UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO:

Detection of *Influenza A virus*, *Respiratory syncytial virus* and *Human metapneumovirus* in guinea pigs (*Cavia porcellus*) raised as livestock in Paute, Ecuador

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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Urcuquí, agosto 2019

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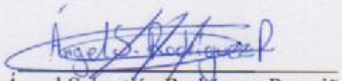

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Ángel Sebastián Rodríguez Pazmiño

Abstract

Viral agents such as *Influenza A virus*, *Respiratory syncytial virus* and *Human metapneumovirus* are some of the most frequent human respiratory pathogens and a concern for the public health. Guinea pigs (*Cavia porcellus*) could be a potential reservoir of these pathogens, because their susceptibility to be inoculated by these microorganisms which has been mainly demonstrated in laboratory conditions. However, it is imperative to make diagnoses from samples obtained from animals raised as livestock, to confirm or ruled out a possible role of the guinea pig as a zoonotic vector. In countries such as Ecuador, where the annual production of these animals is considerably high, this type of studies could have greater relevance for public health. In the present investigation, eighty samples of nasopharyngeal washing of guinea pigs from the Paute canton in the province of Azuay were obtained. Diagnoses of the respiratory viruses *Influenza A virus*, *Respiratory syncytial virus* and *Human metapneumovirus* were made, through protocols known as One Step RT PCR and Two Step RT PCR. All the tests yield negative results for the three viruses analyzed and, therefore, the viruses were not isolated.

Keywords

Influenza A virus, *Respiratory syncytial virus*, *Human metapneumovirus*, *Cavia porcellus*, guinea pig as vector, respiratory zoonotic infections.

Resumen

Los agentes virales tales como *Influenza A, virus sincitial respiratorio* y *metapneumovirus humano* son algunos de los más frecuentes patógenos respiratorios humanos y una preocupación para la salud pública mundial. Los cuyes (*Cavia porcellus*) podrían ser un potencial vector de estos patógenos, debido a su susceptibilidad a ser inoculados por estos microorganismos, lo que ha sido demostrado principalmente en condiciones de laboratorio. Sin embargo, es imperativo hacer diagnósticos de muestras obtenidas de animales criados como ganado, para confirmar o descartar un posible rol del cuy como un vector zoonótico. En países como el Ecuador, donde la producción anual de estos animales es considerablemente alta, este tipo de estudios podría tener una gran relevancia para la salud pública. En la presente investigación, se obtuvieron ochenta muestras de lavado nasofaríngeo de cuyes del cantón Paute (provincia del Azuay). Se realizaron diagnósticos de los virus respiratorios Influenza A, virus sincitial respiratorio y metapneumovirus humano, a través de protocolos conocidos como RT PCR de una etapa y RT PCR de dos etapas. Todas las pruebas produjeron resultados negativos para los tres virus analizados y, por tanto, estos no fueron aislados.

Palabras clave

Virus de Influenza A, virus sincitial respiratorio, metapneumovirus humano, Cavia porcellus, cuy como vector, infecciones respiratorias zoonóticas.

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Introduction

The raising of guinea pigs (*Cavia porcellus*) represents an important income for the economy of many families in Ecuador, especially in rural zones of the highlands. In 2015, 710.000 families would sustain approximately a production of 21 million guinea pigs (El Telégrafo, 2015). Due to this recurrent interaction between humans and these animals, and the notorious susceptibility of the latter to be infected by viruses, bacteria, protozoa and parasites (Brabb, Newsome, Burich, & Hanes, 2012), it can be hypothesized that guinea pigs can be a zoonotic vector, but so far are few studies have been done to prove it. *Dermatophytosis*, *Microsporidiosis*, *Listeria* and *Leptospira* are diseases confirmed as zoonotic having to guinea pig as a reservoir (Rigby, 1976). In Ecuador, a research assessed the existence of *Campylobacter jejuni* and atypical enteropathogenic *Escherichia coli* in children and guinea pigs. Although there were positive cases in both groups, it was not shown conclusive evidence of transmission between them (Vasco, Graham, & Trueba, 2016).

Guinea pigs are a highly valued species for animal experimentation for their ability to be infected by a wide variety of microorganisms. However, just few infections are acquired in a natural way and, of these, only a small portion produces symptoms (Brabb et al., 2012). Some of the pathogens successfully inoculated under laboratory conditions have been precisely respiratory viruses such as *Influenza A*, RSV, and hMPV (Dakhama, Vitalis, & Hegele, 1997; Hegele, Robinson, Gonzalez, & Hogg, 1993; Lowen, Mubareka, Tumpey, García-Sastre, & Palese, 2006). In the context of Influenza A, it is interesting to pay attention to the reports of a study that detected antibodies to this pathogen, in guinea pigs raised as livestock in Ecuador (Leyva-Grado, Mubareka, Krammer, Cárdenas, & Palese, 2012). These results open the question of whether these animals could be a reservoir for the transmission of respiratory pathogens to people.

The objective of this research thesis is focused on detecting the *Influenza A virus*, *Respiratory syncytial virus* (RSV) and *Human metapneumovirus* (hMPV) in samples of nasopharyngeal lavage from guinea pigs. This thesis topic it becomes more relevant when we consider that these diseases are endemic in Ecuador (MSP, 2019), and a topic of concern in the field of global public health, for the particular case of Influenza A, due the high mutation capacity of the virus and its pathogenic potential. In order to provide a contribution to the understanding of Influenza A, RSV, and hMPV circulation, the precepts of "One Health" push us to study the possible vectors and reservoirs of the diseases.

The samples used in this study were obtained of six agricultural farms from the canton Paute, in Azuay province, and these were processed in the *Laboratorio de Investigaciones Biomédicas* of the Escuela Politécnica del Litoral at Guayaquil. The process for the diagnosis was performed through One Step RT PCR and Two Step RT PCR, which included RNA purification of the samples, their complementary DNA synthesis, DNA amplification by endpoint PCR and gel electrophoresis for the visualization of the results. After all the tests, it was shown that there is no presence of the viruses, but this, however, does not rule out that guinea pigs may be zoonotic vectors of these viruses, in agricultural settings.

Objectives

To detect the presence of *Influenza A virus*, *Respiratory syncytial virus*, and *Human metapneumovirus* in the samples of nasopharyngeal lavage collected from guinea pigs (*Cavia porcellus*).

To perform complementary tests to validate the results obtained in the detection of the three viruses diagnosed.

Theoretical framework

Influenza A virus

Influenzavirus A is one of the seven genera belonging to *Orthomyxoviridae* family, characterized by possess a single-stranded, negative-sense and segmented RNA. The genetic material of *influenza A virus* (only species of the *Influenzavirus A* genus) has eight genes that codify for 10 to 14 structural and non-structural proteins, depending of the strain (Eisfeld, Neumann, & Kawaoka, 2015). The virion is pleomorphic, with a diameter from 80 to 120 nanometers (Noda, 2012). The nucleocapsid is protected by a lipid membrane, which is studded with the glycoproteins hemagglutinin (HA) and neuraminidase (NA). These two proteins fulfill important roles in the recognition of host cell and the subsequent virus release within it. Matrix (M2) ion channels complement the lipid membrane. The virion core is made up of M1 protein and protect the eight segments of RNA. These segments that are enveloped with the nucleoprotein (NP) and the RNA-dependent RNA polymerase, make up the ribonucleoprotein complex (RNP) (Bouvier & Palese, 2008).

HA protein recognizes and bound to sialic acid on the host cell membrane, which then is split by serine proteases into HA1 and HA2. This is a crucial step for the virus infectivity because the HA2 fraction participates in the fusion of the virus envelope with the cell membranes. The HA1 fraction is provided of the antigenic and receptor binding sites (Steinhauer, 1999). Antibodies counteract the HA activity blocking the virus infectivity, but due the high mutation capacity of the viral RNA, aminoacidic changes accumulate over time (process called *antigenic drift*). Eventually, this phenomenon will cause new strains by which the immune system of the guests could no longer neutralize them effectively (Bouvier & Palese, 2008). After the process of HA protein attachment to sialic acid, virus is endocytosed. A conformational change in HA is caused by the low pH of the endosome, generating a fusion peptide that merges the viral membrane with the endosomal membrane (Couceiro, Paulson, & Baum, 1993; Matrosovich, Matrosovich, Gray, Roberts, & Klenk, 2004). Through

the M2 ion channel, hydrogen ions are pumped from the endosome to the virion. This internal acidification breaks down protein-protein interactions, releasing the RNPs into the host cell cytoplasm (Martin & Helenius, 1991).

Localization signals induce the proteins from the cell to import the RNPs toward the host cell nucleus (Cros & Palese, 2003). All RNA synthesis occurs in this place. Using the viral negative sense RNA as a template, the viral RNA-dependent RNA polymerase synthesize two positive-sense RNA species: mRNA templates which will serve for new viruses synthesis, and complementary RNA (cRNA) intermediates that subsequently will be transcribed into new negative-strands RNAs. The membrane-bound ribosomes from the endoplasmic reticulum are responsible for the synthesis of the envelope proteins, where they are then drove to Golgi apparatus for post-transductional modifications (Bouvier & Palese, 2008). Once the viral proteins and the genetic material are synthesized, the next step is packaging. Evidence suggests that this is a selective process conducted by discrete packaging signals in order to insure a full genome within the majority of the new virus particles (Bancroft & Parslow, 2002). To complete the virus replication cycle, influenza virus budding take place at the cell membrane (Bouvier & Palese, 2008).

Influenza A virus may be found in humans, pigs, horses, sea mammals, chickens and aquatic birds (Parrish, Murcia, & Holmes, 2015). For the case of birds, transmission pathways are through air or water routes, and domestic birds can suffer fatal consequences when this virus is passed from waterfowl. Betwixt mammals, transmission is usual by air conditions. Rarely avian influenza viruses transmit to mammals, but when occur it can bring fatal consequences (e.g. pandemics of influenza in humans) (Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). In laboratory conditions, guinea pigs have been successfully infected by *Influenza A virus* (Lowen et al., 2006), serving as animal models to study different aspects of diseases provoke for these pathogens. A serological study conducted in Ecuador from samples of these animals, detected antibodies to Influenza (Leyva-Grado et al., 2012), which opens the possibility that, in agricultural conditions, these animals may also be a zoonotic vector.

***Respiratory syncytial virus* (RSV)**

Respiratory syncytial virus appertains to the *Orthopneumovirus* genus and to the recently recognized *Pneumoviridae* family (Grint, 1989). It is characterized by possess a nonsegmented, single-stranded, negative-sense RNA (Kiss et al., 2014). Its genome is provided of ten ORFs that codify for eleven proteins of non-structure and structure. The viral RNA is coated by the phosphoprotein (P), nucleoprotein (N), and RNA-dependent RNA

polymerase (L). All this structure forms the ribonucleoprotein complex (RNP). Further, there are three integral membrane proteins: the large glycoprotein (G), the fusion protein (F), and the short hydrophobic (SH) protein. G is in charge of virus attachment to the host cell and F protein is involved with the fusion. The SH protein is an ion channel presents in the membrane (Gan et al., 2012).

Viral replication starts with the attachment of the virion to the host cells of the upper respiratory tract epithelium. The viral RNA enters together with the enzymes from the virus that guide the synthesis of a new viral RNA and proteins. In the cytoplasm occurs the gene expression and RNA replication. The nucleocapsid and the genome are released once the virion is in the cytoplasm. Viral RNA-dependent RNA polymerase then arrives the genome at its 3' end and the genes are transcribed into mRNAs. Replication generates a complete positive-sense RNA complement, which acts as a template for genome synthesis (Cowton, McGivern, & Fearns, 2006). The negative and positive RNA strands are both coated with the N protein at all the time which serves as the template for RNA synthesis. The M protein is in charge to assembly RSV by cooperating with the envelope proteins F and G and with the proteins N, P, and M2-1. The synthesized proteins then self-assembly and budding take place, having an envelope from the membrane. Many viruses are assembled in the cell, which is finally destroyed (lytic cycle). The elimination of ciliated epithelial cells covering the airways eventually gives rise to the symptoms of the infection (Naim, Billah, & Rana, 2017).

RSV causes the majority of respiratory tract infections around the world. It attacks the mucous membranes via the respiratory mucosa (Hall, Douglas, Schnabel, & Geiman, 1981). RSV is transmitted by exposure to large droplet secretions, and due to contact with polluted materials (CDC, 2019). Complications of this viral infection can produce bronchiolitis or pneumonia. During bronchiolitis, a peribronchial inflammation with lymphocytes (initial stage), characteristic necrosis and sloughing of the bronchiolar epithelium appear. The incubation period of RSV occurs between 2 - 8 days (Zhang, Peeples, Boucher, Collins, & Pickles, 2002). There are RSV versions affecting cows, sheep, goats and mice (Grint, 1989) and although the susceptibility of guinea pigs to human RSV virus infection has been demonstrated in laboratory conditions, there are no reports that it has been isolated in agricultural settings.

***Human metapneumovirus* (hMPV)**

Human metapneumovirus, species from genus *Metapneumovirus*, belong to *Paramyxoviridae* . hMPV has two genotypes: A and B. These two genotypes are divided into A1, A2, B1, and B2 subgroups. Subgroup A2 is divided into A2a and A2b (van den Hoogen

et al., 2004). The genomic structure of hMPV seems to that of *Avian pneumovirus* (aMPV), especially type C. Genomes of hMPV and hRSV are similar, too (Foulongne, Guyon, Rodière, & Segondy, 2006). hMPV, of negative-sense single-stranded RNA genome, has eight genes that codify for nine proteins. There are three surface glycoproteins (F, SH, and G) in the viral membrane, in the form of spikes. The RNA genome is joined with the P, N, L, M2-1, and M2-2 proteins, and forms the nucleocapsid (Biacchesi, Murphy, Collins, & Buchholz, 2007).

With the intervention of the G and F proteins, hMPV joins and combines to heparan sulphate receptors on the cell host membrane. Once the combination process has occurred, the nucleocapsid arrives into the cytoplasm of the host cell, to then starts with the replication. The P, N, L, and M2 proteins from the virus assemble with the new viral genome, and goes to the host cell membrane. Next, the new virion leaves the cell with the F, SH, and G proteins outside the viral membrane. The P protein works as a co-factor to preserve the L protein, permitting the arrangement of the virus ribonucleoprotein (RNP) complex while virus replication occurs. The M protein complies a fundamental activity in virus assembly and budding by cooperating with the RNP complex. The nuclease activity of viral genome is avoided by the N protein. (Schickli et al., 2008).

Studies of serological prevalence have shown that children between 5-10 years old, in a high percentage have already been infected by this virus (90-100%). Re-infection can appear throughout adulthood. Mild flu-like symptoms normally appear in adults when they are infected by hMPV. But in some in some adult cases, it can appear severe complications such as chronic obstructive pulmonary disease (Boivin et al., 2002). There are co-infection reports of hMPV, with respiratory pathogens such as RSV (Greensill et al., 2003), *Influenza B*, and *Influenza A* (Esposito et al., 2013). Also other studies have shown co-infection with bacterial pathogens like *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* (Esposito et al., 2013). The unique known reservoir for hMPV are humans, but experimentally guinea pigs have been infected into ferrets and guinea pigs (Hamelin, Abed, & Boivin, 2004). aMPV reservoirs are related to wild birds (Jardine, Parmley, Buchanan, Nituch, & Ojkic, 2018), without reports for guinea pigs.

Problem Statement

The detection of *Influenza A virus*, RSV and hMPV in guinea pigs (*Cavia Porcellus*) is proposed, because these could represent a potential zoonotic reservoir for these pathogens and, therefore, be a potential concern for public health. Respiratory infections derived from these microorganisms can be related to widespread clinical diseases such as common cold, pharyngitis, epiglottitis, bronchitis, pneumonia, among others (Dasaraju & Liu, 1996). It is estimated that there are around 3,9 million annual deaths related to respiratory infections around the world, specially affecting children in developing countries (WHO, 2018). In the case of Ecuador, where the production of guinea pigs is around 20 million annually (El Telégrafo, 2015), it is important to carry out this type of research that could contribute to the formulation of public health policies focused on the prevention and mitigation of infectious respiratory diseases.

On the other hand, there are reports of pigs and chickens raised intensively for human consumption (as well as guinea pigs in Ecuador) that have been identified as reservoirs of pathogens related to different strains of influenza (CDC, 2018). In the particular case of guinea pigs raised in agricultural conditions in Ecuador, the detection of antibodies related to Influenza A and B has been reported (Leyva-Grado et al., 2012), which is an interesting antecedent that motivates the search for the viral agents proposed in guinea pigs. If positive results are confirmed on this detection, it could be suggested that the guinea pigs would represent a zoonotic vector for these pathogens.

Methodology

Collection of samples

During the months of April and May of 2018, samples were collected from six farms located in canton Paute, Azuay province (Ecuador). Paute, at 2.280 mamsl, has a warm and temperate climate, present an average rainfall of 986 mm, and the average annual temperature is 17.3 °C. It has an economy based on agricultural and livestock activities. The eighty samples were extracted through a nasopharyngeal lavage, using a sterilized saline solution. In this process an operator held the guinea pig using his hands, and another person inserted a syringe with the saline solution through one of the nostrils. The lavage was collected in Eppendorf tubes of 2.0 mL, which contained the appropriate carrier for virus transportation. The samples were transported at 4°C to *Laboratorio de Investigaciones Biomédicas*, of *Escuela Politécnica del Litoral* in Guayaquil (Ecuador), where these remained at - 80°c until the days of their processing.

Sample processing

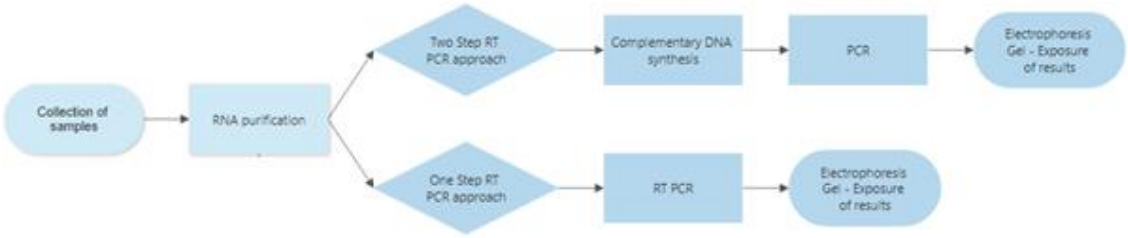


Figure 1. General overview about the methodology applied. *Influenza A virus*, RSV and hMPV detection was subjected to Two Step RT PCR approach. In order to demonstrate that purified RNA samples contained RNA of guinea pig, also it was tested with a One Step RT PCR process, using primers for 18S gene (housekeeping gene of mammals).

Before to start with the first stage, the eighty samples were arranged in groups of five (each group referred to as "pool"), taking 60 µl from each sample to obtain 300 µl by group. Sixteen pools were obtained, where two of them were made up of only four samples, because two Eppendorf tubes appeared shredded and without the sample content. If at the end of the complete process, some of the pools gave positive results, the samples that formed them were going to be processed one by one to determine which of them generated a positive result for the pool. The codification for each sample and its respective organization in pools are described in *table 1*; the two Eppendorf tubes without sample content are highlighted in red.

RNA purification

After pools organization, RNA purification was the next step. In this process, the MasterPure ™ RNA Purification kit (Epicentre) was used. To try to obtain a greater amount of RNA, a double extraction was carried out, that is, 60 µl was taken from each sample, accumulating in an Eppendorf tube 300 µl in total by armed pool, and then dividing this volume into equal amounts in two tubes (150 µl per tube). We also worked with extraction controls, whose test content was ultrapure water. In later stages of diagnosis, these controls were subjected to the same processes like the pools, to verify that there was no contamination at this stage. After each RNA purification process the concentration was measured in nanograms per microliter, using the NanoDrop 2000 spectrophotometer (ThermoScientific). The used methodology for RNA purification is described in *table 2*.

Table 1

Organization of the samples

Farm	Pool	Sample				
1	1	EV1	EV2	EV3	EV4	EV5
	2	EV6	EV7	EV8	EV9	EV10
2	3	RR1	RR2	RR3	RR4	RR5
	4	RR6	RR7	RR8	RR9	RR10
	5	RR11	RR12	RR13	RR14	RR15
	6	RR16	RR17	RR18	RR19	RR20
3	7	RG1	RG2	RG3	RG4	RG5
	8	RG6	RG7	RG8	RG9	RG10
4	9	S1	S2	S3	S4	S5
	10	S6	S7	S8	S9	S10
	11	S11	S12	S13	S14	S15
	12	S16	S17	S18	S19	S20
5	13	A1	A2	A3	A4	A5
	14	A6	A7	A8	A9	A10
6	15	B1	B2	B3	B4	B5
	16	B6	B7	B8	B9	B10

Note: fourteen pools of five samples, and two pools of four were formed. Pool numbers 5 and 7 were arranged using only four samples (excluding those in red).

Table 2

Methodology applied to the RNA purification using the MasterPure™ RNA Purification kit from Epicentre (steps are in accordance to the manufacturer´s protocol)

A. Fluid Samples
1. Dilute 1 µl of 50 µg/µl Proteinase K into 150 µl of 2XT and C Lysis Solution for each sample. 2. Transfer 150 µl of the fluid sample to a microcentrifuge tube and, 150 µl of 2X T and C Lysis Solution containing the Proteinase K, and mix thoroughly. 3. Incubate at 65°C for 15 minutes; vortex every 5 minutes. 4. Place the samples on ice for 3-5 minutes and then proceed with total nucleic acid precipitation in Part B.
B. Precipitation of Total Nucleic Acids
1. Add 175 µl of MPC Protein Precipitation Reagent to 300 µl of lysed sample and vortex vigorously for 10 seconds. 2. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a micro-centrifuge. 3. Transfer the supernatant to a clean micro-centrifuge tube and discard the pellet. 4. Add 500 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times. 5. Pellet the total nucleic acids by centrifugation at 4°C for 10 minutes in a micro-centrifuge. 6. Carefully pour off the isopropanol without dislodging the total nucleic acid pellet.
C. Removal of Contaminating DNA from Total Nucleic Acid Preparations
1. Remove all of the residual isopropanol with a pipette. 2. Prepare 200 µl of DNase I solution for each sample by diluting 5 µl of RNase-Free DNase I up to 200 µl with 1X DNase Buffer. 3. Completely suspend the total nucleic acid pellet in 200 µl of DNase I solution. 4. Incubate at 37°C for 30 minutes. 5. Add 200 µl of 2X T and C Lysis Solution; vortex for 5 seconds. 6. Add 200 µl of MPC Protein Precipitation Reagent; vortex 10 seconds; place on ice for 3-5 minutes. 7. Pellet the debris by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a micro-centrifuge.

8. Transfer the supernatant containing the RNA into a clean micro-centrifuge tube and discard the pellet.
9. Add 500 µl of isopropanol to the supernatant. Invert the tube 30-40 times.
10. Pellet the purified RNA by centrifugation at 4°C for 10 minutes in a micro-centrifuge.
11. Carefully pour off the isopropanol without dislodging the RNA pellet.
12. Rinse twice with 70% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipette.
13. Suspend the RNA in 10-35 µl of TE Buffer.

Two-Step RT-PCR for *Influenza A virus*, RSV, and hMPV viruses

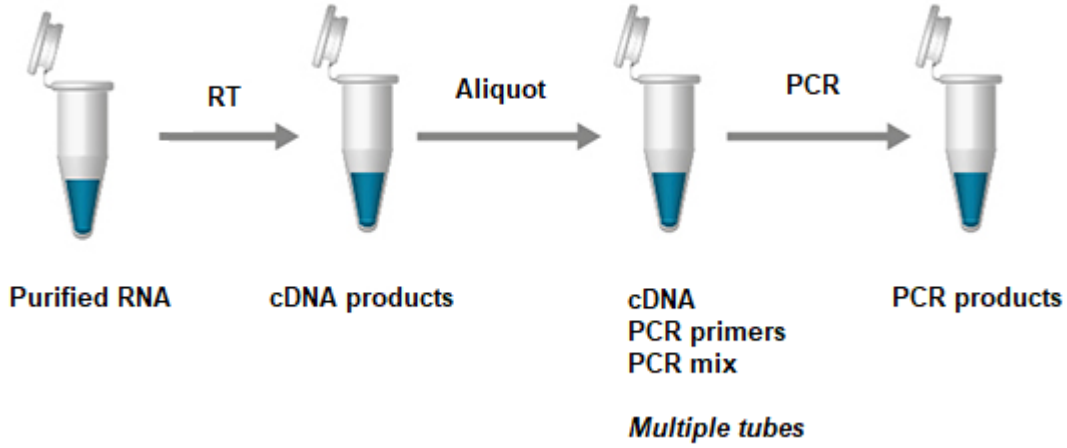


Figure 2. Stages of Two-Step RT-PCR

Through Two-Step RT-PCR approach, *Influenza A virus, Respiratory Syncytial Virus* (RSV) and *Human Metapneumovirus* (hMPV) were subjected to diagnosis. All the tests were evaluated with the appropriate positive controls, provided by the *Laboratorio de Investigaciones Biomédicas* at ESPOL, together with the RNA extraction controls generated in the previous step. Two-Step RT-PCR approach includes reverse transcription reaction using the previously purified RNA, PCR and electrophoresis gel to visualize the results, in that order (look at *figure 2*). The High Capacity cDNA Reverse Transcription (Applied Biosystems) kit was used for reverse transcription reaction (*table 3*). Random primers were used for the retro-transcription (RT) reaction. Thereafter, cDNA stocks were obtained and PCR reactions continued using the corresponding primers for each of the three viruses of interest. As to *Influenza A virus*, in the RT reaction, a stock of cDNA's was also generated using specific primers (specifically of the forward type). For the PCR it was employed TaqMan® Gene Expression Master Mix (Applied Biosystems*)*, where its reagent contains a mix of AmpliTaq Gold® DNA Polymerase, UP (Ultra-Pure), Uracil-DNA Glycosylase (UDG), deoxyribonucleotide triphosphates (dNTP's) with deoxyuridine triphosphate (dUTP), ROX^{TM} Passive Reference, and other buffer components. Components and volumes used for the PCR of the three viruses analyzed are listed in *table 4*.

Table 3

Components used for reverse transcription (RT) reaction

Components	*Volume per reaction (µl)*
10✕RT Buffer	*2*
10✕RT Random Primers	*0,2*
25✕dNTP Mix (100 mM)	*2*
MultiScribeTM Reverse Transcriptase, 50 U/µL	*1*
H2O	*8,8*
RNA sample	*6*
Total	20

Note: the first four components are from the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The RNA samples came from the RNA purification process.

Table 4

Components and volumes used for the PCR of the three viruses analyzed

Components	*Volume per reaction (µl)*
Taqman Gene Expression Master Mix 2x	10
Primer mix 10 µM	0,8
H_2O	8,2
cDNA	1
Total	20

Note: the first two components come from the TaqMan® Gene Expression Master Mix. DNA samples are from the previous RT reactions.

One-Step RT-PCR for diagnosis of 18 S gene

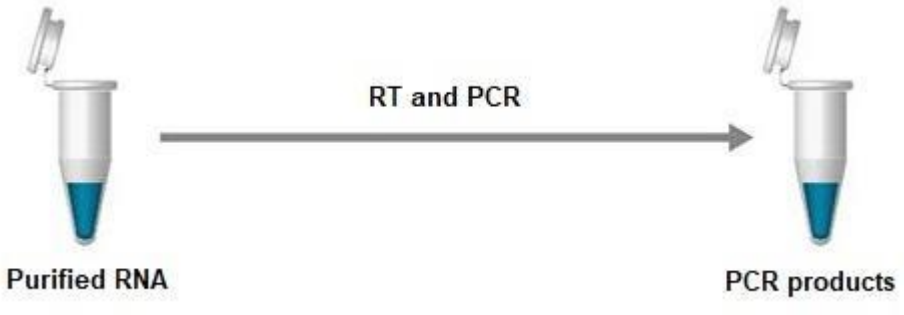


Figure 3. Stages in the One-Step RT-PCR

One-Step RT-PCR includes, in the same reaction, the processes of RNA retro-transcription and DNA amplification by PCR. For the guinea-pig RNA diagnosis, a primer corresponding to housekeeping gene 18S was used. The specifications of these primers and those used for the diagnosis of RSV and hMPV viruses are described in *table 5*, in annexes. The kit used to perform this protocol was the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen™). The components of this kit and the volumes used per reaction are described in the *table 5*.

Table 5

Components and volumes for One-Step RT-PCR applied for diagnosis of 18 S gene using a kit from Invitrogen™

Component	*Volume per reaction (µl)*
2X reaction mix	10,0
50 Mm $MgSO_4$	0,6
SSIII Platinum Taq Polymerase	0,8
10 µM Primer Mix (18S)	0,8
RNA Template (from previous RNA purifications)	1
H_2O Ultrapure	6,8
Total	20,0

Table 6

PCR thermocycler program in Two-Step RT-PCR process for the *Influenza A virus*, RSV and hMPV

		Temperature (°C)	*Time*
Stage 1 x 1	Step 1	50	2:00
	Step 2	95	10:00
Stage 2 x 40	Step 1	95	0:15
	Step 2	60	1:00
Stage 3x1	Step 1	15	∞

Table 7

RT thermocycler program in Two-Step RT-PCR processes

		Temperature (°C)	*Time*
Stage 1 x 1	Step 1	25	10:00
Stage 2 x 1	Step 1	37	60:00
	Step 2	37	60:00
Stage 3 x 1	Step 1	85	5:00
	Step 2	15	∞

Table 8

One-Step RT-PCR thermocycler program for 18 S detection tests

		Temperature (°C)	*Time*
Stage 1 x 1	Step 1	50	30:00
Stage 2 x 1	Step 1	94	15:00
Stage 3 x 35	Step 1	94	0:30
	Step 2	55	0:30
	Step 3	72	1:00
Stage 4 x 1	Step 1	72	7:00
Stage 5 x 1	Step 1	15	∞

Results

After carrying out the One-Step RT-PCR and Two-Step RT-PCR tests, negative results were obtained, for the 100% of the 78 samples processed with the respective quality controls in 16 pools (results are shown in the *Table 9*). As regarded the assays with the 18 S primers, which were run by the One-Step RT-PCR method, bands were obtained for all the 16 processed pools, although only in two of them the bands corresponded to the expected size (560 bp) while multiple (between two and three) bands appeared in five pools.

Calculated RNA concentrations following extraction and their respective absorbance rates at 260/280 and 260/230 nm are shown in *Table 10*. The gels corresponding to the results shown in *table 9* are attached in the annexes.

Table 9

Results corresponding to the One- and Two-Step RT-PCR assays conducted on the *Influenza A virus*, RSV, and hMPV RNA pools

Location	*Pools*	*Specific viral pathogens, (%)*			
		Influenza A	RSV	hMPV	No virus detected
Farm 1 (n=10)	1, 2	0 (0)	0 (0)	0 (0)	10 (100)
Farm 2 (n=19)	3 - 6	0 (0)	0 (0)	0 (0)	10 (100)
Farm 3 (n=9)	7, 8	0 (0)	0 (0)	0 (0)	10 (100)
Farm 4 (n=20)	9-12	0 (0)	0 (0)	0 (0)	10 (100)
Farm 5 (n=10)	13, 14	0 (0)	0 (0)	0 (0)	10 (100)
Farm 6 (n=10)	15, 16	0 (0)	0 (0)	0 (0)	10 (100)

Table 10

Concentrations and absorbance rates of purified RNA pools

Pool	*Concentration (ng/µl)*	*Abs (260/280)*	*Abs (260/230)*
1	23,4	1,63	4,0
2	17,9	1,75	2,6
3	18,3	1,71	2,8
4	23,2	1,61	3,1
5	5,2	1,74	1,4
6	26,3	1,61	3,2
7	21,2	1,59	3,0
8	19,6	1,60	2,8
9	19,8	1,52	2,9
10	20,9	1,56	2,9
11	31,1	1,61	3,0
12	24,1	1,60	3,0
13	20,0	1,63	3,0
14	21,1	1,59	2,9
15	21,3	1,63	3,0
16	21,2	1,62	3,0

Discussion

All the carried out tests were negative for the detection of the respiratory viruses (*Influenza A virus*, RSV and hMPV) under scrutiny. The tests focused on viral diagnosis started from the same stock of cDNA, in which random synthesis primers were used and then, the primers corresponding to each virus were occupied for PCR reactions. We also worked with a stock of cDNA synthesized with specific forward primers for the diagnosis of *Influenza A virus*. This was done to see whether it was possible to obtain a better sensitivity using the specific primers rather than the random primers in the general diagnostic process. Even this change, viral diagnosis continued yielding negative results.

All these tests posited two main concerns for discussion: 1) the reliability of the obtained results; and 2) whether guinea pigs could actually be considered zoonotic reservoirs of these pathogens. Regarding the first one, there are three aspects that were paid special attention throughout the experimentation and that are worth at mentioning.

Validity of the results obtained

Extraction negative and positive controls. During RNA purifications, they were run RNA extraction negative controls that, instead of containing an aliquot of the viral samples, were provided with an aliquot of ultrapure water. These negative controls were subjected to exactly the same One- and Two-Step RT-PCR processing so that they were not supposed to show RNA bands associated with either the viral genes or even genes of any other type. As expected, the run gels showed no bands (see attached annexes), which undeniably confirmed the absence of contamination with nucleic acids during the process of RNA purification. The positive controls, provided by the *Laboratorio de Investigaciones Biomédicas-ESPOL*, were also subjected to the One- and Two-Step RT-PCR processes. Unlike the negative (extraction) controls, the positive (viral) controls showed bands corresponding to prototype viral genes, which gave us confidence about the quality of the performed assays.

Measurement of RNA concentrations. We proceeded to measure RNA concentration (ng/µl) following its extraction. This step was critical, because the High Capacity cDNA Reverse Transcription kit from Applied Biosystems works in an optimal concentration range of up to 2 µg for each 20 µl reaction. Accordingly, if concentrations were below a threshold, purification had to be repeated, whereas if values were too high, dilutions were needed to continue with the diagnosis of the virus. In our samples, concentrations of purified RNA were around 20 ng/µl (see *table 12*). While measuring absorbance rates at 260/280 and 260/230 nm allow determine the concentration of RNA, they also serve as indicators of the purity of the

nucleic acid. According to the spectrophotometer (Thermo-Scientific) guide, a rate of approximately 2.0 is generally accepted as "pure" RNA. Our RNA enriched samples showed absorbance values somewhat below 2.0 (see *table 12*). These values replicated the ones that are routinely observed by our colleagues from Laboratorio de Investigaciones Biomédicas-ESPOL (our laboratory of reference) when performing pathogen virus diagnosis. The absorbance rate at 260/230 nm is a secondary measure of the purity (absorbance usually in the range of 2.0 – 2-2). Values of our samples, were slightly over this absorbance range, which would not apparently represent a technical problem for subsequent diagnostic assays according to in-house experiments conducted in our laboratory of reference.

One Step RT-PCR tests on the 18 S gene. The aim of carrying out molecular diagnostic tests on the 18 S gene in purified RNA samples was to rule out the contamination with RNA from the host organism (i.e., guinea pig) as well as the products of RNA degradation during transportation and storage of the samples. The 18 S gene is a housekeeping gene of eukaryotic cells, whose expected amplicon, should be around 560 base pairs (bp) based on the primers used in this particular set of experiments. It was noteworthy that bands at the expected size (560 bp) appeared in just two out of sixteen pools. In the remaining tests, bands of random sizes were observed, although multiple (two or three) bands appeared in five samples. The reason of the multiplicity of bands was uncertain, but either the lack of specificity of the primers used or similarities in the homology of the 18 S gene with other genes of bacterial origin may account for these experimental artifacts. It was considered that RNA was reasonably preserved during the purification process.

Guinea pigs as a reservoir of *Influenza A virus*, RSV and hMPV

Despite the negative detection of the three viruses, chances are that guinea pigs raised as livestock may still be a zoonotic reservoir for these pathogens, or at least for the *Influenza A virus*. The rational for these negative findings could be the year season when virus samples were collected, which did not coincide with the periods of the greatest expansion of the *Influenza A virus* in Ecuador. According to the historical records from the Ecuadorian Ministry of Public Health (MSP, 2019), there are two seasonal viral momentums. The first begins in January reaching its peak in March, while the second begins in July and reaches its climax in August. Although there is a lack of information about the annual cycles of expansion and circulation of the RSV and hMPV viruses in Ecuador, the same account would be valid for the failure in detecting these viruses since they are also associated with respiratory infections. In this vein, a serological study conducted on a sample of guinea pigs in Ecuador detected high percentages seropositive animals for influenza A antibodies (Leyva-

Grado et al., 2012). It is then guaranteed the repetition of the present research, during the suitable periods with the highest rates of virus circulation according to the information provided by the Ministry of Public Health of Ecuador and using larger sample sizes.

Annexes

Table 11

Primers forward and reverse used for reactions in One- and Two-Step RT-PCR

Oligo Name	Sequence (5' to 3')	Length	Virus	Target gene	Notes	Amplicon (bp)
5WHOMPA	TTCTAACMGAGGTCGAAACG	20	FluA	Matrix	Flu A	232
3WHOMPA	ACAAAGCGTCTACGCTGCAG	20	FluA	Matrix	Flu A	
UR2f	CATGCTATATTAAAAGAGTCTCA	23	hMPV	Nucleoprotein (N)	hMPV	149
UR2r	TCWGCAGCATATTTGTAATCAG	22	hMPV	Nucleoprotein (N)	hMPV	
RSV-ESPOL-F	TGGGAGARGTGGCTCCAG	18	RSV	Nucleoprotein (N)	RSV	343
RSV-ESPOL-R	ACTTGCCCTGMACCATAGG	19	RSV	Nucleoprotein (N)	RSV	
18SF	CTGGTGCCAGCAGCCGCGG	19	-	18 S	Biomarker Vertebrates	560
18SR	TCCGTCAATTYCTTTAAGTT	19	-	18 S	Biomarker Vertebrates	560

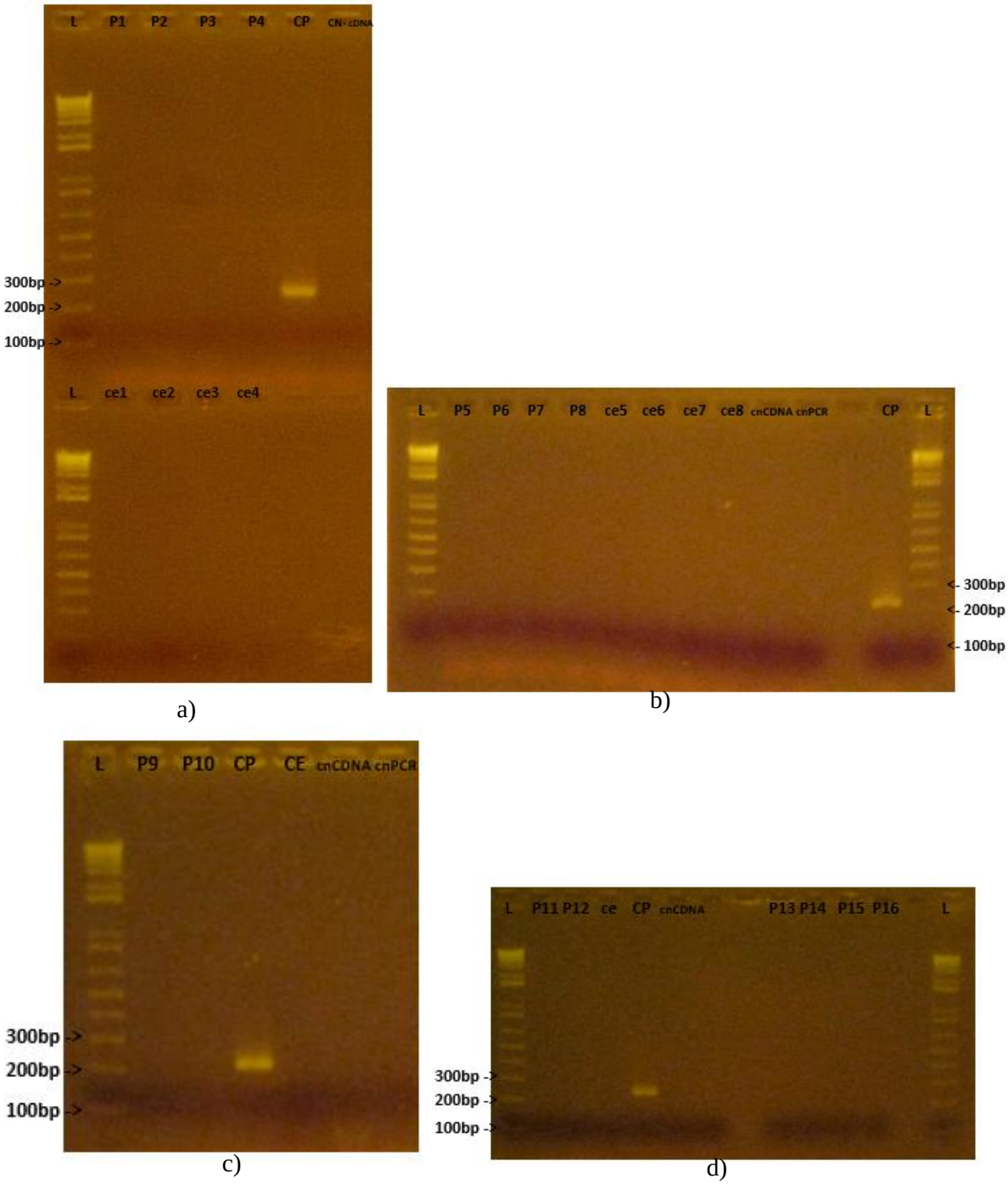
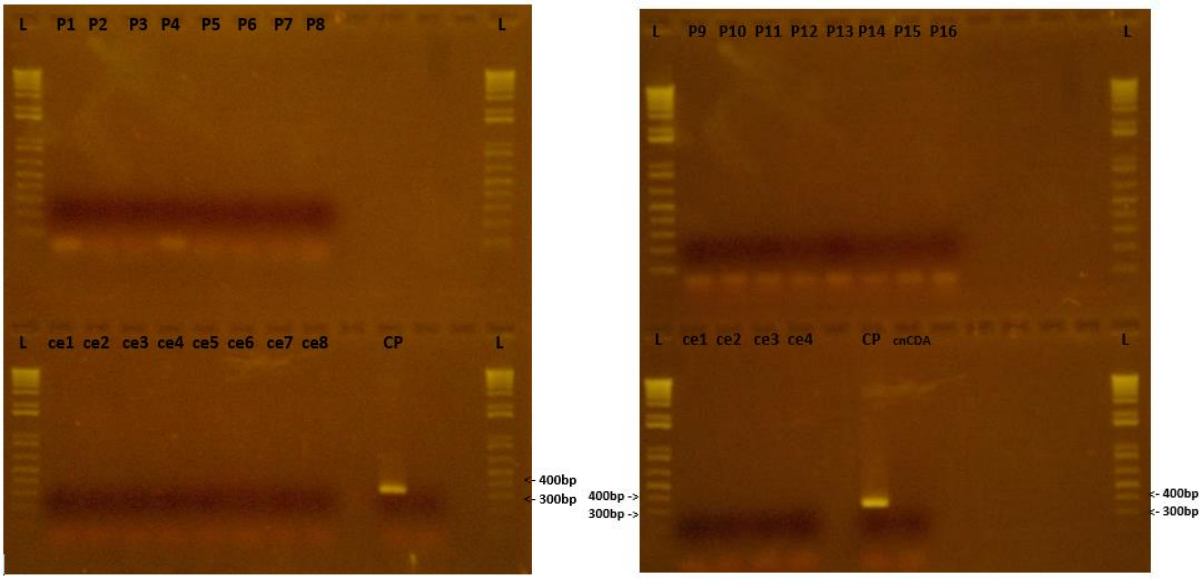
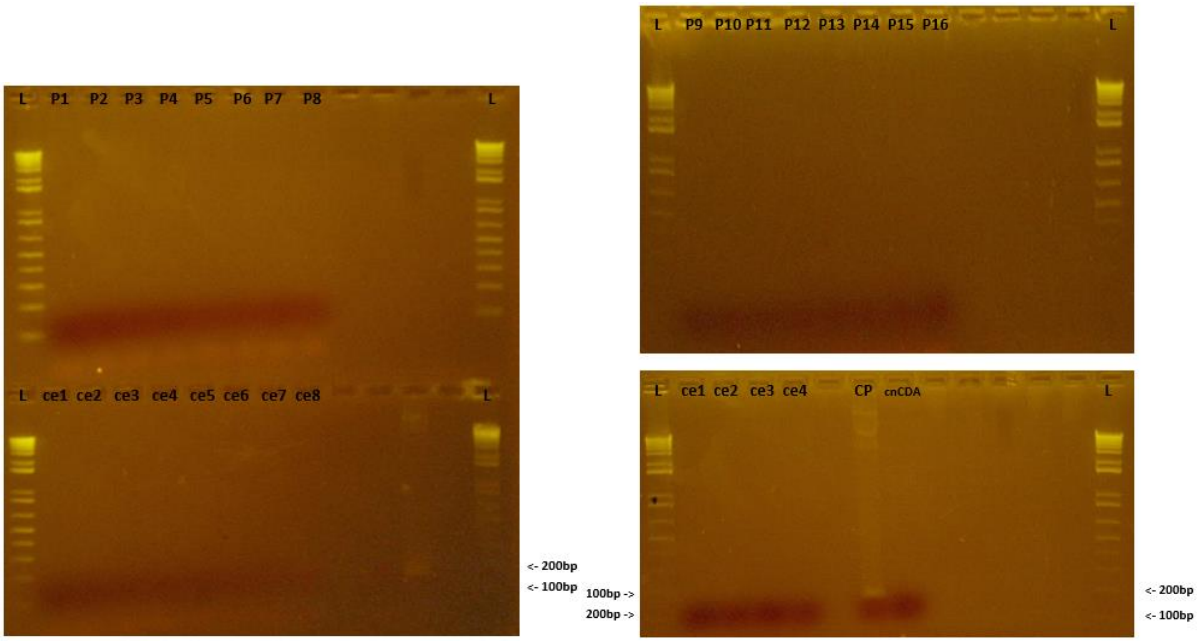


Figure 4. Two step RT PCR for *Influenza A virus* from cDNA stocks generated with random primers. It is observed that the amplicon of the positive control is at an expected approximate height of 232 bp and the rest show no bands. For a), b), c) and d) *L* is ladder; *P* is the pool number; *ce* is extraction control; CP the positive control; and *CN* the negative control.



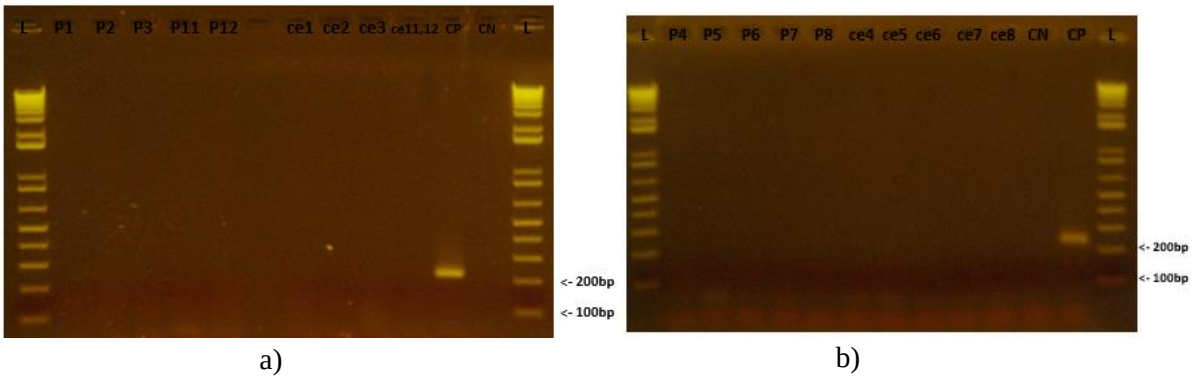
a) b)

Figure 5. Two-step RT-PCR for RSV from cDNA stocks generated with random primers. It is observed that the amplicon of the positive control is at an expected approximate height of 343 bp, while the rest of the samples show no bands. For a) and b) *L* is ladder; *P* is the pool number; *ce* is the extraction control; CP the positive control; and *CN* the negative control.



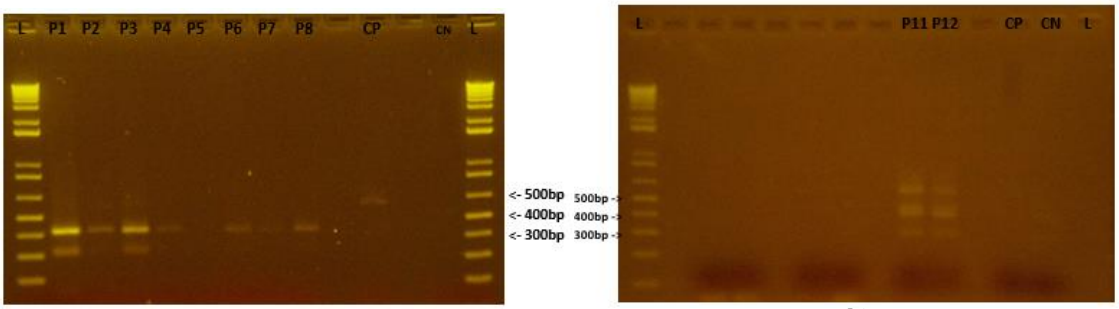
a) b)

Figure 6. Two step RT PCR for hMPV from cDNA stocks generated with random primers. It is observed that the amplicon of the positive control is at an expected approximate height of 149 bp, while the rest of the samples show no bands. For a) and b) *L* is ladder; *P* is the pool number; *ce* is extraction control; CP the positive control; and *CN* the negative control. In b), *ce1* is the control extraction for *P9* and *P10*, *ce2* for *P11* and *P12*, *ce3* for *P13* and *P14*, and *ce4* for *P15* and *P16*.

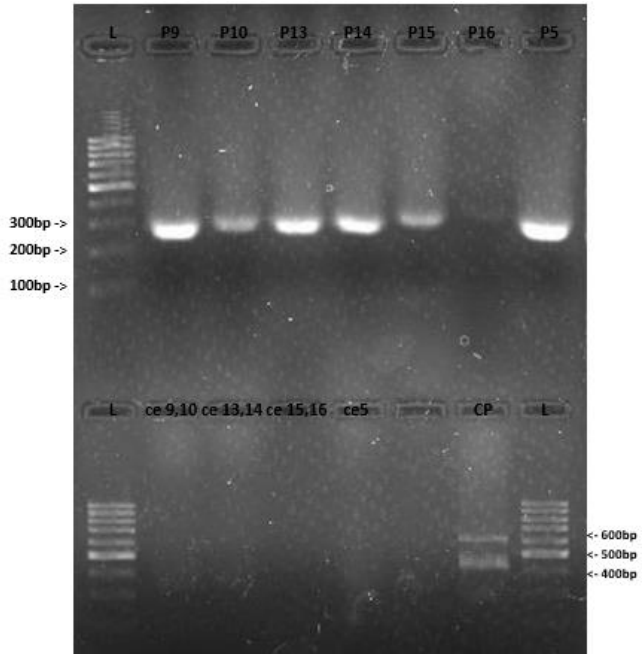


a) b)

Figure 7. Two-step RT-PCR for *Influenza A virus* from cDNA stocks generated with specific primers for this virus. It is observed that the amplicon of the positive control is at an expected approximate height of 232 bp, while the rest show no bands. For a) and b) *L* is ladder; *P* is the pool number; *ce* is extraction control; CP the positive control; and *CN* the negative control.



a) b)



c)

Figure 8. One-Step RT-PCR for 18 S gene. The pools analyzed in this gel show a band at a height close to 300 bp. Pool 16 shows a rather thin band. The extraction controls present no bands while the positive control shows two bands, one of them at the expected height (560 bp). For a), b) and c) *L* is ladder; *P* is the pool number; *ce* is extraction control; CP is the positive control; and *CN* is the negative control.

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