Author's response to reviews

Title: Duration of viral shedding in hospitalized patients infected with pandemic H1N1

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Author's response to reviews: see over
EDITORIAL REQUESTS (ROXANE RAJABI):

- Further consideration of your manuscript is conditional on improvement of the English used. Please ensure particular attention is paid to the abstract.

ANSWER:
The manuscript has been modified to improve the English used, paying particular attention to the abstract, that has been completely rewritten.

- Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/index.html), and any experimental research on animals must follow internationally recognized guidelines. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.

ANSWER:
We realized that the description of the study design was misleading, suggesting that specific laboratory investigation had been performed for the study aim. In fact, this was an observational study, as data analysis was carried out retrospectively, and no specific sampling was performed for the study purpose. In fact, all the clinical samples were collected and tested for diagnostic purpose, during the hospitalization of patients at National Institute for Infectious Diseases “L. Spallanzani”, according to the current clinical practice and to the local policy adopted in respect of the A/H1N1 pandemic. All the data were retrieved from the clinical and laboratory archives. Only for the identification of resistance mutations, the analyses was performed ad hoc if not requested by the clinicians, using residual diagnostic samples. Moreover, the therapy administered to the each subject was decided by the physicians in charge of the clinical management of patients, and was completely independent from the study. According to the current Italian legislation (Gazzetta Ufficiale della Repubblica Italiana serie generale 76, 31-03-2008), no ethics committee approval is required under these circumstances. Some clarifying statements have been introduced in the final part of the introduction (Page 6 Lines 6-10), and in the methods section (Page 6, lines 25 onwards).

- In addition, please can you clarify if the "L. Spallanzani" at the end of the background section of the abstract is part of the hospital's name? If not, please can you remove it from your manuscript.

ANSWER:
"L. Spallanzani" is part of the Hospital name. The statement has been slightly modified.
Referee 1 Comments (Wilina Lim):

- Of the 39 selected patients with serial samples, 23 were classified as SARI and 16 ILI. Detailed breakdown of demographic variables and clinical presentation including days after onset of symptoms and timing of the 175 samples collected from these patients in the study would provide clearer picture and make interpretation of results easier.

ANSWER:
As requested also by the reviewer n. 2, the 39 analysed patients were reclassified according to presence or absence of pneumonia. Detailed breakdown of demographic variables and clinical presentation, including days after onset of symptoms of two groups of patients are shown in Table 1, not considering SARI vs. ILI, but considering pneumonia vs. non-pneumonia, according to the new patient categorization. In addition, the breakdown of the samples collected from these patients has been added to Figure 2 legend. Incidentally, 164 results were available out of the 175 samples collected. This further detail has been added (Page 10, lines22-23).

- There seems some confusion in the classification of patients: SARI implies ILI with breathlessness or difficulty in breathing requiring hospital admission, yet the authors described only 52.2% of their SARI patients presented with dyspnoea. (P9 and Table 1).

ANSWER:
We agree. However the 39 patients have been reclassified according to presence or absence of pneumonia. Consequently, Table 1 has been deleted, and the underlying diseases and the other relevant information have been included in the previous Table 2, now Table 1.

- The authors stated the time course of viral load in NPS from ILI and SARI patients was similar while the proportion of PCR positive NPS was significantly higher in SARI compared to ILI patients. However, in Figure 2B, the proportion of PCR positive patients for ILI cases at 8-9 and >10 days were 21.4% and 0%, similar to the proportion of SARI without pneumonia. (Figure 2D: 25% and 0% at 8-9 and 10 days respectively). Different pattern of viral loads with days after symptoms onset for SARI with and without pneumonia was also evident in Figure 2C. It appeared pneumonia is the deciding factor that caused different viral shedding pattern. The authors in fact stated in p11 that prolonged shedding was correlated with the presence of pneumonia. The authors need to re-group the cases according to clinical presentation, re-organize and re-analyse the data taking this into consideration.

ANSWER:
The reviewer is correct in pointing out that pneumonia appears as the deciding factor that caused different viral shedding pattern. Following the suggestion, the patients have been regrouped and the analysis performed de novo; the results and discussion sections, as well as tables and figures, have been modified accordingly.

- Twenty –nine of the 39 patients received anti-viral treatment. Overwhelmingly they were patients with SARI: 21 SARI and 8 ILI cases. The authors stated that oseltamivir was initiated at varying intervals after onset of symptoms and given in different dosages and duration. It was stated that in untreated patients, “the viral load steadily increased from baseline up to 4 – 5 days after symptom onset, then declined” This is not consistent with authors’ statement that viral load correlated negatively with time since onset of symptoms
(p8 and Fig 1) and also what was shown in Figure 2A. Further stratification of the treated and untreated group into ILI, SARI without pneumonia and SARI with pneumonia may help to clarify the picture.

ANSWER:
The data from Figure 1 show that the highest initial values of A/H1N1pdm RNA concentration were observed in patients presenting for influenza diagnosis on day 2 from start of symptoms, and the lowest on day ≥9. These data are cross-sectional, therefore are not directly comparable to those shown in Figure 2, where the patients were serially sampled, and the time course during follow up is shown. Therefore we think that the contrast is only apparent. Further stratification of Figure 2 patients according to the new classification (pneumonia vs. non-pneumonia) is not possible, as only one patient with pneumonia was untreated. However, we agree that Figure 1 was not properly described. Therefore we modified the axis X title in the Figure 1 and modified the relevant statements, to better clarify the issue (Page 13, lines 9-10 and 18-22).

- Quality of written English: Needs some language corrections before being published.

ANSWER:
The written English was amended along the paper.

Referee 2 Comments (Edward Goldstein):

- As the authors report in the Abstract, “The mean values of viral RNA concentration along the observation period were not significantly different in ILI and SARI patients. However, patients with SARI showed a significantly longer time of viral shedding as compared to patients with ILI”. This point is elaborated upon in the Results section and Figure 2B - viral shedding is defined as time through which viral loads are detectable by PCR; starting days 6-7 since symptom onset, when viral levels are low in both groups, viral levels in the ILI group become particularly low for accurate PCR detection. Thus duration of shedding is to a large extent the artifact of the accuracy of a PCR test, and some reference to that should be made in the Abstract.

ANSWER:
The reviewer is correct in pointing out that PCR accuracy may be low close to the detection limit, so the differences in the duration of viral shedding may be the result of an artifact, if one of the two groups displays particularly low viral levels. However, after regrouping the patients according to pneumonia vs. non-pneumonia, viral levels (Figure 2A) are very similar in the 2 groups, so the accuracy of the PCR test presumably impacted on both groups at the same level. Therefore it is unlikely that the differences in the duration of shedding (Figure 2B) could be now attributed to PCR accuracy artifacts.

- As Figure 2C suggests, SARI patients with pneumonia had different patterns of viral shedding compared to SARI patients without pneumonia, with the latter exhibiting viral shedding patterns similar to patients with ILI (Figure 2A), at least in terms of the sharp drop between days 4-5 and 6-7 in viral load and PCR detectability (Figure 2D). Is the dichotomy for SARI vs. ILI justified in reporting the differences in viral shedding? Perhaps pneumonia vs. non pneumonia cases is a more representative description.

ANSWER:
OK, the suggestion has been received, and the data have been newly analysed accordingly.

- Can any comparison be made between viral shedding of non-hospitalized patients (Figure 1) and different categories of hospitalized patients?

ANSWER:

Figure 1 represents a cross-sectional analysis of viral load values measured in patients referred for Laboratory diagnosis at first presentation, by either the INMI admission department or by other regional hospital, so it does not represent the pattern of viral shedding in serially sampled patients, as it is the case of patients hospitalized at INMI described in the subsequent part of the paper.

For most of the patients described in Figure 1 clinical data are not available, so it is not possible to make any stratification. In addition, the criterion for hospitalization of patients changed along the study period, in accordance with the national policy for pandemic influenza management, therefore hospitalization could not be taken as a marker of enhanced severity.

To address the reviewer point, we compared the viral loads of patients presenting for diagnosis before and after August 2009, that is the time when the national policy for influenza management changed. In fact, before this time the samples analysed in the laboratory were mostly for patients with mild disease, as the laboratory diagnosis was mandatory for all patients with ILI, while from August onward the samples were mostly from severely ill patients, eventually needing hospitalization, for whom the laboratory diagnosis was required. The results indicate no significant differences between the two groups in the viral load values at diagnosis (5.37±1.20 before, vs. 5.23±1.26 after August 2009, p=0.220), suggesting that clinical presentation did not affect viral load values at first clinical observation, at variance with what has been reported (Li et al., EID 2010).

In addition we included the mean viral load values observed at diagnosis in the hospitalized patients with serial samples available, who were further analysed in the subsequent part of the study and categorized according to presence or absence of pneumonia. These values were not significantly different in the two groups (5.49±1.35 in patients without pneumonia vs. 5.01±1.66 in patients with pneumonia, p=0.360), again supporting the concept that, at first sampling, the viral load in the NPS was not influenced by the severity of clinical presentation.

These results have been included in the results and in the discussion (Page 9, lines 5-12; Page 10, lines 17-19; Page 13, lines 18-22).

- Did all SARI patients with pneumonia had pneumonia upon admission to the hospital? Was there any relation between progression to pneumonia and antivirals?

ANSWER:

Most patients with pneumonia (9/11) already showed this complication at presentation. Only two patients developed pneumonia after the hospitalization, despite early antiviral treatment: one of them resulted positive to *Haemophilus influenzae*, the other resulted positive to *M. Tuberculosis*. A statement has been added (Page 0, lines 20-23).

Referee 3 Comments (Michael Ison):

- Careful review and revision by a native English speaker. Not suitable for publication unless extensively edited.

ANSWER:
The manuscript has been modified to improve the English used.

**Presentation of some details as to why serial samples were collected in the individuals presented; it is possible that there is some bias (i.e. patients that did worse or had more protracted courses than non-included patients).**

**ANSWER:**
For the hospitalized patients, the local policy for pandemic influenza management included the assessment of viral shedding during the hospitalization period, in order to tailor the individual isolation measures, and to monitor the efficacy of treatment in patients receiving antivirals. A statement has been included in the methods section (Page 6, lines 25 onwards).

We agree that there is the possibility of some bias, due to the fact that patients with more protracted disease course were sampled for longer times. This possibility has been mentioned in the discussion (Page 14, lines 7-9).

- Present some details about other co-factors that could explain prolonged viral shedding: steroid use, antibiotic use, immune suppression as an underlying condition, underlying lung disease, etc. Many additional analyses (i.e. time to initiation of treatment, specific treatment, dose, etc) would provide significant insight and improve the quality of the paper.

**ANSWER:**
- The requested analyses have been performed, and the new data included in Tables 1 and 2 (previous Tables 2 and 3), and in the results section (Page 12, lines 3-5 and 8-12).

- Several groups have documented that viral shedding is shorter and not always representative of lower airway shedding - this is important since a large number of patients had obvious pneumonia - how was this assessed/controlled for? Were there BAL specimens available?

**ANSWER:**
Due to the observational character of the study, the requested comparison between upper and lower respiratory tract samples could not be performed. In fact, sampling of BAL was performed on the basis of clinician’s judgement, so viral shedding in the lower respiratory tract was not regularly assessed. In fact, serial BAL samples were analysed only for the patient with leukaemia, who showed severe pneumonia and received enhanced antiviral treatment. In this patient the BAL returned negative earlier than NPS, but this observation could not be correctly interpreted, as it is anecdotic. A statement has been included in the discussion concerning this point (Page 16, lines 6-11).

- Clarify the discussion - what does this study add to the existing data? In my opinion the resistance piece is particularly important and novel.

**ANSWER:**
The discussion has been modified for better emphasizing the novel piece of information added to the existing data. In particular, the resistance issue has been more extensively addressed. Particularly, the immunosuppression status of our patients has been reported (Page 12, lines 18-19) and a number of comments have been added (Page 14, lines 22 onwards, Page 15, lines 6-12, Page 16, lines 21-23).