pakistan, were analysed for CCHFV infections. The ticks belonged to the genera *Hyalomma* and *Rhipicephalus*. *Hyalomma* spp. infestation was higher as compared to *Rhipicephalus* spp. infestation, which is in accordance with previous studies from Pakistan (Ahmad et al., 2014; Ali, Maqbool, Muhammad, Khan, & Younis, 2013; Rehman et al., 2017; Sajid et al., 2011). However, two other studies from Pakistan reported a higher infestation of *Rhipicephalus* spp. as compared to *Hyalomma* spp. (Ahmed, Numan, Manzoor, & Ali, 2012; Kakar et al., 2017). Moreover, among the genus *Hyalomma*, highest infestation was of *H. marginatum*, followed...
by *H. excavatum*, *H. dromedarii*, *H. anatolicum* and *H. scupense*. This is in contrast to previous studies in Pakistan, which reported that *H. anatolicum* was most frequently found as compared to other *Hyalomma* species (Karim et al., 2017; Rehman et al., 2017).

We detected *Hyalomma* ticks in all districts in the study area. Previous studies in this area reported *Hyalomma* ticks (Iqbal & Nawaz, 2007; Kakar et al., 2017; Karim et al., 2017; Rafique, Kakar, Iqbal, Masood, & Razzaq, 2015); however, *H. marginatum* had not been found so far as to our knowledge. Moreover, there was hardly any information regarding the geographical distribution of the ticks in the area, in particular at the district level. The climate in the study area is mainly arid with low precipitation. It is mostly comprised of rangelands with grasses and shrubs (Ahmad & Islam, 2011), with abundant livestock (Government of Balochistan, 2016) which provides a favourable habitat for ticks. In addition, livestock farms in this area have mainly open type of housing with crevices and cracks in the walls where ticks can breed and hide (Muhammad, Naureen, Firyal, & Saqib, 2008). *Hyalomma* ticks have the ability to adapt, when introduced into new environments.

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**FIGURE 2** Phylogenetic tree of a partial S segment (180 nt) from the genome of Crimean-Congo haemorrhagic fever virus (CCHFV) (isolate from this study in bold) with the maximum likelihood method using the Kimura two-parameter model. Bootstrap values at the nodes of above tree (percentage of replicate trees in which the interrelated taxa clustered together) obtained from the bootstrap test (1,000 replicates).
environments, especially in areas with dry climate. They also accustom easily to new animal housing facilities (wall crevices, under dried dungs, etc.) (ECDPC; Latif & Walker, 2004). Furthermore, livestock in this area is mainly fed by grazing on rangelands. This compels the farmers to move in search of pastures from one area to another, especially by nomadic and transhumant flock owners who migrate in winter from the northern part of province towards the southern part and return back to the north in spring (Ahmad & Islam, 2011). *Hyalomma* ticks are known as "hunting ticks", because they actively run towards their hosts (humans or animals) for distances of up to 400 m (Bente et al., 2013). Also, previous studies reported questing *Hyalomma* ticks on grazing pastures (Gunes, Poyraz, & Vatansever, 2011; Sherifi et al., 2018). Livestock can support high infestation of up to 100 *Hyalomma* ticks on one single animal (ECDPC; Estrada-Pena, Jameson, Medlock, Vatansever, & Tishkova, 2012).

In this study, a prevalence of 4% CCHFV was detected in ticks in Balochistan. CCHFV was first diagnosed in ticks in Pakistan in 1970 in the north-eastern part of the country (Begum, Wiseman, and Casals, 1970). We are not aware of any other published record of CCHFV detection in ticks from any other part of the country. We found that the CCHFV prevalence was higher in *H. marginatum* as compared to *H. dromedarii, H. excavatum, H. anatolicum* and *H. scupense*. This result is in accord with previous reports from Turkey (Gargili et al., 2011; Gunes et al., 2011; Ozdarendeli et al., 2008; Tekin, Bursali, Mutluay, Keskin, & Dundar, 2012; Tonbak et al., 2006), Iran (Fakoorziba et al., 2012; Zakkyeh et al., 2008) and Bulgaria (Gergova & Kamarinchev, 2013). *Hyalomma* ticks play a crucial role in the maintenance of CCHFV-endemic foci in nature. Moreover, it has been suggested that an increase in the population of *H. marginatum* is followed by an increase in CCHFV infections in humans in the affected area (Gargili et al., 2017). We found CCHFV-positive ticks in the districts of Kalat, Quetta and Killa Abdullah, where CCHF cases among humans have previously been reported (Alam, Khurshid, Sharif, Shaukat, Rana, et al., 2013; Khurshid et al., 2015). Also in a recent study, CCHFV genomes in sheep, and CCHFV-specific antibodies in sheep and goats were found in these areas (Kasi et al., 2019). This region is close to the border with Afghanistan, which is also endemic for CCHFV (Khurshid et al., 2015; Sahak, Arifi, & Saeedzai, 2019). Balochistan is considered as a corridor for the trade of ruminant skins from Iran (also endemic for CCHFV) and Afghanistan for the leather industry, and also importation of livestock from Afghanistan to Pakistan is common (Raziq, Younas, & Rehman, 2010).

In the current study, all sequenced amplicons of the CCHFV-positive ticks clustered in the genotype Asia 1. The Balochistan-42-2017-Pakistan sequence shows high identity with CCHFV strains from Oman, Iran, and Pakistan. The results are in accord with previous phylogenetic studies conducted with human CCHFV isolates from Pakistan, which also clustered in this genotype (Alam, Khurshid, Sharif, Shaukat, Rana, et al., 2013; Khurshid et al., 2015). Genotype Asia 1 includes CCHFV strains from Pakistan, Iran, Afghanistan, Middle East and China (Alam, Khurshid, Sharif, Shaukat, Rana, et al., 2013). However, Alam, Khurshid, Sharif, Shaukat, Suleman, et al. (2013) found a CCHFV isolate in Pakistan that clustered in the genotype Asia 2 with strains from Tajikistan and Dubai. This sequence was detected in a human CCHF patient from Balochistan. Genotype Asia 2 includes CCHFV strains from China, India, Kazakhstan, Tajikistan, Uzbekistan, Azerbaijan and Middle East (Alam, Khurshid, Sharif, Shaukat, Suleman, et al., 2013; Yadav et al., 2013).

Our phylogenetic analysis was conducted with a partial S segment sequence of the CCHFV genome. Similar (partial) sequences were also used in other studies for the classification of CCHFV into genotypes (Abdiyeva et al., 2019; Alam, Khurshid, Sharif, Shaukat, Rana, et al., 2013; Alam, Khurshid, Sharif, Shaukat, Suleman, et al., 2013; Drosten, Minnak, Emmerich, Schmitz, & Reinicke, 2002; Khurshid et al., 2015; Papa et al., 2002). Further research is needed to obtain the full-length CCHFV sequences of S, M and L segments, to determine possible genetic re-assortment and recombination in the genome of CCHFV strains circulating in the area. Genetic re-assortment has been reported in European CCHFV strains (Lukashev et al., 2016). In Iran, which borders in the south-west with Balochistan, the circulation of genomic variants of CCHFV has been reported (Biglari et al., 2016).

5 | CONCLUSIONS

This study confirms the circulation of CCHFV in ticks in the south-western part (Balochistan) of Pakistan. *Hyalomma* and *Rhipicephalus* ticks infesting livestock were identified. The CCHFV strain found in *Hyalomma* ticks belongs to genotype Asia 1. The most dominant tick species infected with CCHFV was *H. marginatum*, followed by *H. dromedarii, H. excavatum, H. anatolicum* and *H. scupense*. It is imperative to take effective tick control measures in this area, especially to...
control livestock infestations with ticks to prevent CCHF outbreaks among the human population.

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ETHICAL STATEMENT
Serum samples were collected according to fundamental ethical principles for diagnostic purposes in context of national surveillance studies. Ethical Statement is not applicable if sample collection or questionnaires from animals/human have been gathered.

DATA AVAILABILITY STATEMENT
The data supporting the results of this study are available upon request from the respective author, M.H. Groschup. The data are not publicly accessible due to the privacy of the research participants, especially the contributing pastoralists and herdsman.

REFERENCES
Abbas, T., Younus, M., & Muhammad, S. A. (2015). Spatial cluster analysis of human cases of Crimean Congo hemorrhagic fever reported in Pakistan. Infectious Diseases of Poverty, 4, 9. https://doi.org/10.1186/2049-9957-4-9
Abdiyeva, K., Turebekov, N., Dmitrovsky, A., Tukhanova, N., Shin, A., Yeraliyeva, L., ... Essbauer, S. (2019). Seroepidemiological and molecular investigations of infections with Crimean-Congo haemorrhagic fever virus in Kazakhstan. International Journal of Infectious Diseases, 78, 121-127. https://doi.org/10.1016/j.ijid.2018.10.015
Ahmad, I., Khawja, A., Shams, S., Ayaz, S., Khan, S., Akbar, N. U., ... Zakir, M. (2014). Detection of babesiosis and identification of associated ticks in cattle. International Journal of Bioassays, 3, 3195-3199.
Ahmad, S., & Islam, M. (2011). Rangeland Productivity and Improvement Potential in Highlands of Balochistan, Pakistan. Retrieved from https://www.researchgate.net/publication/221916004_Rangeland_Productivity_andImprovement_Potential_in_Highlands_of_Balochistan_Pakistan (accessed 10 April 2018).
