In situ Molecular Identification of the Influenza A (H1N1) 2009 Neuraminidase in patients with severe and fatal infections during a pandemic in Mexico City

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Abstract

Background. In April 2009, public health surveillance detected and increased number of influenza-like illness in Mexico City's hospitals. The etiological agent was subsequently determined to be a spread worldwide novel influenza A (H1N1) triple reassortant. **Objective.** The purpose of the present study was to demonstrate that molecular detection of pandemic influenza A (H1N1) 2009 strains is possible in archival material such as paraffin-embedded lung samples. **Methods** In order to detect A (H1N1) virus sequences retrospectively, eight paraffin-embedded lung biopsies were collected from patients who died of pneumonia and respiratory failure being tested for influenza A (H1N1) Neuraminidase (NA) mRNA using *in situ* RT-PCR. **Results.** The expression pattern of the NA viral gene in lung biopsies obtained from patients with pneumonia was determined by *in situ* RT-PCR. We detected NA transcripts in 100% of the previously diagnosed A (H1N1)-positive samples as a cytoplasmic signal. No expression was detected by *in situ* RT-PCR in both previously diagnosed AH1N1-negative patients using standard protocols and in non-related cells. *In situ* relative transcription levels correlated with those obtained when *in vitro* RT-PCR assays were performed. Partial sequences of NA gene from AH1N1-positive patients were obtained by the *in situ* RT-PCR-sequencing method. Sequence analysis showed 98% similarity with influenza viruses reported previously in other places. C**onclusions** We have successfully amplified specific A (H1N1) NA sequence by *in situ* RT-PCR using stored clinical material**.**. These results **s**uggest that this strategy could be useful when clinical RNA samples are quantity limited, or when poor quality is obtained. In addition, amplification signal provided evidence of the cellular type that is susceptible to A (H1N1) viral infection. Here, we provide a baseline of retrospective data of the first influenza A (H1N1) outbreak in Mexico City.

Background

During April 2009, the number of atypical pneumonia cases increased in Mexico City's hospitals and spread to almost all boroughs in the city; at the same time, a new influenza A (H1N1) virus strain was identified as the etiological agent [1-4]. In less than a month the virus had spread worldwide. On June 11, 2009 the World Health Organization (WHO) declared the start of the first 21st century influenza pandemic.

Influenza A viruses belong to the *Orthomyxoviridae* family; they are characterized by a unique genome structure, the RNA viruses are segmented, single-negative stranded, which codifies, between others, two transmembrane proteins: hemagglutinin (HA) and neuraminidase (NA) [5-7]. HA plays an important role during the cell entry of influenza viruses. This protein is essential during the initial steps of infection because it is responsible for attachment of the virus to sialic acid (SA), the cellular receptor. This interaction explains, at least in part, the host range and tissue tropism of influenza viruses [5, 8]. The NA of influenza viruses is a homotetrameric glycoprotein anchored by a fibrous stalk in the viral membrane. The protein possesses a globular head comprised of four monomers that constitute the active site composed of nine conserved residues. Its primary role in the infectious cycle is to liberate the viral progeny from infected cells. Its enzymatic activity catalyzes SA removal from its linkage to galactose, thereby destroying the receptor and allowing the virus to disseminate and infect other cells [8, 9]. Furthermore, this protein is also the main target of the antiviral drugs zanamivir and oseltamivir. These drugs closely resemble the structure of the natural substrate of the neuraminidase and thus prevent the removal of the SA residue from the glycopeptide receptor by the viral neuraminidase [5]. In addition to the increased transmissibility and the lack of immunity of the human population, the fact that new

reassortment events may alter the pathogenicity of circulating strains, makes it crucial to monitor the progress of the pandemics at the molecular level [2, 10, 11].

Molecular methods are becoming more widely used for the detection of respiratory pathogens, in part because of their superior sensitivity, relatively rapid turnaround time, and ability to identify pathogens that are slow growing or difficult to culture. The recent novel H1N1 influenza A pandemic served to underscore how quickly new molecular tests can become available for clinical use [12]. Over the years, PCR has been the dominant amplification method. Recently, modifications of this technology have emerged, some of which allow for the rapid detection of multiple pathogens in a single test such as multiplex molecular technologies, reverse transcriptase-PCR, real-time PCR, microarrays and nucleic acid sequencing-based amplifications [12-14]. Other studies have also shown the usefulness of rapid immunoassays for seasonal influenza virus [15]. These methods have greatly enhanced the capability for surveillance and characterization of influenza viruses and their clinical utility for the detection of respiratory pathogens. However, these methods can not be easily applied for the analysis of paraffin-embedded tissues. *In situ* RT-PCR has some major strengths for the detection of nucleic acid sequences. First and foremost, one can detect specific sequences on archival material; second, this technique combines the extreme sensitivity of PCR with the cell localizing ability similar to *in situ* hybridization [16]. A third strength of *in situ* RT-PCR relates to the issue of sample contamination in solution-phase PCR. Sample contamination, which can lead to false-positive results in PCR and has limited its value as a diagnostic test for viral infections, is not encountered in *in situ* RT-PCR. Fourth, this technique is the only amplification technique that allows target-specific incorporation of a reporter nucleotide (such as DIG-digoxigenin-dUTP-labeled nucleotide) [17, 18, this work], thus eliminating the need for a hybridization step.

Clearly, *in itu* RT-PCR technique has been useful for any target that is low copy and, thus, difficult to detect with standard *in situ* hybridization, which has a detection threshold of 10 to 20 copies per cell [16].

The purpose of the present study was to demonstrate that molecular detection of pandemic influenza A (H1N1) 2009 strains is possible in archival material such as paraffin-embedded lung samples. This strategy would be useful to perform retrospective studies in a specific and reproducible manner.

Materials and methods

Biopsies

The project was approved after being checked for the Science and Bioethics Committee. From April 2009 to February 2010, 8 lung biopsies were taken from patients who died of pneumonia and respiratory failure at Instituto Nacional de Enfermedades Respiratorias-Ismael Cosío Villegas (INER) and Clinica-32 of Instituto Mexicano del Seguro Social (IMSS), Mexico City. Samples were paraffin-embedded and sectioned to obtain 5 μm thick sections mounted on electrostatically charged slides.

Molecular detection of A (H1N1) viruses

The detection of pandemic influenza A (H1N1) 2009 viruses was done using RT-PCR standard protocols as a part of the Virology Department's surveillance routine and this detection method has been described elsewhere [http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html; 2, **19**]. All specimens were accompanied by a Standard Form within formation on age, sex, date of illness onset, date of specimen collection, place of residence, clinical features of each patient, travel history, vaccination history and administration of antiviral treatment.

Primers

Pre-validated Neuraminidase specific primer sequences were selected according to the need of the *in situ* technique: NA/AH1N1F (sense) 5'-ACCATTGGTTCGGTCTGTATG-3' and NA/AH1N1R (antisense): 5'-GAGGCCTGTCCATTACTTGGPU-3'. As internal constitutive expression control, β2-microglobulin (β2-m) transcripts were detected using the following primers: β2mF (sense): 5'-ACCCCCACTGAAAAAGATGAGTAT-3' and β2mR (antisense): 5´-

ATGATGCTGCTTACATGTCTCGAT-3´. *In situ* detection was performed as previously described [20; see above]. All primers were purchased from Invitrogen (U.S.A.).

***In situ* RT-PCR**

Direct *in situ* RT-PCR was performed as previously described with some modifications [17, 18, 20-22] (Fig. 1). Briefly, dried dewaxed sections on electrostatically charged slides were incubated with protein lysis buffer (0.1 M Tris-HCl pH 8.0, 50 mM EDTA pH 8.0) containing 0.5 μg/ml Proteinase K for 30 min at room temperature. After Proteinase K digestion, tissues were treated with 50 μl of a solution containing 1 U/ sample of DNase I, RNase-free (Roche, U.S.A.) during 48 h at room temperature. After thoroughly washing with DEPC-treated water, reverse transcription was performed using the SuperScript II reverse transcriptase (Invitrogen, U.S.A.), following the manufacturer's specifications. In brief, 70 μl DEPC-treated water containing 3 μg of random primers, 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), 5X first-strand buffer, 10 mM DTT, recombinant ribonuclease inhibitor (40 U/μl) and reverse transcriptase (100 U/section) (Invitrogen, U.S.A.) were added to each section. Slides were incubated at 42 °C for 1 h in a sealed humidified chamber. After thoroughly washing with ultrapure water, 50 μl of the PCR master mix solution containing 100mM digoxigenin-11-(2'-deoxy-uridine-5')-triphosphate (DIG-11-dUTP; Roche, U.S.A.), 10X PCR buffer, 50 mM $MgCl_2$ and primer mix (10μM each) were added [21]. To reduce primer-dimer formation the PCR solution was heated to 70 °C for 10 min before Taq DNA polymerase (5 U per reaction) was added. Negative controls were made without primers or Taq. *In situ* PCR was performed using the system provided by Perkin Elmer (U.S.A.). The slides were preheated to 70 °C on the assembly

tool included in the *in situ* Perkin Elmer equipment, 50 μl of PCR master mix were added to each sample and the reaction was sealed using AmpliCover discs and clips (Perkin Elmer, U.S.A.). After assembly, slides were placed at 70°C in the GeneAmp In situ PCR system 1000 (Perkin Elmer, U.S.A.) until running was started. PCR amplification was performed running 18 cycles. After cycling was complete, the temperature was kept at 4 °C until disassembly. Clips were removed and AmpliCover discs were very carefully lifted from the slides without moving them sideways and slides were washed for 5 min in PBS followed by 5 min in 100% EtOH before they were air dried [20]. Controls included substituting one of the primers by H_2O in the PCR reaction or omission of the reverse transcription reaction. For either NA or β2-m amplification, samples were first heated to 94°C (3 min) and then subjected to 18 cycles of: 94°C/ 1 min, 60°C/ 1.5 min, 72°C/ 1min. After amplification a 10-min elongation step at 72ºC was carried out. To ensure consistency and reproducibility and to eliminate PCR artifacts, all assays were performed on a minimum of three separate occasions. Slides were processed in order to detect *in situ* PCR products (see below).

Detection of *in situ* PCR products

An indirect immunolabeling method using a primary Anti-Digoxigenin antibody (Fab fragments; Roche, U.S.A.) conjugated to alkaline phosphatase was chosen to detect the PCR product. Briefly, blocking was carried out in 5% BSA (Sigma, U.S.A.) in PBS for 30 min. Slides were then drained and an Anti-DIG antibody diluted 1:200 in 100 mM Tris-HCl pH 7.4 and 150 mM NaCl, was applied (100 μl per sample) for 2 h at room temperature. As a negative control the primary antibody was omitted. Detection of alkaline phosphatase was carried out for 10-30 min using NBT/BCIP kit (Zymed,

U.S.A.). After detection, slides were rinsed in distilled water for 5 min and air dried before mounting in Permount histological mounting medium (Fisher Scientific, U.S.A.).

Digital Image Capture, analysis and quantification

All photomicrographs were obtained using a DFC290 HD digital camera (Leica Microsystems, USA). The following method of quantification was used both for immunohistochemical and *in situ* RT-PCR analyses. After acquisition of the images using the digital camera, the experimental image files were opened in the PhotoImpact software (Ulead PhotoImpact SE version 3.02; Ulead Systems, U.S.A.). The images were digitally processed in order to obtain the better and homogeneous signal and then selected for analysis of relevant regions. The selected regions were then digitally analyzed using the Image-ProPlus Analysis Software (Ver 4.5.0.19, Media Cybernetics, Inc., U.S.A.). The amount of signal was quantified using a pixel matrix data (the color contained within a pixel inside an image at specific location). Therefore, chromogen quantity was determined by calculating the norm of the matrix file for that image. This allows pixels of similar "color" immediately adjacent to the index pixel to be included for analysis. All pixels falling within the selected threshold parameters were quantified, recorded and used to generate a graphic. The file for the control image was similarly generated: the control slide is acquired and treated identically as the experimental slide except that negative controls were included: A (H1N1)-negative biopsies; reactions without reverse transcriptase; reactions without anti-DIG antibody. Beta2-microglobulin signal was used to normalize data in case of *in situ* RT-PCR quantification.

Sequencing of *in situ* RT-PCR products

The nucleotide sequences of the PCR products were determined using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) and analysed on the ABI Prism 3100 Analyzer Sequencer (Applied Biosystems). The obtained sequences were aligned and analysed using Basic Local Alignment Search Tool (Fig. 1) (BLAST; http://blast.ncbi.nlm.nih.gov). As expected, sequences corresponded to the segment between the middle part and the 3' end of the NA gene.

***In vitro* Duplex amplification technique**

In vitro duplex amplifications were performed using 2 μL of cDNA as template and reaction mixtures (25 μL) containing: 1x PCR buffer, 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 400 nM of each primer, 2.5 units of Taq DNA polymerase (Invitrogen, U.S.A.) and specific primers (see above). Amplifications were carried out using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, U.S.A.). The duplex protocol included a 3 minutes incubation at 95ºC followed by 40 cycles of 1 min at 94ºC, 1.5 min at 60 ºC and 1 minute at 72ºC. A final extension of 10 minutes at 72ºC was performed. Finally, the duplex PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

RNA isolation

As a control of *in situ* determinations, total RNA was isolated from biopsies obtained from patients with influenza illness using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The RNA preparations were used for cDNA synthesis and Real-Time PCR.

Preparation of cDNA for Real-Time PCR

Three micrograms of total RNA were reverse transcribed in a 20 µl reaction using 100 U SuperScript II Reverse Transcriptase, following the manufacturer´s specifications (Invitrogen). In parallel, cDNAs synthesized during the *in situ* reverse transcription reaction were recovered and used for sequencing (Fig. 1).

Relative mRNA Quantification by Real-Time PCR (RTqPCR)

The relative quantification of the A (H1N1) viral load was determined by RTqPCR using a 7300 Real Time PCR System (Applied Biosystems, USA). PCRs were processed through 35 cycles of a 3-step PCR, including 10 sec of denaturation at 95 °C, a 10-sec primer dependent annealing phase (60 °C), and a 10 sec template-dependent elongation at 72 °C. The amplification of each template was performed in duplicate in one PCR run. The relative viral load was calculated as the ratio normalized to β2-microglobulin.

RTqPCR data analysis using $2^{-\Delta\Delta CT}$ method

Real-time PCR was performed on the corresponding cDNA synthesized from each *in situ* sample. The data were analyzed using the equation described by Livak [22]. Validation of the method was performed as previously reported [22, 23].

RESULTS

Clinical features of patients with Influenza-like Illness (ILI)

A total of 8 samples obtained from influenza-positive patients (3 female and 5 male) were analyzed using *in situ* RT-PCR for influenza A (H1N1) (2009). The most common symptoms among the infected subjects were fever over 39 °C (100%), cough (94%), headache (84%), sore throat (72%), rinorrhea (71%), myalgia (69%), chills (50%), nasal congestion (44%), and conjunctivitis (40%) (Table 1).

Expression levels of Neuraminidase A (H1N1) influenza gene in patients with ILI

Specific primers recognizing the NA A (H1N1) influenza gene were used to establish expression levels in RNA samples. Expression level was determined using the β2-microglobulin housekeeping gene as internal control (Fig. 2). Primers and duplex amplification technique were validated analyzing a large number of nasopharyngeal swabs obtained from ILI patients (Manuscript in preparation). In addition, cDNA was successfully amplified and analyzed by quantitative RTqPCR (Fig. 2b).

NA A (H1N1) influenza gene can be detected in biopsies using in situ RT-PCR

The expression pattern of the NA viral gene in lung biopsies obtained from patients with pneumonia was determined by *in situ* RT-PCR. We detected NA transcripts in 100% of the previously diagnosed A (H1N1)-positive samples as a cytoplasmic signal (Fig. 3; Table 1). As expected, no expression was detected by *in situ* RT-PCR in both previously diagnosed AH1N1-negative patients using standard protocols and in non-related cells. *In situ* relative transcription levels correlated with those obtained when *in vitro* RT-PCR assays were performed.

Sample sequencing

Partial sequences of NA gene from AH1N1-positive patients were obtained by the *in situ* RT-PCR-sequencing method (Table 2). Sequence analysis showed 98% similarity with influenza viruses reported previously in places such as New York/San Salvador (98% homology; one of eight patients); Texas/Wisconsin/Roma/Colombia District (98% homology; one patient); Ontario/Mexico City/Auckland/Jalna (98% homology; 3 patients); Viena/Yaroslavl/Texas (98% homology; one patient). Lower sequence homology was determined with Jalna/Auckland (97% homology; one patient) and Puerto Rico/IvPR8 (96% homology; one patient).

Discussion

In this study we describe a successful method for the molecular detection of A (H1N1) influenza virus in lung sections obtained from persons who died during the epidemic outbreak of influenza A (H1N1) 2009 in Mexico City. More than 100 countries have officially reported more than 7 million people infected with the influenza A (H1N1) 2009 virus, from which at least 13,500 patients have died [4]. During the initial outbreak, Mexico reported most cases and the highest number of casualties around the world [1, 2, 4, 8, 9]. Although the current diagnostic technologies are reasonably effective for sporadic and epidemic influenza [24], new strategies including more sensitive and more specific tests have been developed enhancing diagnostic capability. These new developments promise to improve not only the management of sporadic and epidemic influenza in both individual patients and communities, but also to address the need for retrospective testing. Laboratory diagnosis of influenza virus has become a cornerstone for the prevention, containment, surveillance, and treatment of the associated illness. In addition, conventional tests for influenza have historically been of questionable value for the management of patients, because of limited test sensitivity and long turnaround times [24]. The main goal of this work was the standardization of virus detection in patients where sample collection such as good-quality RNA could not be performed. This method for diagnostic testing could be efficient in monitoring and therefore diminishing the propagation of the virus among the population.

In order to demonstrate that A (H1N1) Neuraminidase transcript was expressed in infected biopsies, we decided to use *in situ* RT-PCR. This technique combines the extreme sensitivity of PCR with the cell localizing ability similar to *in situ* hybridization and allows comparing epithelia with stroma. *In situ* RT-PCR results demonstrated that A (H1N1) influenza virus could be detected in the same tissue as that in which

histopathological lesions had been observed. Viral nucleic acid was demonstrated in the bronchial and bronchiolar epithelial cells, pneumocytes and macrophages (Fig. 3A). Furthermore, sequencing data showed that, *in situ* technique was capable to specifically detect viral sequences. In addition, these data suggest that Mexico City could have played an important role for the dissemination of some variants throughout the world as indicated by the match between the Mexican samples included in this study and sequences described in other Countries such as New York, San Salvador, Texas, Wisconsin, Roma, Colombia District, Ontario, Auckland and Puerto Rico. Although the entire genome from the viruses should ideally be sequenced, the partial NA sequences generated by our group are of importance as they could provide a baseline of NA sequences from where the pandemic emerged.

On the other hand, we have found that *in situ* PCR products can be used to asses a relative viral load by quantitative real time methods (Fig. 3B). Patients showing high NA transcript levels were consistent with clinical observations from INER, Mexico. The fatalities we have reported here could not be **c**orrelated neither to the NA sequence similarity nor to viral load (not shown).

Interestingly, our results suggest that AH1N1 influenza virus infects mainly Type I and Type II alveolar cells (ATI and ATII). ATI and ATII cells have distinct physiological and functional properties within the lung. The alveolar surface is critical to gaseous exchange and is under continuous exposure to environmental and microbial insults, and 95% of the alveolar epithelium is covered by the flattened ATI cells [25, 26]. Thus, ATI cells are likely to be a cell type first infected by influenza viruses within the lung parenchyma and critical for effective gas exchange. ATII cells have the capacity to proliferate, differentiate into ATI cells and restore the epithelium after damage [**27**]. Both cell types play an important role in maintaining the alveolar fluid equilibrium by

transporting sodium and fluid from apical to basolateral surface of the alveolar epithelium [28]. Therefore, the dynamic interplay between ATII and ATI cells is crucial to maintenance of the fragile physiological balance crucial to efficient gas exchange in the lung. Recently, immuno-histopathological analyses of autopsy lung from patients with viral pneumonia revealed that the alveolar epitheliums as well as the alveolar macrophages are key target cells infected by influenza [27, 29-31]. Similar conclusions have also been drawn from experiments where *ex vivo* lung tissue has been infected with H5N1 virus [32]. In addition, massive infiltration of macrophage has been observed in the lungs of H5N1 infected humans and mice [33, 34]. It has been reported that H5N1 virus was a more potent cytokine and chemokine inducer than seasonal influenza H1N1 virus in both ATI and ATII cells [27]. These inflammatory mediators are likely to be important in initiating neutrophil and macrophage recruitment into the lung following infection. The overly exuberant chemokine responses elicited by H5N1 may explain the massive macrophage infiltration seen in the lung of H5N1-infected patients and contribute to the severe viral pneumonia and acute lung injury associated with this virus [35]. Probably, A (H1N1) virus detected in this study has acquired certain pathogenic characteristics exhibited by H5N1, contributing to fatal outcome.

In situ RT-PCR has the main advantage of allowing us to detect specific viral sequences in tissue sections as compared to other methods such as ultrastructural analysis with electronic microscopy [36]. Molecular strategies such as *in situ* hybridization (ISH) had been used before to identify cells infected with influenza virus in lung and other tissues [37-40]. One of the main features of the *in situ* RT-PCR is to take advantage of the *Taq* DNA polymerase to amplify a very low number of copies of a specific sequence in comparison with ISH in which the results depend on one-to-one strand recognition. To the best of our knowledge, the *in situ* RT-PCR strategy has been used in a limited

number of cases for detecting influenza viral sequences in tissues such as myocardium and placenta [41, 42]. One novel advantage that we have developed performing *in situ* RT-PCR is the ability of recover and subsequently to amplify the cDNA synthesized during *in situ* reverse transcription reaction. This amplification product can be then analyzed by quantitative Real Time PCR (RTqPCR) to validate expression levels [18, present work]. In Figure 2, transcription levels of Neuraminidase viral gene was determined using Real Time quantitative PCR, and in general, similar results were obtained with both methods. In addition, another advantage of our strategy is that it allowed us to obtain cDNA from tissue samples and to obtain the nucleic acid sequences. Our findings correlated with other groups that previously had localized A (H1N1) viral sequences to type II pneumocytes in the respiratory tract [31, 43, 44] and provide insights into the viral tropism of the main cell types found within the lung and may be relevant to understanding the pathogenesis of severe human influenza disease.

In summary, retrospective detection of influenza AH1N1 virus arising in Mexico City could provide important information to study the natural viral spread of influenza virus and, if it is possible, to correlate this data with emergence of new pathogenic influenza virus pandemic. The development of accurate tests for the detection of influenza in archival paraffin samples besides being an alternative tool for the detection of the virus, enables the laboratory to provide a prompt, definitive diagnosis, which would allow clinicians to initiate preventive methods, implement appropriate infection-control measures eventually decreasing the incidence of influenza cases, diminishing duration of hospitalization, reducing ancillary testing, and decreasing health care costs.

Figure Captions

Fig. 1. Experimental design used to detect AH1N1 virus by *in situ* RT-PCR. (1) Tissue fixed on electrostatically charged slides was permeabilized and genomic DNA was eliminated using RNase-free DNase (2). After *in situ* reverse transcription (3), synthesized cDNA was amplified and detected on tissue (4). Reaction mix was recovered for sequencing (5).

Fig. 2. Neuraminidase A H1N1 expression levels in clinical samples. (a) Duplex RT-PCR of A (H1N1) Neuraminidase (NA) and β2-microglobulin (β2-m) in A (H1N1)-positive samples. RNA preparations, obtained from nasopharyngeal swabs of AH1N1 positive cases were assayed using NA or β2-m specific primers (729 and 100 bp amplification product, respectively). In all experiments, negative Influenza-like illness patient [Neg ILI (-)] and positive (H1N1 Puerto Rico/Puerto Rico/IvPR8/Puerto Rico; 98% homology) controls were included. MWM: Molecular weight marker. (b) Neuraminidase A H1N1 expression levels determined by quantitative RTqPCR.

Fig. 3. AH1N1 detection in lung biopsies obtained from patients with influenza-like illness and pneumonia using *in situ* RT-PCR. Tissue was processed and *in situ* RT-PCR was done with NA specific primers as described in the Materials and Methods section. Arrows indicate intense cytoplasmic signal in positive samples (P01-P03) in comparison with a sample obtained from an AH1N1-negative patient. As expected, signal was detected in pneumocytes (black empty arrows) or macrophages (red empty arrow; patients P01 and P02) and tracheal epithelium (patient P03). Recovered cDNA were subsequently amplified and PCR products were validated using sequencing reactions. Neg ILI (-): Negative Influenza-like illness patient.

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Author ´s contributions.

ROD. Participated in the study design, coordination and draft the manuscript ; Carried out and interpretation the molecular studies.

MA and EG. Participated in sequence alignment and drafted the manuscript.

MAG. Participated in the clinic studies and patient data case studies.

CC. Participated in the clinic studies and patient data case studies.

DR. Carried out and interpretation the molecular studies.

FM. Assistance on characterization of the isolates with reference strains

MEM. Conceived of the study and participated in its design and draft the manuscript. Participated in clinic and case studies.

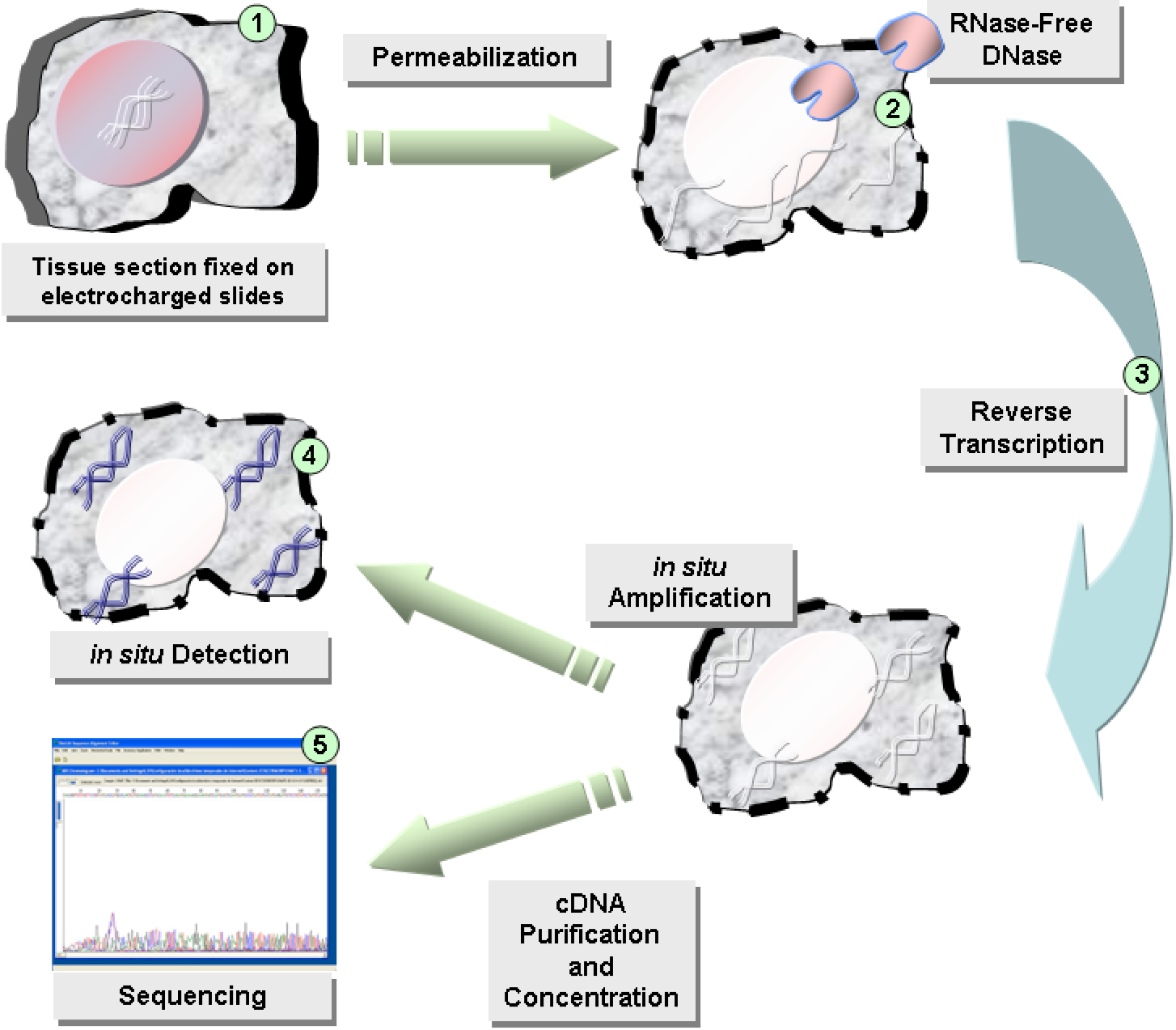
COA and RRR. Obtained the samples and performed the data analysis.

PG. Conceived of the study and participated in its design and draft the manuscript.

All authors read and approved the final manuscript.

Conflict of interest

The authors declare they do not have conflict of interest.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in situ Detection
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Sequencing
cDNA Purification and Concentration

Figure 1

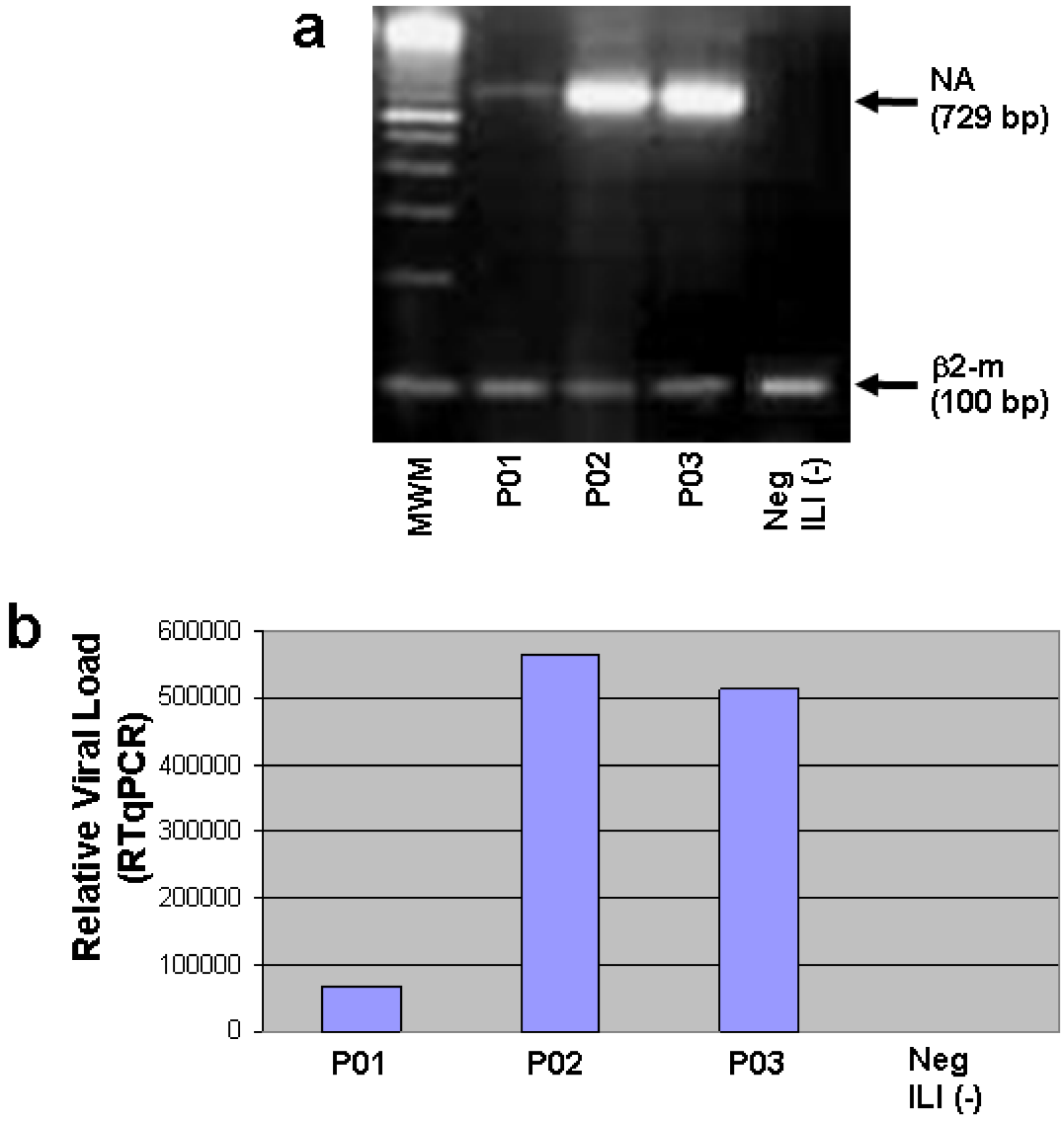
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Figure 2

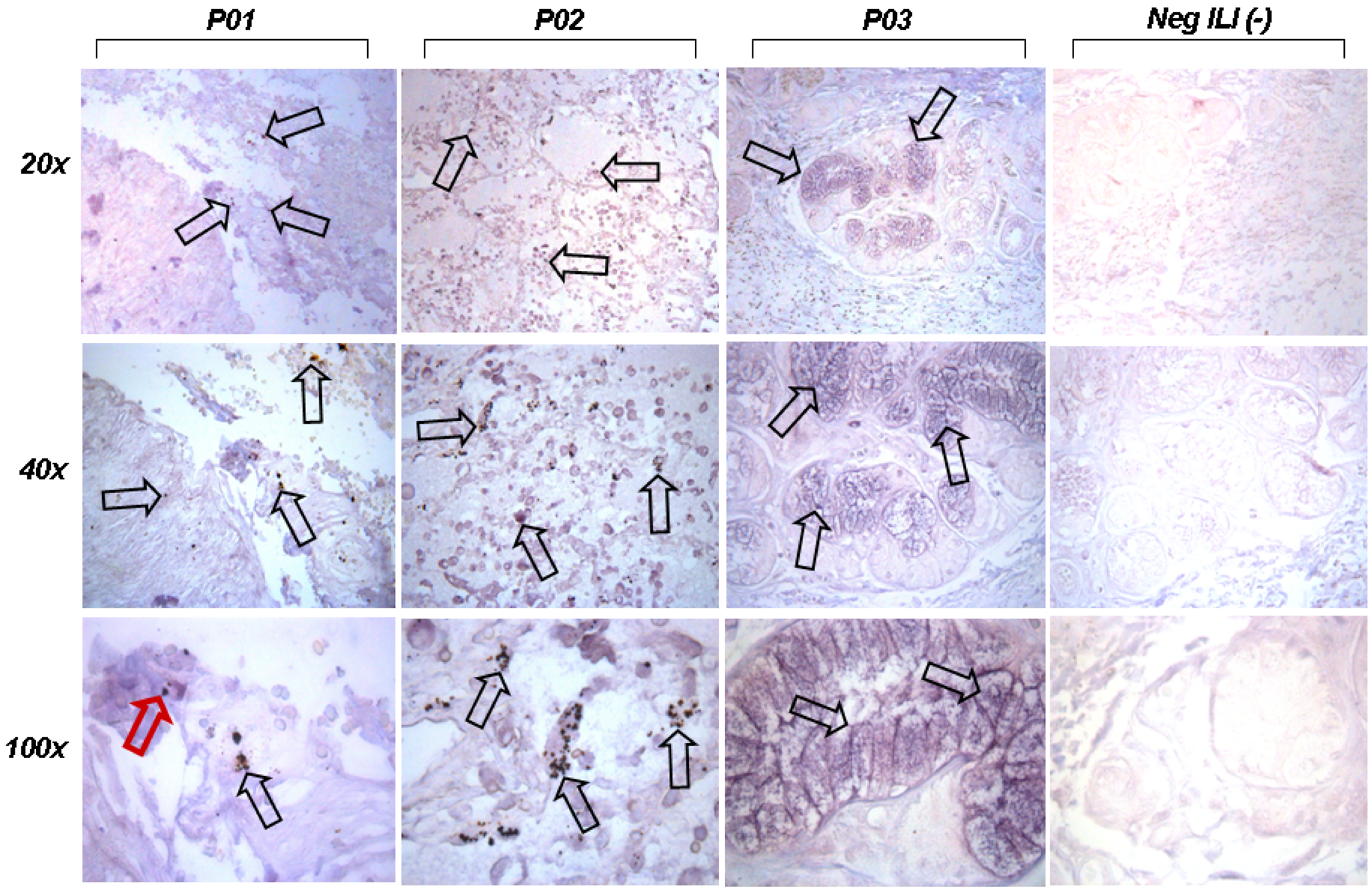

Figure 3

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