SUPPLEMENTARY MATERIAL

Sample handling procedures

All NPAs were sent on ice to the laboratory, where they were treated with 1% antibiotic-antimycotic solution (30 units of penicillin G, 30µg of streptomycin and 0,075µg of amphotericin-B; from Thermo Scientific, Carlsbad, CA, USA) in phosphate-buffered saline (PBS) plus 30µg of ciprofloxacin (Bayer, Leverkusen, Germany). Each sample was distributed in aliquots: two 250µl aliquots of NPA were mixed with 750µl TRIzol® (Thermo) and kept at -70ºC for nucleic acid extraction; two 500µl backup aliquots were kept at -70ºC in virus freezing medium, which consisted of Eagle´s minimum essential medium (MEM; GIBco) supplemented with 20% (w/v) fetal bovine serum (FBS; GIBco) plus 15% glycerol and 1% antibiotic-antimycotic solution. The remainder of the sample was used for HRSV screening using rapid test.

HRSV isolation

Semi-confluent (70%) HEp-2 cell (human nasopharyngeal carcinoma) monolayers prepared on 24-well plates were incubated with PBS pH=5 for 30 min at room temperature. Next, 100µl of HRSV-positive NPA plus 100µl of MEM supplemented with 2% (w/v) FBS (MEM2%) were added per well. Negative control cultures were inoculated only with 200 µl of MEM2%. After a 1h absorption period at room temperature, the inocula were removed by aspiration and monolayers were washed once with PBS pH=7.2 to minimize sample cytotoxicity. Next, cultures were replenished with 1ml/well of fresh MEM2% and maintained at 5% CO_2 at 37ºC. Cultures were harvested at 24h post infection (p.i.) regardless of the presence of CPE and tested for HRSV by indirect immunofluorescence (IF) assay. Briefly, cells seeded on glass slide were fixed by heat following by 5 minutes immersion in pure and cold acetone. The slide was stained with HRSV specific antibody MAB858-4 (Chemicon International Inc., Temecula, CA, USA) for 30 minutes at 37°C in a humid chamber. After three 5-minute washes in PBS pH=7, slides were incubated with blocking solution for 15 minutes at 37°C in humid chamber. Next, slides were washed three times for 5 minutes with PBS, and then incubated with secondary antibody for 30 minutes at 37°C in a humid chamber. Finally, slides were washed three times with PBS for 15 minutes, mounted in glycerol and examined in the fluorescence microscope. All positives were considered primary HRSV isolates, and called passage 1 (P1).

Regardless of IF results, all P1 harvests were blindly passaged onto fresh HEp-2 cell cultures that had also been previously treated with low-pH PBS (pH=5). The resulting P2 passages were monitored daily and harvested upon visualization of CPE, followed by confirmation by IF. To make viral stocks, two additional passages (P3 and P4) were done in fresh HEp-2 cell monolayers, with confirmation by IF. The clinical HRSV isolates that were passaged beyond P2 in HEp-2 cells were named clinical HRSV strains (P3 or P4).

Colorimetric syncytia assay (CSA)

Semi-confluent (70%) HEp-2 cell monolayers seeded onto glass coverslips were pretreated with PBS pH=5 for 1 hour at room temperature and then infected with HRSV isolates (P3 or P4) in triplicate. When cytopathic effect (CPE) was discernible at 24h p.i., the medium was removed, monolayers were fixed overnight with 200uL/well of fresh 4% paraformaldehyde and at room temperature. Paraformaldehyde was discarded, coverslips were rinsed with distilled water and stained with Giemsa (3% of Giemsa, 39% glycerol and 60% of methanol) for 30 seconds, followed by another rinsing in distilled water. Coverslips were then drained, dehydrated in ethanol series, treated with xylene and mounted on slides with Permount. Each coverslip was examined three times by three different viewers, by light microscopy, which allows for easy identification of HRSV syncytia (Figure S1). The number of syncytia per coverslip was determined at 100 × magnification. In order to verify the medium size of syncytia, a fourth person took three random pictures of different areas on each coverslip using under 400 × magnification, all three observers determined the mean number of nuclei per syncytium on each picture. The geometric means of counts by the observers were obtained.

Flow cytometry syncytia assay (FCSA)

This protocol was modified from the previously published for HIV by Wünschmann and Stapleton [11]. The harvested cells were re-suspended in 1mL of PBS pH=7.2 (GIBco, Thermo Scientific) and then pelleted at 500 x g for 5 minutes. The pellet was re-suspended in 1mL of 4% parafolmaldehyde for 15 minutes, and pelleted again at 500 x g for 5 minutes. Then the pellet was re-suspended in 1mL of PBS with 0.02% Tween 20 and 0.5% bovine serum albumin (PBST) for permeabilization of cell membranes, and then centrifuged for 5 minutes at 500 x g. The pellet was then treated with 1mg/mL of RNAse A (Thermo) diluted in 500µL of PBST for 15 minutes at 37^{o}C, re-pelleted, and re-suspended in 300µL of PBST with 50µg/mL of propidium iodide (PI), and stored

protected from light at -20°C until being analyzed in a FACS Scalibur Cytometer (Becton-Dickinson) using the FL2 channel and the software FCS Express V3. Cells with more than two nuclei gate in the R3 region of the histogram (Figure S2). The quantity of syncytia per sample was determined by the value of R3 obtained with HRSV-infected cells minus the R3 obtained with negative control noninfected cells, and then normalized for 1000 cells.

Determination of HRSV group by real time PCR

To identify HRSV-isolates as group A or B, a qPCR by the TaqMan® method was done, directed to the N gene, generating a final product of 80pb. Reverse transcription was carried out with 180ng of total RNA primed with random hexamers, using "High Capacity cDNA Reverse Transcription kit" (Applied Biosystems - Thermo) as per manufacturer's protocol. Three microliters of cDNA were used in the qPCR with 5µL of TaqMan® Universal PCR Master Mix, 1µL of forward primer (10pmoles), 1µL of reverse primer (10pmoles), 1µL of probe (10pmoles). The thermocycler was a 7300 Real Time PCR System (Applied Biosystems - Thermo) and the temperature conditions were 50ºC for 2min, 95ºC for 10min, and 40 cycles of 95ºC for 15sec and 60ºC for 1min. To detect HRSV-A and -B, primers and probes were: RSV A21 forward (5'-GTCCTTAGCAAAGTCAAGTTGAATGA-3'), RSV A102 reverse (5'-TGCTCCGTTGGATGGTGTATT-3'), RSV A48 probe (5'-/56-FAM/ACACTCAACAAAGATCAACTTCTGTCATCCAGC/36-TAMSp/-3'), RSV B17 forward (5'-GATGGCTCTTAGCAAAGTCAAGTTAA-3'), RSV B120 reverse (5'-TGTCAATATTATCTCCTGTACTACGTTGAA-3') and RSV B45 probe (5'-/56-JOEN/TGATACATTAAATAAGGATCAGCTGTCATCCA/36-TAMSp/-3').

Quantitation of genomic RNA and F-protein mRNA of HRSV-A by qPCR

Both genomic RNA and F-protein mRNA from clinical HRSV-A isolates were quantitated by qPCR. Reverse transcription (RT) was carried out using 180ng of total RNA and the "High Capacity cDNA Reverse Transcription kit" (Thermo), following the procedure proposed by the manufacturer. Each RT was done using 20 pmol/reaction of primer specific for HRSV-A genome (Lea/NS1-forward 5'-/Bio/CGAAAAAATGCGTACWACAAACTTGC-3') or for the mRNA of the F-protein (5'-end biotinylated oligo-dT). Next, the obtained biotinylated cDNAs were purified by magnetic separation using streptavidin-conjugated beads ("Dynabeads kilobase binder M-280 Dynal Biotech", Thermo) as per manufacturer's procedure. The final product was eluted in 200µL of pure water.

The qPCR for HRSV-A genomic RNA was carried out using the SYBR® Green strategy, with the primers Lea/NS1-Forward and Lea/NS1-Reverse (5'-CAATGCTACTTCATCATTGTCAAACA-3'), directed to the NS1 gene, generating a 153bp product. The qPCR for HRSV F-protein mRNA was done using F-

Forward (5'-TGCTCATGCAAAGCACAACA-3') and F-Reverse (5'-CCAACACCTAACAAAAAACCAAGA-3') primers, generating a 151bp product. Both PCR reactions were done using 5µL of "SYBR® Green Master Mix" (Thermo), 1µL (10pmoles) of each primer, and 3µL of cDNA, on a 7300 Real Time PCR System (Applied Biosystems - Thermo), with the following cycling conditions: 50ºC for 2min, 95ºC for 10min, 50 cycles of 95ºC for 15sec, and 60ºC for 1min.

For absolute quantification, serial decimal dilutions of NS1 and F genes cloned in plasmid pGEM-T were prepared using PCR products and the pGEM-T Easy Kit (Promega). DNA was quantitated at 260nm and calculation of the number of molecules was made considering 6,02 x 10^{23} molecules of 1pb as 660g. The results were analyzed with the 7300 System software (Applied Biosystems - Thermo). The sensitivity of the qPCR for both HRSV-A genomic RNA and F protein mRNA was 1 copy of DNA.

HRSV titration by plaque assay