Author’s response to reviews

Title: The First Clinical Case due to AP92 like strain of Crimean-Congo Hemorrhagic Fever Virus and A Field Survey

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Author’s response to reviews: see over
Reviewer: Chris CW Whitehouse
General Comments
The current focus of the manuscript, and as written in the title, is the identification of a “new CCHFV strain” in Turkey. While it is true that the particular strain has not been seen in Turkey previously, it is not a new strain, as suggested by the title.

The title was revised.

The truly interesting and significant data of this report is the identification of a strain so closely related to AP92 (based only on the S segment, however) that is causing clinical disease. The AP92 strain was first identified in Greece in 1980 and had not been associated with clinical disease since. This is the first report of clinical disease being caused by an AP92-like CCHFV strain. This should be the main focus of the manuscript. The fact that this strain is circulating in the Balkanian part of Istanbul, given the close proximity to Greece is interesting, but should be a secondary point. I would suggest a change in the title to reflect this focus: “Case report of Crimean-Congo hemorrhagic fever caused by an AP92-like strain and serosurvey for the disease in Istanbul, Turkey.”

The revisions were done accordingly.

Also, it needs to be made clear that the serological data obtained here is NOT specific for this particular strain of CCHF, but reflects overall CCHF infection/exposure; this should be mentioned in the Discussion. Future work needs to be targeted at culturing the virus and/or obtaining the full-length sequence to fully determine the relatedness to the AP92 strain. Also, overall, the English grammar of the manuscript needs improvement.

Specific Comments

1. Title needs modified to reflect proper focus of the manuscript

The title was revised.

2. Abstract: The “Background” as written is not correct. This is not background, and furthermore, the authors did not attempt to describe the extent of this strain in Turkey, but only regions around Istanbul.

The background was revised as:
“Crimean-Congo Hemorrhagic Fever (CCHF) is a fatal infection, but no clinical case due to AP92 strain was reported. We described the first clinical case due to AP92 like CCHFV.”

3. Methods: “The sera was re-studied for IgM and IgG.” This was a second collection of sera, so it was not “re-studied”

The related sentences in Methods were revised:
“The individuals, who were positive for IgM and/or IgG were prospectively surveyed, and four months later the second sera from these individuals were collected, and were studied for IgM and IgG. The initial sera were re-tested simultaneously with the second samples of sera.”

The sentence was deleted.

5. Methods: “RNA extraction, PCR procedures and construction of phylogenetic tree” should be “RNA extractions, PCR, and phylogenetic analysis”

Revised

6. Not sure why the authors decided to design their own CCHF RT-PCR assay? There are several validated assays in the published literature. This brings into question the performance of this assay as there is no indication that it was fully validated. Also, nested PCR assays are notoriously problematic and prone to contamination. If there are any validation data on this assay, suggest including it.

We intended to detect the AP92 like strain in Turkey. Therefore, we designed 2 separate primer sets targeting specifically AP92 like strains and those circulating in epidemic region. Since we did not have AP92 or like strains previously, there was no risk for cross contamination.

7. “0.25 ul Taq” need to include the Unit concentration.

This was replaced by 1.25 U Taq DNA.

8. “ABI 310 Foster City, USA” need to include the state (Calif.)

revised

9. Results: Useful to include the normal ranges for the laboratory results (these should be the normal ranges specific for the laboratory where the tests were performed)

The normal ranges for the laboratory results were incorporated:

“In his laboratory investigation, alanine aspartate transaminase (AST) was found to be elevated (89 U/L, normal range: 5-42), activated partial thromboplastine time 43.7 second (normal range: 23-35 second), and prothrombin time was 20.1 seconds (normal range: 12-15 seconds).”

10. Was the tick that bit the patient recovered and tested? Was it identified? This would be important information to include if known. Although Hyalomma ticks are the most important vector of CCHF, it should be kept in mind that AP92 strain was originally isolated from Rhipicephalus bursa ticks, thus these species should also be targeted in any future tick surveillance studies as well as Hyalomma ticks.

No, the tick was not brought to the medical center.
11. Conclusions: Not appropriate to refer to an “attack rate” of a virus. More appropriate to refer to an “infection rate”

The rate that we presented was the attack rate. The nominator is the patients, and the denominator is the number of infected individuals, who were IgM positive in the first analysis and became IgG positive in the second run four months later. But, there is no tool yet to discriminate antibodies against different clades CCHFV.

12. Figure 2. Need to indicate significance of the shading in the figure legend. Perhaps it would be more appropriate to shade only the Turkish strains, instead of the entire clade?

Turkish strains were shaded in the figure 2.

Reviewer: Anna Papa
Reviewer's report:
I have a few suggestions: A paragraph with “Material” will be useful. It will include all information about humans and ticks, that means total number, area and time. You can start “A mild CCHF case….”, which is the first sentence of the Methods. Also add the date of the index case. Then, please, write all information concerning humans: the total number of sera tested for the serosurvey, the season and year of sera collection, the region where the survey was performed … (figure 1) and include the two sentences which are in the Methods: “The informed consents ………Helsinki declaration”. In the Material section, please, write the total number of ticks tested (in total 56 ticks) and the region where they were collected. Add the season and the year of tick collection.

All the information demanded by reviewer was described in Methods and results sections. The admission date of the case and the season of the serologic studies were inserted to the Methods.

Discussion
1st paragraph:

Accordingly, and the literatures were inserted.

Accordingly, individuals infected with AP92 with no recognized disease were reported from Greece [13]. This is not scientifically valid, because AP92 was not detected by PCR or isolation or neutralization; therefore, it is not known whether the antibodies found in Greek population
were against AP92 strain or any other. I suggest writing: Accordingly, 4 individuals among 65 tested in the same region where AP92 strain was isolated in Greece, had antibodies against CCHF virus without recalling any symptom resembling CCHF (the exact reference here is: Antoniadis A, Casals J. Serological evidence of human infection with Congo-Crimean hemorrhagic fever virus in Greece. Am J Trop Med Hyg. 1982 Sep;31(5):1066-7.).

Revised, and the literatures were inserted.

In the same paragraph, where is the seroprevalence of 1.1% with a range from 0 to 6.3%, the exact reference is: Antoniadis A, Alexiou-Daniel S, Malisiovas N, Doutsos J, Polyzoni T, Le Duc JW, Peters CJ, Saviolakis G. Seroepidemiological survey for antibodies to arboviruses in Greece. Arch Virol 1990 [Suppl. 1]: 277-285.

Revised, and the literature was inserted.

Figure 1.
For an easiest comparison among the distribution rates in the four regions, my suggestion is to write in the following way: 14/420 (3.33%) instead of Positive 14, Negative 406, etc.

Positive results were given as proportions.

Reviewer: Masayuki Saijo
Reviewer's report:
Major compulsory revisions: The paper entitled as #Emergence of a new Crimean-Congo hemorrhagic fever virus strain in Turkey# describes that Crimean-Congo hemorrhagic fever (CCHF) virus (CCHFV) that was genetically and closely associated with CCHFV AP92 strain that had been isolated in Greece caused CCHF in a patient in Istanbul, Turkey. The PDF version of this paper might be mistranslated from the original form, resulting in difficulty in reading. I cannot understand how does this happen? It is evident that CCHFV genome (KMAG-Hu-07-01) amplified from the index case was phylogenetically associated with AP92 strain. This evidence is interesting. The authors conducted the serological survey among the residents living in the region from where the index case was reported. They demonstrated that approximately 5% of the 741 subjects showed positive reaction in both the IgG and IgM antibody tests. All the sera collected from the 741 subjects with IgM-positive reaction showed a IgG-positive reaction, indicating that all the CCHFV-IgG-antibody positive subjects were infected with this virus very recently and that the residents had never been infected with CCHF before. It is highly possible that CCHFV-IgG antibody induced the cross positive reaction in the IgM antibody test. To exclude this assumption, IgM-capture ELISA assay should be carried out.

Response:
It is not likely to have cross positive reaction between IgG and IgM. IgM capture ELISA is not a necessary method. We have already indicated this issue clearly in the text; “Since the original kit didn’t contain any reagent for hindering false IgM seropositivities due to IgG type antibodies, before IgM detection, IgG was eliminated using a commercial IgG removal kit (Zeus®, USA). The individuals, who were positive for IgM and/or IgG were prospectively surveyed, and four months later the second sera from these individuals were
collected, and were studied for IgM and IgG. The initial sera were re-tested simultaneously with the second group of sera.”

CCHFV genome was not amplified from ticks collected in the region. Therefore, there is no evidence that the IgG and IgM-antibody positive subjects were infected with CCHFV KMAG.

Response:
Tick survey could be a plus to the study, but not a necessary. We did not claim that all the seropositivities were due to KMAG strain.

The conclusion that CCHFV KMAG is less virulent than other CCHFV isolates in Turkey is open question. Further study is needed.

Response:
This conclusion is based on the reported cases from Greece (15,16,17). There is no doubt that, further studies will bring more data on the issue.

The methods for CCHFV IgG and IgM antibody detection should be mentioned. At least, the sensitivity and specificity of the commercial kit used in this study should be described.

Response:
Evaluation of the ELISA kits was out of the scope of this manuscript. In this study, we did not develop a new ELISA kit, we used existing commercial kit.

Minor comments
1. Title: The term #emergence# in the title is misleading.
2. Page 2: The first sentence in the Background section: CCHF is a fatal viral infection reported in Turkey five years ago# is wrong. CCHF has already been reported before the identification of CCHF in Turkey. Define the year when CCHF was first identified.

The sentence was revised:
“The first Crimean-Congo hemorrhagic fever (CCHF) case in Turkey was reported five years ago”

3. Page 3, the last sentence in the Background section: Cite the paper for this sentence.

Reference was inserted.

4. Page 3, Methods section: How was the index case diagnosed as having #mild# CCHF?

A reference was already cited (by Ergonul) for the severity criteria of the cases.

5. Page 6, Discussion section: Is the rate 17.43 % (200/218 nt) correct?

The sentence was corrected as:
The nucleotide sequence divergency between KMAG strain and AP92 was 8.63% (38/440).
6. Page 7, Discussion section: As mentioned in the general comments section, the IgG and IgM positives could not be confirmed as having infection with CCHFV KMAG. Therefore, this reviewer considers that the attack rate cannot be calculated.

The attack rate was given for overall CCHF infection, not specific for CCHFV KMAG.

7. Statistical analysis: There are not enough data to assess the acceptability for statistical analyses.

This comment is wrong. There is data to do statistical analyses as we showed in Table 1 and 2.

Reviewer: Christian Drosten
Reviewers report:
This important article presents highly relevant facts that may change our understanding of CCHFV ecology and epidemiology. However, because of their importance these data deserve a better methodological foundation (see point 5). If for any reason authors cannot provide extra testing in due time, they might want to report these important data in a more preliminary format.

Major compulsory points:
1. Authors should be more careful about claims regarding circulation and traits of the AP92-like virus. They have no identification of virus from vectors and reservoir, and they have no exact idea regarding time of exposure and reliability of reporting in people. Definition of infected persons only relies on an IgM result in a single EIA test that has not been in use in many studies.

Response:
No. Definition of infection does not only rely on IgM result in a single EIA test. All individuals were re-tested four months later for both IgG and IgM, simultaneously with the initial serum samples. By this study, IgM positive individuals except one were found to be seroconverted to IgG four months later. This is a strong evidence for the recent infection.

In this light, sentences like "This difference can be explained by different virulences among the circulating strains" (Page 8) sound overstated. If authors want to raise hypotheses, these should be clearly identified as such, and developed a bit further (see below).

Response:
As indicated, further studies are necessary for this issue. In this context, our study is the first and a significant step.

2. The Abstract suggests that antibody testing was done specifically against the new AP92-like strain, which is almost certainly not the case. Please identify the test antigen in the EIA test. Authors should also keep in mind that there are no differential CCHFV serotypes, and should avoid any indirect implications into this direction unless they have proof of it.

Response:
The antigen used in the EIA kit was not given in the package insert of the kit.
3. Has virus isolation from patient blood been attempted? It would be a very high priority to have an isolate of an AP92-like strain (pathogenicity modelling / vaccine template). This should be discussed.

Response:
No, because of lack of BSL-4 facility in the country. Yes, we agree with the comment, but this issue should be elaborated in further studies.

4. Page 5 "Since the original kit didn’t contain any reagent for hindering false IgM erospositivities due to IgG type antibodies, before IgM detection, IgG was eliminated using a commercial IgG removal kit (Zeus®, USA)." - To my knowledge IgG is removed from plasma before IgM detection in order to prevent shielding of antigens by IgG if both isotypes are co-existing. To exclude the effect of rheumatoid factor, authors should conduct a rheumatoid factor test in all patients positive for IgM. Positives should not be counted as anti-CCHFV IgM positives.

Response:
The explanation in methods section was revised according to the instruction of the kit as: “A commercial variant capture ELISA kit (vectorbest®, Russia) was used for detection of IgM antibodies of CCHFV.”

5. All antibody-positive sera (or ideally, all sera) should be re-tested by a second EIA test, or by IFA.

This is ideal for more robust results, but there is no alternative commercial EIA kits. In such epidemiologic studies, usually second way of control could be, a second test such as IFA, or a control group like healthy individuals in urban part, or two step testing of the same population (re-test) as we did in our study. Under these circumstances, we preferred to perform retesting.

6. Figure 2: This seems to be an unrooted cladogram. The phylogeny should be rooted with Dugbe virus, which is a well acceptable outgroup.

The cladogram is rooted and AP92 strain was assigned as out group.

Minor essential points:

7. Page 6, "Thirty four out of 35 (97.4%) IgG positive patients were also IgM positive." – Please state how many patients had IgM positive / IgG negative status in total (I calculate five).

There are four patients, who are IgM positive but IgG negative, as it was indicated in the first paragraph of the “survey in the region” section.

8. Page 6, "All the IgM positivities except one, converted to IgG positivity." – Does this mean that all patients who had only IgM / no IgG in the first test seroconverted to IgG positive?

Yes. This is a strong point for the study, which shows a recent infection.
9. Page 7: In the population survey, have people been asked for tick morphology and have they been shown Boophilus / Rhipicephalus / Hyalomma ticks as examples? Can any conclusions been made in this direction?

**No. People were asked for the history of tick bite.**