RESEARCH ARTICLE

Serological surveillance of Crimean-Congo Hemorrhagic Fever Virus in animals using targeted Enzyme Immuno Assay

Maaza Sana[1], Sercan Keskin[2], Muhammad Tahir[3], Muhammad Faheem[4], Ali Zohaib[5], Mehmet Ziya Doymaz[2] and Aneela Javed[1]*

[1]Department of Biomedicine, Atta-ur-Rahman School of Applied Biosciences, National University of Science and Technology, Sector H-12, Islamabad, Pakistan; [2]Department of Biotechnology, Institute of Health Sciences, Bezmialem Vakif University, Istanbul, Türkiye; [3]Department of Agricultural Sciences and Technology, Atta-ur-Rahman School of Applied Biosciences, National University of Science and Technology, Sector H-12, Islamabad, Pakistan; [4]Department of Biological Sciences, National University of Medical Sciences, Abid Majeed Road, Rawalpindi, Punjab 46000, Pakistan; [5]Department of Microbiology, Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Pakistan
*Corresponding author: javedaneela19@asab.nust.edu.pk; javedaneela19@gmail.com

ABSTRACT

Crimean–Congo hemorrhagic fever (CCHF) is endemic to many countries including Pakistan and remains the second most important disease on the WHO's diseases priority list. With a mortality rate of 10 - 40%, there is a dire need to develop indigenous diagnostic methods for surveillance of asymptomatic infections in animals. The current study employed CCHFV Nucleoprotein (NP), for the development of a targeted Enzyme Immuno Assay (EIA) and was tested on 1034 animal serum samples collected throughout the country. The CCHFV-NP was purified using Fast Protein Liquid Chromatography (FPLC) and confirmed through western blotting. *In vivo* immunogenicity of the purified antigen was tested in BALB/c mice and was subsequently used for establishing a sensitive in-house indirect Enzyme Immuno Assay. CCHFV immunized rabbit serum and CCHFV positive human serum were used as positive controls while uninfected rabbit and human sera and untransformed BL21 lysate were used as negative controls. Finally, the assay was used to test Asymptomatic, domestic and wild animal serum samples collected from all four provinces including CCHFV endemic and non-endemic regions. Our results indicate that from Baluchistan 10% (20/200), Khyber Pakhtunkhwa 9.3% (14/150), Sindh 4% (8/201), Kashmir 2% (1/50) and Punjab 13.6% (55/434) samples were found to be positive. These findings suggest a potential disease risk in the mentioned regions, some of which had not previously reported any human cases or outbreaks. The assay can be further used for screening animals for potential disease risk in the country and to mitigate the required diseases response strategies.

INTRODUCTION

Crimean–Congo hemorrhagic fever virus (CCHFV), a member of the *Nairoviridae* family, *Hareavirales* order and *Bunyaviricetes* class (Taxon Details | ICTV, 2024), is transmitted to humans via tick bites or contact with infected blood. CCHFV is a zoonotic virus and circulates between three reservoirs: ticks, animals and humans. Infected ticks of Hyalomma (predominantly), Amblyomma and Rhipicephalus either directly or indirectly via wild and domestic animals (Gonzalez *et al*.,1991) infect humans. Human to human transfer occurs by nosocomial or other means. In humans, infection is symptomatic and results in severe disease. Whereas in animals it is asymptomatic (Gonzalez *et al*., 1991; Akıncı *et al*., 2013; Flick and Whitehouse, 2005).

There are seven distinct genotypes of CCHFV and Genotype 1 is predominantly reported in Pakistan, however, a comprehensive animal survey is yet required to identify the other possible genotypes circulating among animals and humans (Leblebicioglu *et al*., 2014). There is a 2–14-day period of viral incubation period in humans post infection, before the symptoms start to appear. In a 1-7 days pre-hemorrhagic phase at the onset of symptoms, few

individuals can clear the infection however in other individuals, it develops into hemorrhagic phase, and if not properly managed, ultimately leading to the death.

CCHFV is ssRNA, enveloped virus with a tripartite genome i.e. small (S), medium (M), and large (L) segments. The S segment encodes the nucleocapsid protein (N/NP), while the M segment encodes the Gn and Gc proteins within the glycoprotein precursor, and the L segment encodes viral RNA-dependent RNA polymerase (RdRp) (Morikawa, Saijo, and Kurane, 2007).

NP plays a critical role in the entire viral life cycle of CCHFV. The NP encoded by the S segment oligomerizes by forming a ribonucleoprotein (RNP) complex. This RNP complex encapsidates all the three RNA segments of the genome and thus participates in viral replication, transcription, and assembly (Šantak and Matić, 2022). It has also been reported that, in the case of Orthonairoviruses, RNP complex is controlled through a close interaction between the RNP and viral glycoproteins (Carter *et al*., 2012; Šantak and Matić, 2022). NP triggers interferon induction cascades and remains highly conserved throughout the Nairoviridae family (Carter *et al*., 2012; Zivcec *et al*., 2016; Šantak and Matić 2022). The NP has shown to interact with host immune element MxA protein there-by contributing to the immune evasion by the virus (Andersson *et al*., 2004). NP is highly immunogenic, inducing strong B and T lymphocytes responses in mammals and crucial for viral clearance (Bergeron *et al*., 2009; Zivcec *et al*., 2015; Šantak and Matić, 2022). Thus, qualifies to be selected as preferred candidate protein for the development of Enzyme Immuno Assay (EIA) as a diagnostic method in current study.

Despite a significantly lower incidence rate compared to COVID-19, CCHF has a considerably higher mortality rate of 10 - 40%, (Greene *et al*., 2022) and CCHFV can cause severe symptoms, including hemorrhagic fever. WHO has included CCHF in its list of blueprint priority diseases (Oygar *et al*., 2023) and has classified it as a high-priority pathogen due to its high fatality rate, absence of effective medical countermeasures and its pandemic potential. In addition to its high mortality rate, the limited knowledge about the disease makes it a serious global health concern. Furthermore, there are no standard diagnostic tests available for the detection and screening of CCHFV (Frank *et al*., 2024), specially in animals where the infection remains asymptomatic. CCHF is endemic in Pakistan, exhibiting a case fatality rate (CFR) of 7.4%, and is exerting significant pressure on the country's healthcare system (Karim *et al*., 2017; Zohaib *et al*., 2020; Oygar *et al*., 2023; Tabassum *et al*., 2023). However, control of tick vectors has been challenging task for the control of CCHFV spread in Pakistan (Alam *et al*., 2013).

The disquieting increase in both geographical distribution and number of cases of CCHF can be attributed to the lack of epidemiological research in animals, where the disease being asymptomatic, goes unchecked and can in-turn be linked to the sporadic human cases of severe symptoms and deaths. Absence of affordable and proficient diagnostics as well as therapeutics (both animals and humans) in CCHF-endemic countries (Akıncı *et al*., 2013; Leblebicioglu *et al*., 2014) is the major challenge faced and needs urgent attention.

This further strengthens the idea of an effective mitigation strategy for CCHFV is addressing the issue using "*One-Health*" approach of identifying and eliminating the pathogen in animal reservoirs before its spread to humans. However, there are severe limitations in animal surveillance due to lack of specific and cost-effective diagnostic assays for surveillance in animals.

Current study aims to develop an in house, more specific and cost-effective diagnostic assay—EIA that can be used for massive CCHFV surveillance in Pakistan enabling a more realistic assessment of the status of CCHFV in Pakistan and the magnitude of the threat it may pose, to mitigate future outbreaks.

MATERIALS AND METHODS

Plasmid Containing Antigen Protein (NP): The codon optimized sequence of NP (reported from Pakistan with GenBank Accession No. U88414.1) cloned into pDual GC plasmid expression vector (pDual-GC-CHFV-NP) was kindly provided by Prof. Dr. Linfa Wang (Professor & Director, Programme in Emerging Infectious Disease, Duke-NUS Medical School, 8 College Road, Singapore 169857).

Transformation of pDual GC plasmid into *E. coli*: The plasmid pDual-GC-CHFV-NP was transformed into One Shot BL21 Star (DE3) chemically competent *E.coli* cells (Thermo Fisher Scientific, Waltham, MA, USA) using heat shock method (Froger and Hall, 2007;). 350 µl of super optimal broth with catabolite repression medium (SOC) (Kalkan-Yazıcı *et al*., 2021) was added and set for 2 h shaking (200 rpm) incubation at 37℃. For kanamycin selection, 100 µl of transformed cells in SOC media were plated on kanamycin-agar plates and incubated overnight at 37℃. A single colony was picked, and colony PCR was performed (Bio-Rad T100 PCR Thermal Cycler, USA). A 0.8% EtBr (Ethidium Bromide) gel was used to visualize the products.

After confirming the transformation, the expression culture was incubated until the optical density OD 600 of 0.4 to 0.6 for the 200 ml culture was reached. the expression induction was carried out with 2 mM IPTG (Biomatik, Cambridge, ON, Canada) (Kalkan-Yazıcı *et al*., 2021; Wang *et al*., 2023). The entire process was repeated for untransformed *E.coli* BL21 DE3 for the purpose of its use as a negative control.

Nucleoprotein expression and lysate preparation: The cultures were then harvested, washed (with PBS) and resuspended in urea lysis buffer (10 mM Tris-HCl, 8 M urea, 1% Tween 20, 300 mM NaCl, pH 8.5). They were then sonicated and incubated at room temperature with agitation for 30 min. The lysed cells were centrifuged at $30,000 \times g$ for 30 min and expression of NP in supernatant and pellets were confirmed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Untransformed *E.coli* BL21 was processed as negative control.

Nucleoprotein purification: The Supernatant from ultra-centrifugation was filtered using 0.45µm Chrom Tech F30-NY045 Syringe Filter (Control Specialties Inc. Gainesville, GA, USA) and subjected to Fast Protein Liquid Chromatography (FPLC) using ÄKTA pure

chromatography system (GE Healthcare Life Sciences, Glattbrugg, Switzerland and HisTrap excel columns (GE Healthcare Life Sciences) under denaturing conditions. The FPLC fractions were eluted 250 mM imidazole containing buffer and. loaded on 12% SDS-PAGE to assess their purity (Kalkan-Yazıcı *et al*., 2021).

The FPLC purified elution fractions were dialyzed using 20% sucrose-1X PBS solution (Wang *et al*., 2009; Oluka *et al*., 2023) with SnakeSkin™ Dialysis Tubing (Thermo Fisher Scientific, Waltham, USA) on Bio-Rad Digital Hot Plate Stirrer magnetic stirrer (Biorad System, USA) at 400rpm overnight stirring (4°C) (Bio-Rad Laboratories, Inc., USA). Protein quantification was done using NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Untransformed BL21 strain was processed to be used as negative control.

Western blotting: NP transformed BL21 cells were lysed and centrifuged to obtain supernatant and pellet. The supernatant and pellet were electrophoresed on 12% SDS-PAGE gels and proteins were transferred to a 0.45 μm nitrocellulose (Amersham Protran) membrane by electroblotting (BioRad) for 1 h at 100 V. Subsequently the membranes were saturated in a 5% non-fat milk solution prepared with TBST (20 mM Tris HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween) for 1 h. Afterwards the membranes were incubated in 5% TBST-milk overnight at 4°C with gentle agitation, for the detection of NP, with primary antibodies sources (1:1,000 dilution of CCHFV-inoculated mouse sera (ın-house) as well as Antı-Hıs Antıbody from Invitrogen (6x-His Tag Monoclonal Antibody (HIS.H8); Thermo Fisher Scientific Inc.)). Finally, 3× washings of 10 min using TBST (Freitas *et al*., 2020), membranes were then incubated with horseradish peroxidase-conjugated anti-mouse anti IgG (sc-2005; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) and anti-6×His tag antibody (HRP; Abcam, UK, as per manufacturers instructed dilutions). Quantification of proteins was performed with chemiluminescence detection (WesternBright Sirius chemiluminescence detection kit; Advansta, CA, USA, according to the manufacturer's instructions) on Vilber Chemiluminescence/Fluorescence Gel Doc Systems (Ade Lab Scientific; 36 Holland Street, Thebarton, 5031, South Australia). The size of FPLC purified elution fractions were also electroblotted using the same method, for the purpose of revalidation (Raheel *et al.*, 2015; Begum *et al.*, 2022) (Fig 4).

Mice immunization: The purified NP was used for immunization of 6 - 8 weeks old (Fig 1), 1 male and 2 female BALB/c mice (obtained from National Institute of Health, Islamabad, Pakistan). One male and one female mouse was immunized with (20 μl) NP (1.723 μg/μl) (Maira-Litrán, 2017), while one female mouse treated with (20 μl) 1XPBS used as negative control. Booster shots were given on Day 15 and Day 30. After 4 - 5 days of last immunization, the serum was separated from the blood for EIA.

Indirect *In-house* Enzyme Immuno Assay (EIA): The flat-bottomed polystyrene 96-well microtiter plates (Immulon1B; Dutscher Scientific) were coated with serially diluted NP obtained from both FPLC and whole lysates, using the coating buffer (35 mM NaHCO$_3$ [1,465

mg], 15 mM Na$_2$CO$_3$ [765 mg] in 500 ml distilled water, pH 9.6) with overnight incubation at 4°C. The standard CCHFV-NP controls from D-5052 VektoKrym-KGL-IgG Crimean–Congo hemorrhagic fever virus Kit (Vector-Best, Novosibirsk, Russia) were diluted in coating buffer as per manufacturer's instruction. When testing the rabbit, goat, sheep, cow and rat sera, the concentrations chosen for serum IgG were 10 μg/well (Deelder and Kornelis, 1980; Emmerich *et al.,* 2013; Emmerich *et al.,* 2018; Shaffer *et al.,* 2021).
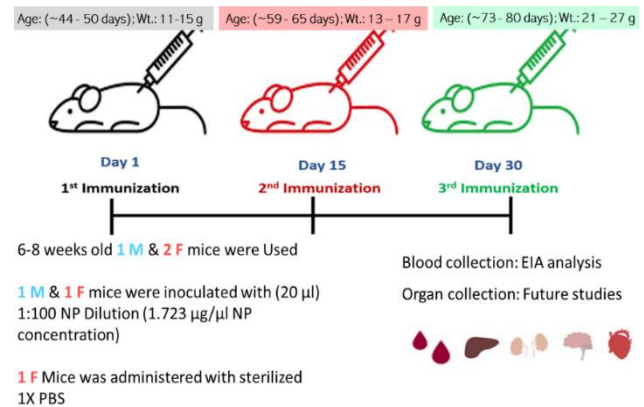
Fig 1: The rationale for mice immunization indicating successional immunizations from day 1 to day 30, followed by serum collection for EIA analysis.

The plates were blocked with 200 μl 5% nonfat dairy milk in PBS with 0.2% Tween 20 (PBST; pH 7.2) for 2 h at room temperature (RT). Wells were aspirated and washed with 1 × 0.2% tween-20 containing PBS. Subsequently, 100 μl of 200-fold diluted anti-CCHFV rabbit sera or 100 μl of 1000-fold diluted anti-CCHFV human sera (positive controls), in 5% nonfat dairy milk was added was added and incubated at 37°C for 1 h. The original diagnoses of the infections in all positive controls were made by the Public Health Laboratories of the Turkish Ministry of Health with by using a reverse transcription-PCR test directed to specific for CCHFV detection (of Kelkit strain challenged rabbit or human) and were further confirmed immunologically by a commercial serological CCHFV-IgG EIA (Vector-Best, Novosibirsk, Russia). The Serum samples as well as FPLC purified NP were serially diluted and tested against CCHFV+ and CCHFV- rabbit serum samples (controls).

The plates were washed 3× with PBST and incubated for 1 h at 37°C with 100 μl of 1/5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (sc-2030; Santa Cruz Biotechnology, Inc.). While for the goat, sheep, cow and rat samples PierceTM Recombinant Protein A/G, Peroxidase Conjugated (Thermo Fisher Scientific Inc.) secondary antibody was used according to manufacturer's prescribed dilutions. The plates were washed 3× with PBST, and 100 μl 3,3′,5,5′-tetramethylbenzidine (TMB; Abcam, Cambridge, UK) substrate was added per well and incubated in the dark at 37°C for 10 min. The Reaction was terminated by addition of 100 μl of 2 N H$_2$SO$_4$ and OD was measured in an iMark microplate reader (Bio-Rad Laboratories, Inc.) at 450 nm. All washing steps were performed using a Well wash Versa microplate washer (Thermo Fisher Scientific, Waltham, USA). The EIA was repeated using the same conditions in three independent experiments.

Sample collection: The targeted sampling sites for animal testing were identified keeping in view the previously unreported areas, the areas where human cases were reported recently but lacking any animal study and some endemic areas previously reported to have CCHFV positive animals. Therefore, Balouchistan province — previously reported a highly endemic region, the districts Gwadar, Quetta and Kalat (highly endemic), while district Awaran (previously non reported district of highly endemic Balouchistan), were selected for goat samples collection. Similarly, Khyber Pakhtunkhwa (KPK), a highly reported endemic region, especially known for very high human disease outbreaks, Peshawer, Mardan and Lakki Marwat districts were chosen for tick ridden goat samples collection. While the province of Sindh and independently administered Azad Jammu and Kashmir (AJK) region lack any animal study, therefore, from Sindh, districts Malir, Shikarpur, Khairpur and Kashmore were selected for goat sample collection. While from AJK, Bagh district was chosen for tick infested goats blood sample collection (Fig 2). In the case of Punjab, many districts were considered (Fig 2). Therefore, the districts, Attock (wild rodents), Jhang, Rawalpindi, Jhelum, Lahore, Bahawalpur, Dera Ghazi Khan, Mianwali, Multan, Fatehjung and Khanewal were chosen for both wild and farm animal samples collection (Fig 2). A total of 1034 animal serum samples included 949 Goats, 44 Sheep, 22 Cows, 4 Water Buffalos and 15 Rats, were collected and for screening by using the developed EIA.

Fig 2: District map of Pakistan indicating the geographical location of sampling areas. Key: Blue pins: Balouchistan, Dark green pins: Khyber Pakhtunkhwa, Light blue pins: Azad Jammu and Kashmir, Red pins: Punjab, Light green pins: Sindh (Adapted and Modified from, https://map.comersis.com.).

The 2 - 5 ml whole blood of the animal was drawn by venipuncture of jugular vein, in the yellow capped clot activating collection tubes and kept undisturbed at room temperature for 30 - 60 min, then stored at 4°C for transportation. The serum was separated by centrifugation (1000 -2000 × g) for 10 min at 2 to 8°C. The separated serum was stored at -80 °C till testing.

Animal serum sample testing: Following the development and optimization of the in-house EIA, pilot-scale testing of 134 domestic and wild rodent specimens was done, followed by screening large-scale animal samples for the presence of CCHFV antibodies. The secondary antibody utilized in the field study EIA was HRP Rabbit Anti-Goat IgG (H+L) (AS029) (ABclonal, Woburn, MA, United States), following the manufacturer's recommended dilutions. All samples that were positive for CCHFV antibodies, when screened with NP antigen, were subsequently cross-verified using a previously established EIA by Kalkan-Yazıcı *et al*., 2021.

Data analysis and graphing: The data obtained from *in-house* EIA in terms of optical density (OD) at 450nm were analyzed using GraphPad Prism Version 6.0 (GraphPad Software, San Diego, CA, USA; URL: https://www.graphpad.com/). The true negative value was calculated by establishing by taking the average of the OD of negative control group (negative rabbit NP and negative rabbit BL21 untransformed) and average of their corresponding standard deviations (SD) and using the following formula (Eyre *et al.,* 2021; Oluka *et al.,* 2023).

$$Cutoff = Mean\ OD + (10 \times SD)$$

Consequently, any value below the threshold cut-off was classified as a true negative, while any value over this cutoff was classified as a true positive.

RESULTS

CCHFV-NP expression and purification: The pDual-GC-CHFV-NP plasmid was transformed into *E. coli* strain (BL21). Successful transformation was confirmed by colony PCR. Large scale CCHFV-NP protein expression was carried out in BL21 and preliminary expression of CCHFV-NP was done using 12% SDS-PAGE. A band size of 62 kDa was observed. The concentration of NP after FPLC purification was found to be 1.723 mg/ml.

After the confirmation of large scale CCHFV-NP expression (Fig 3 a, b), it proceeded for FPLC pu rification. The dialyzed FPLC purified eluted NP was loaded on 12% SDS-PAGE gel for confirmation and again electroblotted for size confirmation (Fig 4). The Western Blot analysis confirmed the size of the NP to be 62 kDa.

Immunization in mice: Two females and one male mouse was immunized using purified CCHFV-NP (Fig 5). One male and one female mouse immunized with NP showed OD at 450 nm as 0.575 and 0.582 respectively, which is notably higher than calculated cut-off of 0.524. These results indicate successful production of CCHFV antibodies in NP immunized mice sera. On the other hand, PBS negative control showed OD of 0.453 which is significantly lower than the cut-off and serum being negative for the CCHFV specific antibodies.

Pilot scale testing of Enzyme Immuno Assay (EIA): The pilot-scale farm and wild animals were tested using 1:1000 NP dilution ($1.723 \times 10^{-3}\ \mu g/\mu l$) as an antigen coating (Table 1) of each well of ELISA plates for EIA diagnostic analysis. There was a steady decrease in OD upon dilution (Fig 6) which is the expected outcome of this ELISA. Therefore, a

total of 23.13% (31/134) animals serum samples (9 cows, 6 sheep, 12 goats and 4 wild rats) were tested positive for the presence of CCHFV antibodies (Fig 7).

(a) Anti -His Antibody

(b) CCHFV Inoculated Mice Sera Antibodies
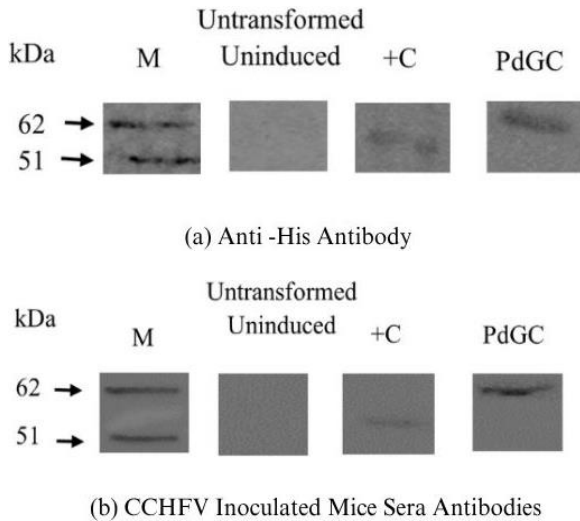
Fig 3: (a) In Western blot analysis after Opti-Protein Marker (10 kDa - 175 kDa), Membrane containing NP probed with anti-his tag antibody, indicating the Band Size of 62 kDa. While NP obtained from Ankara Kelkit Strain Turkey was used as positive control (+C), band size was between 51 and 62 kDa. (b) In this western blot analysis after opti-protein marker (10 kDa -175 kDa), membrane containing np was probed with CCHFV immunized mouse sera antibodies indicating the band size of 62 kDa As Well. And NP obtained from Ankara Kelkit Strain Turkey was used as positive control (+C), band size between 51 to 62 kDa.


CCHFV Inoculated Mouse Sera Antibodies
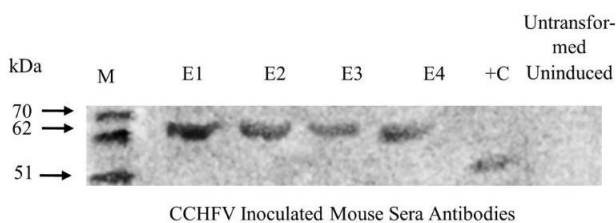
Fig 4: Membrane containing elution fractions E1, E2, E3 and E4 (eluents having purified NP) was probed with "CCHFV inoculated mouse sera containing antibodies against antibodies confirming the band size of 62 kDa. And NP obtained from Ankara Kelkit Strain Turkey was used as positive control (+C), band size between 51 to 62 kDa.

Table 1: The comparison of different dilutions of FPLC purified NP w.r.t. their concentration and OD

FPLC Purified NP Dilutions	NP Concentration Per Well (µg/µl)	OD at 450nm
1:1	0.865	2.7465
1:10	0.1723	2.488
1:100	0.01723	2.431
1:1000	0.001723	0.7875

Animal serum samples testing: Large scale EIA testing was performed on field collected samples from all four provinces as well as independently administered region of Pakistan (Fig 9). The results indicate that 10% 20/200 from Balouchistan; Awaran 4% (2/50), Quetta 16% (8/50), Kalat 4% (2/50) and Gwadar 16% (8/50), goat serum samples tested positive for presence of CCHFV antibodies. While from Khyber Pakhtunkhwa 9.3% (14/150); Peshawar 16% (8/50), Mardan 6% (3/50), Lakki Marwat 6% (3/50) goat samples tested positive for presence of CCHFV antibodies. A total of 4% (8/201) goat serum tested positive for CCHFV antibodies from Sindh — Malir 8% (4/51), Khairpur 8% (4/50), Shikarpur 0% (0/50) and Kashmore

0% (0/50). Whereas 2% (1/50) from Bagh, Kashmir and 8% (24/300) Punjab; Bahawalpur 6% (3/50), Dera Ghazi Khan 6% (3/50), Khanewal 0% (0/50), Mianwali 12% (6/50), Multan 12% (6/50) and Fatehjung 12% (6/50), goat samples were tested positive for presence of CCHFV antibodies (Fig 10 b). Therefore, 67/900 goats collected from all over Pakistan, were positive for CCHFV antibodies during large-scale screening (Fig 9). In conclusion a total of 98/1034 wild and farm animal samples were tested for presence of CCHFV antibodies for the entire current study (Fig 10 b).
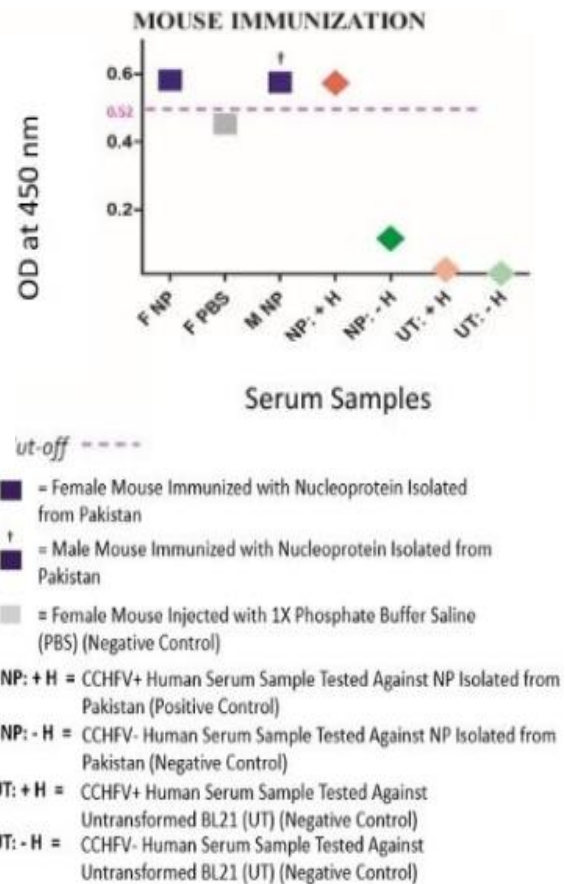

Fig 5: EIA performed on BALB/c mice immunized with NP. Male and female mice immunized with NP isolated from Pakistan showed OD higher than the cut-off, indicating successful CCHFV antibodies production in NP immunized mice sera.

It is pertinent to mention that three samples from Awaran district with the highest (Arn 31; OD = 0.573) moderate (Arn 33; OD = 0.292) and lowest OD (Arn 33; OD = 0.051) (Fig 8 a) when diluted to 1:2000 (of serum) showed a steady fall in OD values with increasing dilution factor (Fig 8 b), indicating the further validating the detection limit of EIA.

Cross verification of positive samples: The serum samples tested positive for the presence of CCHFV antibodies from *in-house* EIA were further cross verified through another already established and previously tested (Kalkan-Yazıcı *et al*., 2021) Enzyme Immune Assay (Fig 10 a). All positive samples (as tested by in house EIA were found to be positive (average OD 0.660 vs 0.667 respectively) (Fig 10 a), further confirming the reliability of the assay.
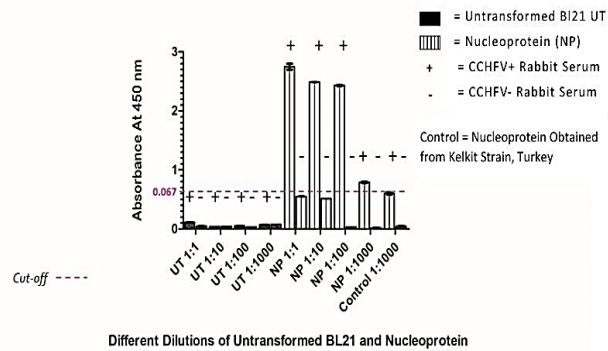
Fig 6: EIA analysis for optimization of FPLC purified (NP) obtained from Pakistan. Comparison was set-up between different dilutions of untransformed BL21 vs different dilutions of nucleoprotein (NP). NP obtained from Ankara Kelkit Strain Turkey (NPt) was used as a positive control which was obtained from CCHFV immunized rabbit with Kelkit strain of CCHFV. Different dilutions of BL21 also served as negative control.
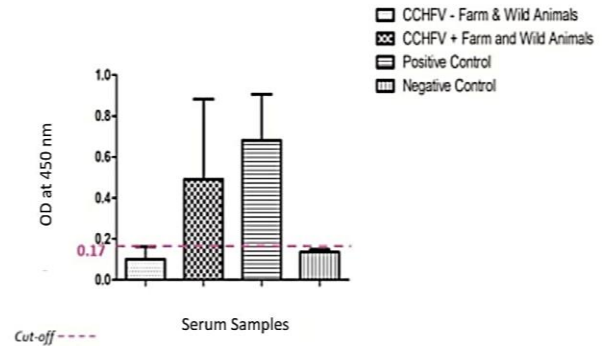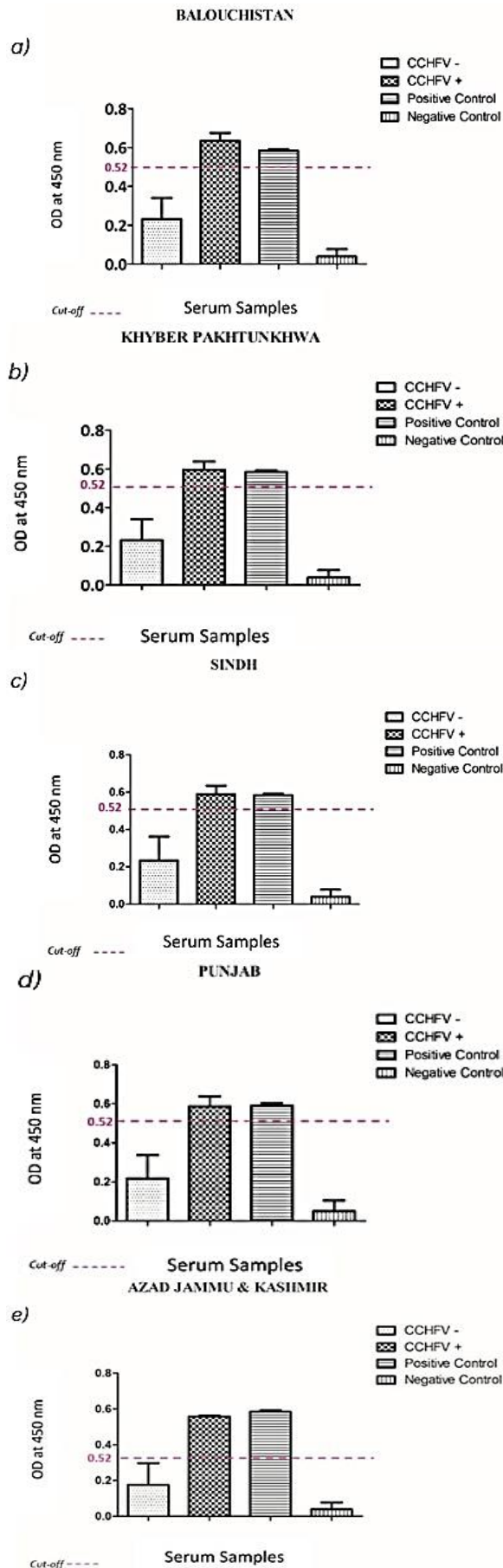

Fig 7: EIA analysis for pilot small-scale animal samples collected from Punjab, indicating OD of positive samples and controls above the cut-off while OD of negative samples and controls are lying below the cut-off.
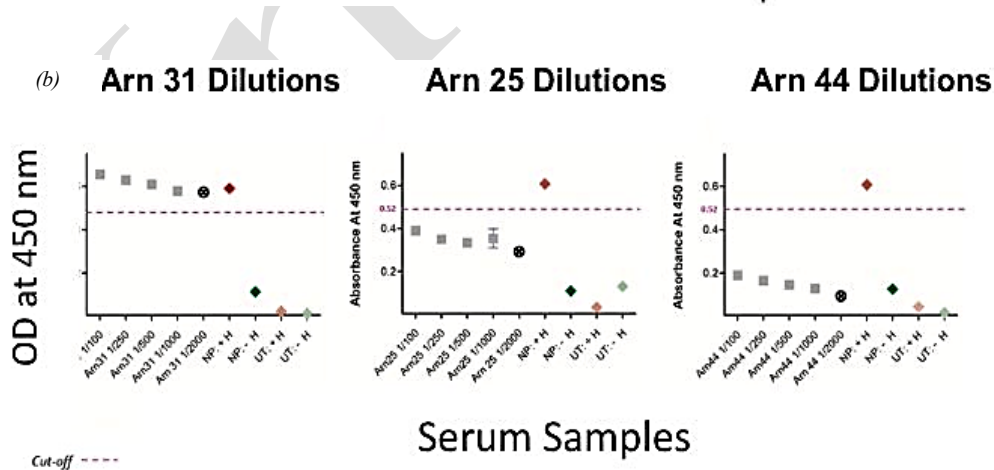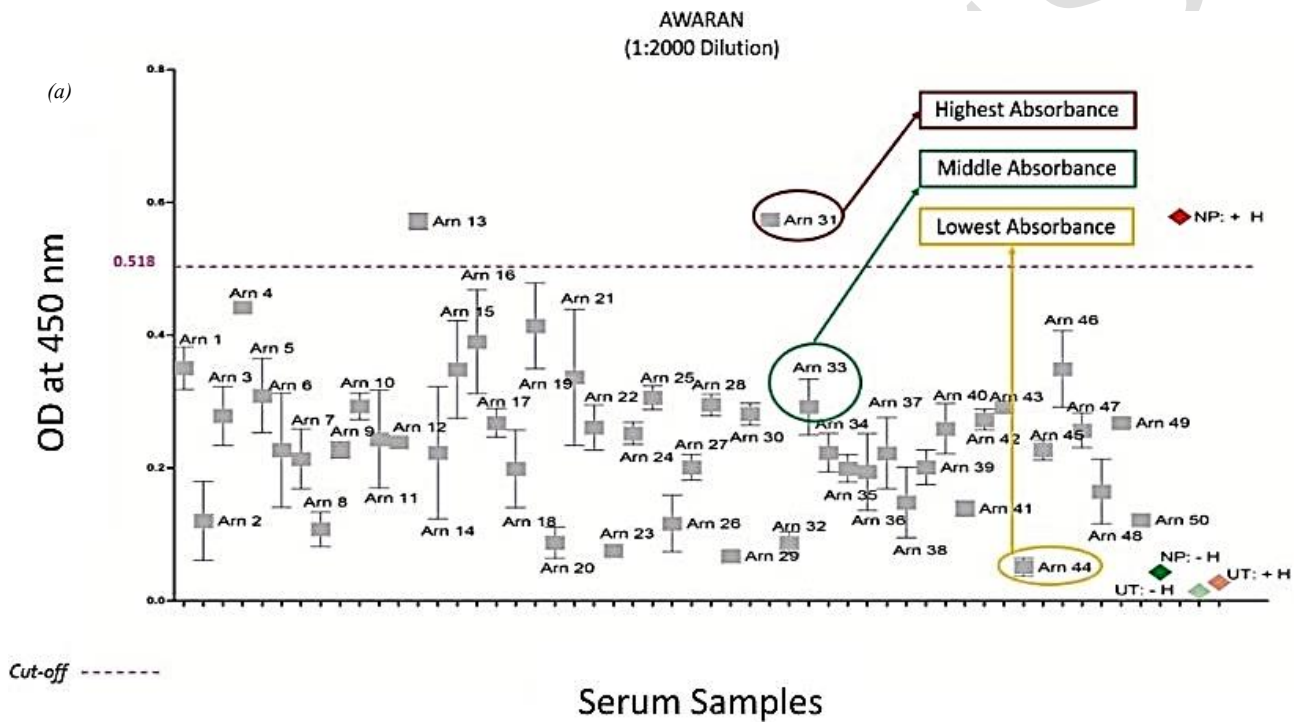

Fig 8: (a) Additional optimization EIA analysis performed on samples collected from Awaran before implementation on large-scale animal screening. (b) Dilution Analysis on Awaran serum samples that showed highest, moderate and lowest Optical Density (OD) for detecting the serum dilution limit for the EIA.
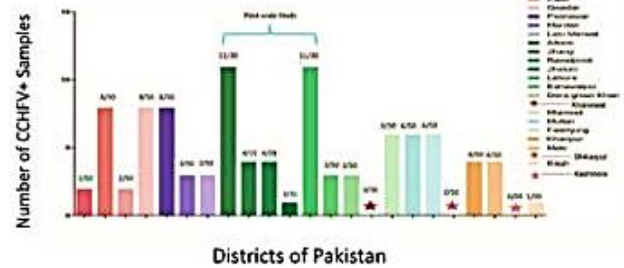
Fig 9: (a, b, c, d, e) The *in-house* EIA experiments used for screening animal samples collected from all the provinces of Pakistan. The OD of positive samples and controls above the cut-off while OD of negative samples and controls are lying below the cut-off.
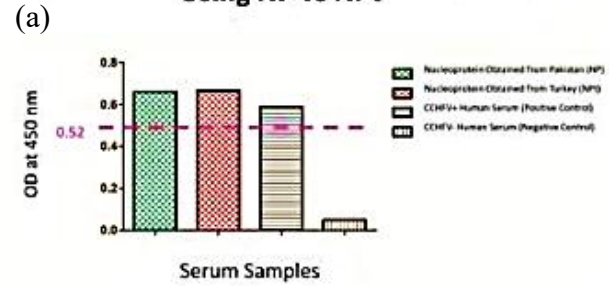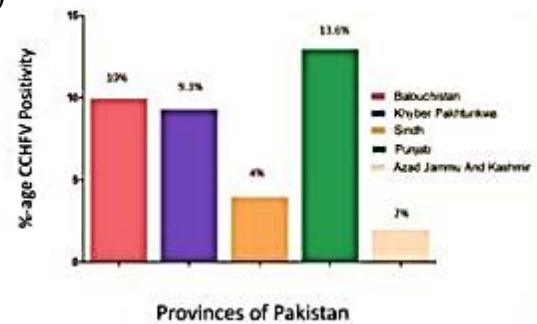

Fig 10: (a) Comparison of *in-house* EIA performed for all CHFV+ samples screened using the sequence of CCHFV Nucleoprotein (NP) isolated from Pakistan as antigen vs *in-house* EIA performed for CCHFV+ samples using nucleoprotein (NPt) obtained from Turkey as antigen. (b) District wise distribution of CCHFV+ serum samples after *in-house* eia analysis. (c) Percentage distribution of CCHFV+ asymptomatic animal serum samples in each province of Pakistan.

DISCUSSION

Livestock, a cornerstone for Pakistan's economy by significantly contributing to agricultural GDP (61.9%) value addition, food security, rural income (8 million rural families deriving 35-40% of income via livestock production), foreign exµchange earnings and national GDPs (14.0%) (Rehman *et al.*, 2017). Among other challenges, Disease outbreaks in livestock are of pronounced consequences impacting productivity and livelihoods. Addressing this challenge is crucial for sustainable economic growth and development. Animal and related zoonotic infections especially of asymptomatic nature, such as CCHFV, are critically important to be evaluated regularly. Thus, cost effective, in-house, more specific CCHFV diagnostic tests are critically important for mass animal screening and potential hotspot identification for the prevention of human transmission and fatality. The current study intent was to develop a cost effective, in-house, regional strain specific and reliable

serological testing assay for continuous and regular annual or biannual screening of the animals for identification of possible CCHFV hotspots and surveillance.

CCHFV nucleoprotein was cloned in pDual GC vector for expression in Bl21 *E. coli* followed by its purification by FPLC method. The purified protein had a high yield and was used for development of in-house EIA. Pilot testing was done on serum samples collected from farm as well as wild rodents and the results were cross verified by using already well-established EIA (Kalkan-Yazıcı *et al*., 2021). Thereafter the EIA was applied for screening a large number of samples collected from all over the country specifically targeting the previously untouched districts/regions.

Over the past two decades, multiple CCHFV outbreaks have been reported in Pakistan. But during the last five years CCHF incidents have increased exponentially, and the virus has also spread to previously non-endemic areas (Zohaib *et al*., 2020; Kasi *et al*., 2020; Yamin *et al*., 2022). It is thus imperative to screen as many animals as possible to identify the potential regions where asymptomatic animals can be a potential threat for any future human outbreaks.

In the case of Baluchistan, the present study targets both the "most endemic" Quetta, Kalat, Gwadar as well as previously non-reported region of Awaran for sample collection. In the current study from Awaran 4%, Quetta 16%, Kalat 4% and Gwadar 16% goat serum samples tested positive for presence of CCHFV antibodies (Fig 10 b). Whereas the overall percentage prevalence of CCHFV antibodies for Balouchistan province found to be 10% (Fig 10 b). These findings are in line with previous studies reporting a higher than average prevalence in Balouchistan as compared to the rest of the provinces (Zohaib *et al*., 2020). Another study documented the presence of CCHFV antibodies in 18.6% (149/800) sheep and 4.6% (37/800) goat serum samples (Kasi *et al*., 2020). It is pertinent to note that during successive years, in November 2023, a severe human outbreak was reported in Quetta, Baluchistan, affecting 12 healthcare workers and one causality ("Crimean-Congo Hemorrhagic Fever Infects Health Workers in Pakistan Outbreak," 2023). Indicating that animal screening has a direct impact on forecasting the human infections in hotspots and thus timely public health interventions can save human lives.

In previous studies from the Awaran region of Balouchistan was not reported to be surveilled for CCHFV prevalence in animals (Kasi *et al.,* 2019). Thus, the current study is the first one to report on the prevalence of CCHFV in animals from district Awaran, 4% (2/50) demanding a larger scale animal screening to avoid any potential threat of future human infections.

Similarly for Khyber Pakhtunkhwa, the current study screened animals from Peshawar, Mardan and Lakki Marwat, where 16, 6 and 6% goat serum samples were tested positive for CCHFV antibodies respectively (Fig 10 b). The overall prevalence percentage of CCHFV for Khyber Pakhtunkhwa for this study was found to be 9.3% (Fig 10 c). Zohaib *et al*., 2020 reported a 52.4% (230/439) CCHFV prevalance in animals. While Zia *et al*., 2024, reported a 26.7 % positivity rate of CCHFV for humans and further stipulated that in July 2022, a significant spike (31.6 %) of CCHFV in humans. Despite these alarming reports

of human infections, less or no data was available from stated districts for CCHFV prevalence in animals. Therefore, the current study is the first report of animal screening and points toward the required large scale animal screening in these regions.

Sindh has been least reported for the presence of CCHFV in humans as well as animals and considered as a non-endemic region. However, several ticks' species have been reported from this province, such as Hyalomma (27.5%) followed by Rhipicephalus (24.3%) and Boophilus (22.8%). The Amblyomma presence was also noticed (14.2%) (Shaikh *et al*., 2021). Therefore, in the current study two districts from north (Kashmore and Shikarpur), one district from east (Khairpur) and one district from South (Malir) were included in the study. The overall percentage of animals positive for antibodies against CCHFV in Sind was 4% (Malir 8%, Khairpur 8%, Shikarpur 0%, Kashmore 0%, Shikarpur 0%). (Fig 10 b). However, Zohaib *et al*., 2020 reported 16.2% (64/396) CCHFV positive anima samples from Sindh (Fig 10 c).

Although various ticks' species such as Hyalomma (39.2%), Haemophysalis (27.5%) and Otobius (26.3%) and Rhipicephalus (7%) were previously reported (Sultana *et-al.*, 2023). But no data regarding CCHFV surveillance in animals was reported from AJK. Our study for the first time reported 2% CCHFV antibody prevalence positivity rate in animals tested by EIA, for AJK (Fig 10 b and c).

Punjab is the most populated province of the country and thus current study included 11 districts of Punjab for sampling. In that regard animal samples collected showed the presence of CCHFV antibodies accordingly in districts: Attock 36.6% (11/30), Jhang 26.6% (4/15) (wild rodents), Jhelum 3.2% (1/31), Lahore 36.6% (11/30), Rawalpindi 14.3% (4/28), Bahawalpur 6% (3/50), Dera Ghazi Khan 6% (3/50), Khanewal 0% (0/50), Mianwali 12% (6/50), Multan 12% (6/50) and Fatehjung 12% (6/50) (Fig 10 b). The overall prevalence percentage for Punjab in the current study was found to be 13.6% (59/434) (Fig 10 c). A previous study reported from the Punjab province (Zohaib *et al*., 2020) stated the CCHFV prevalence to be 52.4% (230/439) in domestic animals and 12.1% (265/2183) in ticks.

The comparison between study by Zohaib et al., 2020 vs the current study, presented a significant observation. It was noted that all provinces showd a concordant trend of higher CCHFV prevelance percentage in study by Zohaib in comparison to the current study. This difference can be explained by the fact that Zohaib used more cattle samples while the present study used more goat samples for surveillance.Therefore, indicating the possibility of cattle having higher rates of CCHFV antibodies than goats.

Overall, our Study indicated that animal samples from Punjab (13.6%) have the highest prevalence of CCHFV antibodies compared to Balouchistan, Khyber Pakhtunkhwa, Sindh, Azad Jammu & Kashmir (10, 9.3, 4 and 2% respectively) (Fig 10 c). This high positive rate from Punjab can be attributed to the fact that Punjab is highly urbanized, inhabits largest economic centers as well as farming industry and is the largest province in terms of human population.

Furthermore, during the pilot phase of the current study, the prevalence of CCHFV antibodies in rodents collected from Jhang, Punjab is indicative that wild animals

should also be screened. The presence of CCHFV positive samples from Sindh, Punjab, Azad Jammu and Kashmir is highly noteworthy because the Eastern provinces (Fig 2) were previously less reported for CCHFV.

The use of in-house EIA for identifying the hotspots, have emerged as significant tool in controlling outbreaks of Crimean–Congo hemorrhagic fever (CCHF) in other countries of the region with highest burden of CCHFV including Iran (Chinikar *et al.*, 2008) and Turkey. These assays have proven particularly valuable for enhancing diagnostic capabilities of these countries, thus enabling timely detection of CCHFV-specific antibodies in infected animals crucial for outbreak management. Therefore, the development and application of in-house EIA diagnostic assay proposed in current study for screening asymptomatic animal samples highlights the necessity of establishment of CCHFV monitoring programs for hotspot identification and control of CCHFV in Pakistan. Therefore, this in-house diagnostic assay can be utilized by public health departments of the country for timely and targeted diagnostics.

Furthermore, Pakistan has emerged as a significant player in the export sector in the global livestock market, currently ranking as the sixth largest exporter of livestock. This position is largely attributed to the country's vast livestock population, which is one of the largest in the world Trade Development Authority of Pakistan (Trade Development Authority of Pakistan (TDAP), 2022). Owing to this insight, CCHFV is a great threat to livestock all over Pakistan and its economy as well as food security. the rising concerns regarding increasing CCHFV cases in Pakistan Also demand the efficient diseases surveillance mechanisms and accurate.

Conclusions: The outcome of this study demonstrates the successful development of Enzyme Immuno Assay for detection of CCHFV infection in asymptomatic animal serum samples collected from Pakistan. The Nucleoprotein (NP) can detect antibodies produced in both Turkish and Pakistani CCHFV infected animals. Similarly, this study also revealed that Turkish Nucleoprotein was also successful in screening Pakistani animal serum samples. The presence of CCHFV antibodies in rats is a novel discovery. Therefore, the study needs to be extended to larger number of rat samples as well as other wild animal serum samples to accurately ascertain the CCHFV prevalence in wild animals in Pakistan. The study showed that the CCHFV infection in animals is creeping Eastwards toward the non-endemic region of Pakistan which is an alarming discovery. Furthermore, the study also needs to be extended to a larger animal pool of other farm animals like cows, sheep and buffalo other than goats for comprehensive analysis in the future. Ultimately, this serological diagnostic assay if becomes successful in its efficacy, then it can be enacted into a policy for a compulsory routine testing of animals throughout Pakistan. This will help in minimizing losses incurred to both humans and livestock as well as on meat export of Pakistan due to CCHFV infection.

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