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First report of molecular detection of chicken astrovirus (CAstV) in chicks affected with enteric disease in Ecuador through a fast RT-qPCR assay based on SYBR® Green

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Abstract

Background Chicken meat production has expanded considerably on a global scale due to its ease of production compared to other species. As a result, the prevalence of avian viruses has grown. Chicken astrovirus (CAstV), an RNA virus with roughly 7 kb in length that is disseminated globally and exhibits both horizontal and vertical transmission, is one of the most important enteric pathogenic avian viruses. CAstV and some enteric viruses' infections causes significant economic losses because they are associated with high mortality in chickens. CAstV cause a variety of pathologic changes such as runting and stunning syndrome, nephritis, and white chick syndrome, making early detection critical. For this purpose, the objective of this study was to determine the presence of CAstV in chickens affected with enteric disease through a fast RT-qPCR assay based on SYBR® Green. For this, 120 samples of jejunum from seven-day-old chicks that succumbed to enteric disease characterized by pronounced lethargy, apathy, diarrhea and cloacal pasting were subjected to investigation.

Results At necropsy, the intestines of all chicks appear pale and filled with yellow or green liquid content, thin wall and presence of gas; at the jejunum it was evidenced the presence of remanent yolk sac. The liver, kidneys, spleen did not show any alteration. CAstV RNA was detected and quantified in 85 samples, revealing significant levels (9.6 × 10^6) of CAstV gene copies. This indicates the presence of the virus in Ecuadorian chicks from a few days of age, suggesting vertical transmission and potential sources for the virus's dissemination. The phylogenetic analysis clustered all Ecuadorian sequences in one group related to sequences from India and Brazil. The comparison identity of NT sequences in part of Orf 1b gene showed 83.27 – 93.5% with other sequences of CAstV from India and Brazil. Additionally, the LoD and LoQ were determined in 10^1 gene viral copies. The standard curve showed an efficiency of 97.3% and a melting curve showed a single peak without any alterations and a melting temperature of 77.5 °C. The assay was specific for amplification of the CAstV genome and no amplification was shown from other viral genomes (aMPV, NDV, IBV, AReV, ARoV, ANV) or from the negative controls.

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Conclusions Presents, for the first time, the detection of CAstV in chicks suffering from enteric disease in Ecuador. It also demonstrates that the assay developed herein is an effective tool for the early detection and quantification of CAstV in diseased chickens, while being a reliable, specific, cost-effective, and rapid diagnostic method.

Keywords qPCR, Astrovirus, SYBR, Diagnostic, Chicken

Background

Astroviruses are small spherical viruses with a diameter of 25 to 35 nm, whose genome contains positive-sense single-stranded RNA of approximately 7 kb in length. In addition, their genome encodes 3 proteins: the non-structural polyprotein (NS), the RNA-dependent RNA polymerase (RdRp) and the capsid protein. The NS polyprotein and the capsid protein are encoded by individual open reading frames (ORFs), namely ORF1a and ORF2, whilst the RdRP that acts as a fusion protein for the NS protein is encoded by ORF 1b [1].

This group of viruses is transmitted through the fecal-oral route, causing gastroenteritis in a variety of mammals and different diseases in some poultry including turkeys [Turkey Astrovirus (TAstV)], ducks [Duck Astrovirus (DAstV)], and broilers [Avian Nephritis Virus (ANV) and CAstV)] [2, 3]. Particularly in chickens, it is related to growth retardation, runting and stunting syndrome (RSS), nephritis [4–9], and white chick hatchery disease, all of which infect their progeny by vertical transmission [10–12], although viral particles have also been isolated from clinically healthy birds [13, 14]. The distribution of CAstV is extensive, with reports of its prevalence in regions such as North America, Brazil, China, Poland, Finland, the United Kingdom, and India [3, 8, 10, 15–22].

Initially, the diagnosis of astroviruses was carried out by electron microscopy [23, 24] or by immunoassays that detect viral antigens [4]. However, recent studies in the last two decades have presented some assays for viral detection by RT-PCR [25–27] and molecular characterization allows us to understand the relationship and genetic identity of the different strains isolated around the world [28, 29]. In several countries documented instances of chickens affected with enteric disease associated to CAstV [2, 17, 22, 28, 30–34]. Additionally, poultry farmers in the same country have reported similar cases of enteric diseases (unpublished data), where no bacterial pathogens were isolated, and the etiology of the disease remains undetermined. The high specificity and sensitivity of molecular assays established them as fundamental tools for pathogen identification, while the advancement of innovative techniques facilitates early diagnosis and curtails disease transmission. Thus, the aim of this study was to determine by the first time the presence of CAstV in chicks affected with enteric disease in Ecuador through a rapid and sensitive diagnostic assay that enables the early detection and quantification of CAstV using SYBR® Green-based RT-qPCR.

Results

Macroscopic findings

At the necropsy of received death chicks, pale beaks and claws, and cloacal pasting was observed (120/120 birds). Distended intestines (duodenum, jejunum and ileum) with thin wall, filled with yellow liquid with and gas were observed at the opening of the celomatic cavity (Fig. 1). At the jejunum was evidenced the presence of remanent yolk sac (120/120 birds). Heart, lungs, liver, spleen, kidneys, proventriculus and gizzard did not show any macroscopic abnormalities (120/120 birds).

Primers

The designed primes could amplify 74 bp region flanked the part of CAstV's ORF 1b gene. The primer CASTV-F is located between nucleotide (NT) 4191–4209, and the primer CASTV-R is located between NT 4241–4264 based on the sequence OQ685946.1 (Fig. 2).

Determination of standard curve

The ten dilutions of plasmid DNA were used to build a standard curve demonstrating an efficiency of 97.3%, a slope of -3.389, and a correlation coefficient of 0.999 (Fig. 3A). No primer dimers were detected in any run.

Limit of detection and quantification

The standardized method presented herein was able to detect up to 10^8 copies of the cDNA. The limit of detection (LoD) and limit of quantification (LoQ) was 10^1 target gene copies per µL (Fig. 3A and B). The melting curve showed a single peak without any alterations (Fig. 3B), and a melting temperature of 77.5 °C. No curves were formed in the no template control, and no primer dimers were present.

RT-qPCR run time

The running time of the RT-qPCR assay in fast conditions is approximately one hour, and when the melting curve analysis is included, it takes approximately one and a half hours. The same assay run under standard conditions extends to two hours approximately without the melting curve analysis. The assay run in fast conditions showed high sensitivity, amplifying a few (10^1) to a high number (10^8) of gene copies.

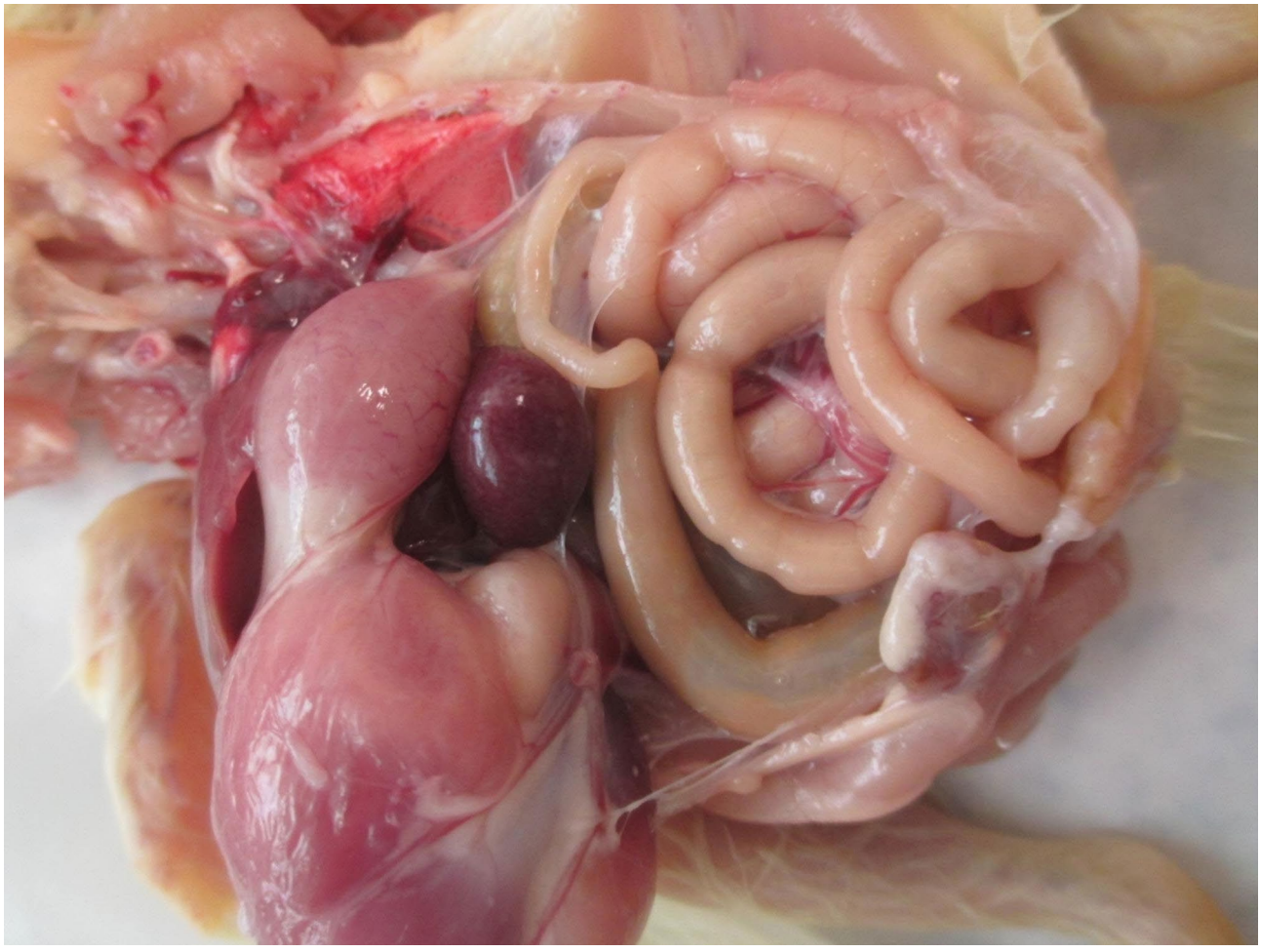

Fig. 1 Necropsy of chick showed intestines filled with liquid and distended with gas

Repeatability of assay

Repeatability analysis performed with 10-fold serial dilutions of standard curve from 10^8 to.

10^4 plasmid copy numbers showed an inter-assay coefficient of variation (CV) of 0.126 to.

0.334%, and an intra-assay CV of 0.298 to 0.485% (Table 1).

Detection and quantification of CAstV and evaluation of the RT-qPCR assay

The RT-qPCR assay based on SYBR® Green developed herein was able to detect cDNA of CAstV in 85 samples (70.83%) of the 120 tissue samples (jejunum) analyzed (Fig. 4). In 35 samples (29.17%) of jejunum CAstV RNA was no detected. Among the samples where CAstV was detected, we could observe a variability in the concentration of viral gene copies (GC) concentration.

Thus, 24 chicks showed a range of 10 to 100 GC, 11 chicks had a range of 101 to 500 GC, 14 chicks had a range of 501 to 5000 GC, while from 5001 GC and beyond the number of particles increased until reaching extremely high viral concentrations (Fig. 5). The highest viral concentration detected was 96582242,47 GC in the jejunum of one of the analyzed chickens (Fig. 5). All positive samples analyzed presented the same melting temperature of 77,5 °C. No primers dimers were detected during any run. When non-template controls or other viruses were submitted in the same assay no primer dimers or any amplification whatsoever were detected.

Phylogenetic analysis

The analysis of obtained sequences with the NCBI BLAST tool showed high identity of nucleotides (NT) with other sequences of CAstV deposited in the GenBank. The phylogenetic analysis of sequences of CAstV showed two well-defined groups, one group (clade bootstrap value = 100%) formed with the sequences of CAstV from India, Brazil, and Slovakia, and the sequences from Ecuador were branched into a clade (clade bootstrap value = 94%) separate from the sequences of the other countries in the group (Fig. 6).

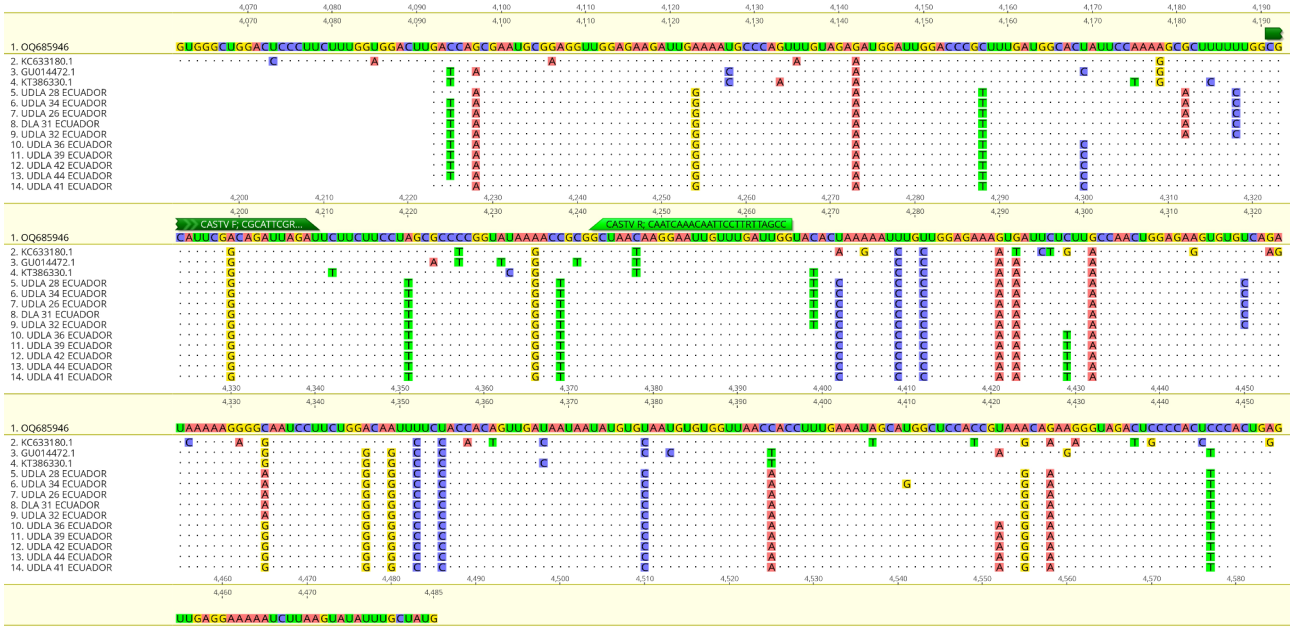

Fig. 2 Alignment built with the sequences used for primer design and the sequences here obtained. The bars in green (different shades) on the reference sequence indicate the location of the primers

The Ecuadorian sequences have a NT identity of 97.5– 100% between them, and these sequences when compared with those from other countries showed 90.7–98.5% identity of NT (India); 83.3– 92.9% identity of NT (Brazil); 77.6– 78% identity of NT (Slovakia); and 34.7–37.9% identity of NT (China) (Fig. 7).

Discussion

The production of poultry meat is steadily rising worldwide since it is an easily manageable source of animal protein for human consumption [20]. Nevertheless, viral diseases in poultry significantly affect the poultry sector economically due to elevated death rates [20, 30], and there are no commercial vaccines available for several enteric viruses [ANV, CAstV, Avian Rotavirus (ARoV), Avian Orthoreovirus (AReV), or Chicken Parvovirus (ChPV)] that could prevent these diseases. One of the most prevalent enteric viruses that cause high mortality especially in young birds are astroviruses [9, 12, 28]. CAstV has a worldwide distribution, and studies indicate that this virus is detected in 96% of chicken flocks that exhibit growth problems [9] and both breeder and broiler flocks demonstrate an equally high prevalence of this virus, regardless of disease status [9, 31]. The present study showed the first report of molecular detection of RNA from CAstV in chicks affected with enteric disease in Ecuador; these birds that showed mainly diarrhea, cloacal pasting, depression and impairment, clinical signs described in birds affected with RSS, a syndrome described for the first time by Olsen [32] in 1977, and since then CAstV has been detected worldwide, and reported as an agent involved in the pathogenesis of disease [2, 17, 27, 33]. The principal macroscopic lesions in the analysed chicks were distended intestines filled with undigested food, yellow to green liquid, think walls and presence of high rate of gas bubbles; these lesions have been described in birds suffering RSS [18, 35–38]. On the other hand, the liver did not show any macroscopic lesion including necrotic foci as observed in CAstV B iv infection [7, 11, 29, 39], which are significant for future research aimed at identifying the circulating genotypes in Ecuadorian chicken populations. The presence of lesions in the intestines of all analysed chicks suggests that CAstV cannot be associated as an etiological agent of these lesions, as 35 chicks tested negative for CAstV. In these birds, other enteric viruses may be present and could be responsible for macroscopic lesions.

Infections caused by CAstV generally occur in the birds first days or weeks of life [8], so it comes of no surprise that among the birds sampled in this study we were able to detect and quantify the virus in seven-day-old birds in a percentage by 70.8% (85/120) (Fig. 5). Interestingly, multiple studies indicate that the more rapid the infection of poultry with the virus, particularly through vertical transmission, the more severe the disease progression can become. Moreover, additional factors can influence this development such as maternal antibodies, viral load, and the presence of other enteric pathogens such as ANV, AReV, and avian adenovirus (FAdV), which are frequently found as pathogens that cause co-infections in birds [2, 5].

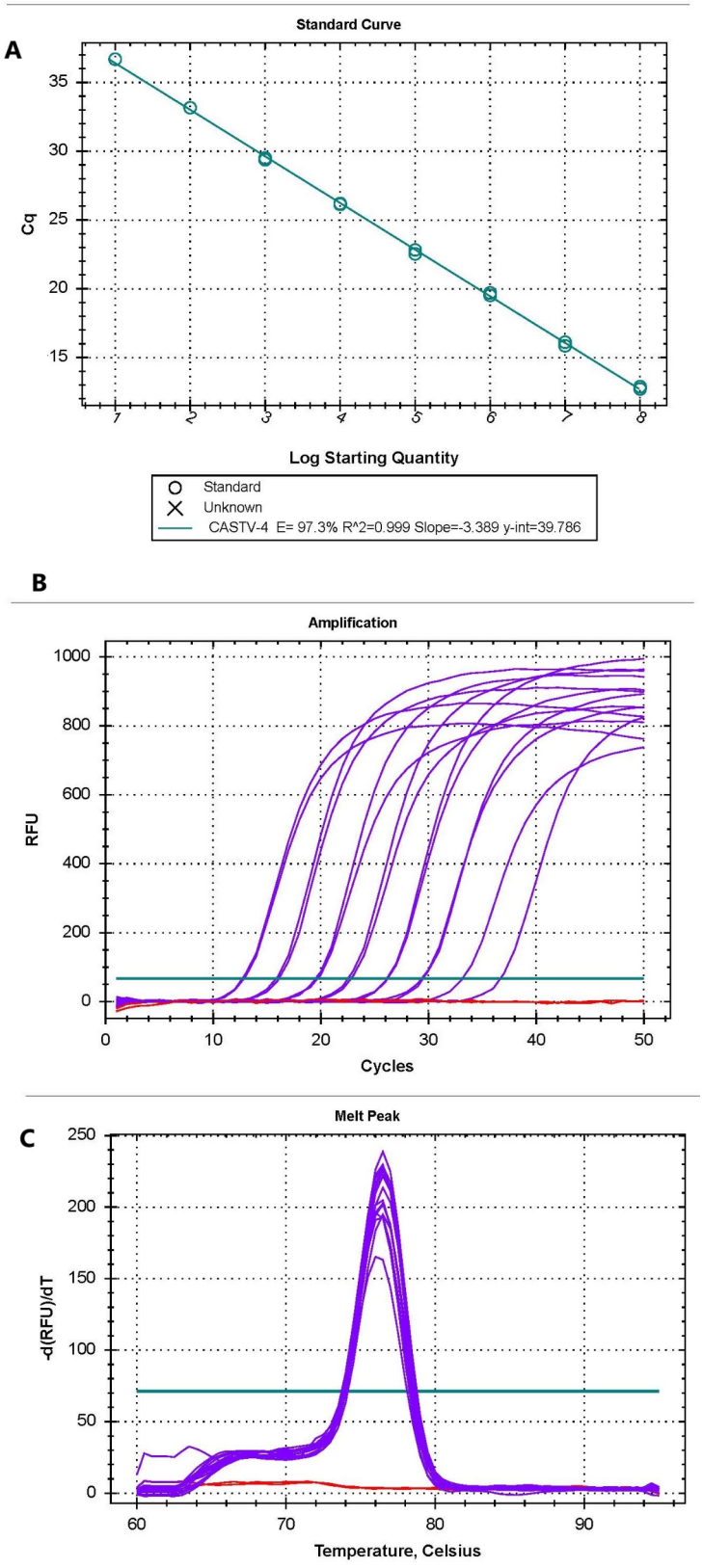

Fig. 3 Real-time PCR with SYBR® FAST PCR double-strand DNA intercalating for specific detection and quantification of the conserved region ORF1b of CAstV: (**A**) Efficiency curve, (**B**) Amplification plot, and (**C**) Melting curve

Table 1 Repeatability analysis performed with standard curve points from 10^8 to 10^4 copies

Copy Number	Inter-Assay		Intra-Assay	
	Cq Mean	Cq St Dev	Cq Mean	Cq St Dev
10^8	13,93	0.138	14,193	0,356
10^7	14,99	0,23	15,250	0,344
10^6	16,75	0,126	17,055	0,485
10^5	21,01	0,321	21,280	0,333
10^4	24,95	0,334	25,165	0,298

Cq = Cycle quantification

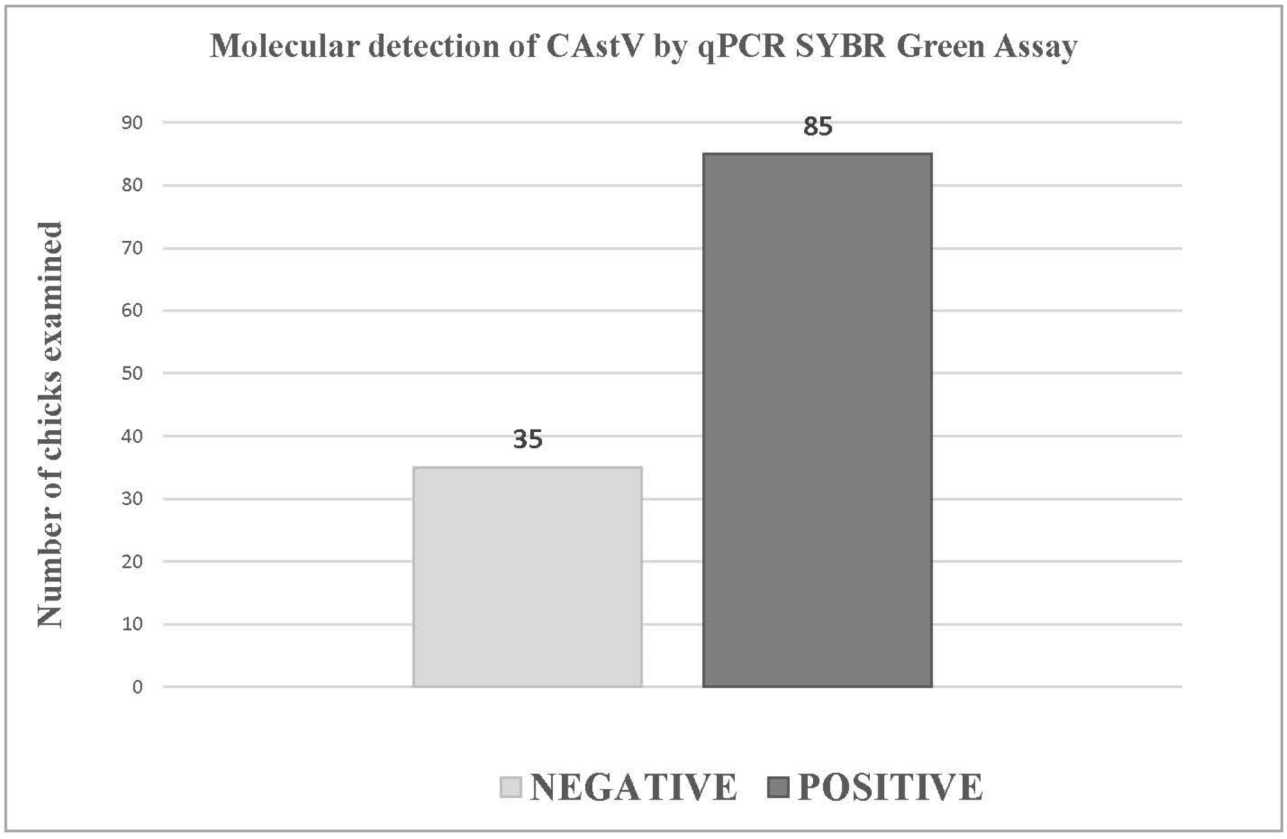

Fig. 4 Number of positive and negative samples for CAstV diagnosed in the investigation

Existing diagnostic methods for the study of astroviruses range from electron microscopy [23, 24], immunoassays [4], isolation in embryonated eggs using different inoculation routes [18] and isolation in cell culture [4], while the most frequently used methods are based on the RT-PCR. However, given the variability of astrovirus genomes, attention should be placed on the proper selection of the reactions to be used [3]. Additionally, RT-qPCR [34] and next-generation sequencing detection methods have also been reported [20]. In our study, we developed a RT-qPCR for the detection and quantification of CAstV targeting the ORF-1b gene that encodes a non-structural protein that is an RNA-dependent RNA polymerase [1, 5, 10, 35]. Our findings show that this RT-qPCR method based on the assay SYBR® Green assay is fast and sensitive, and the amplification time has been reduced compared to qPCR assays with hydrolysis probe technology-based qPCR assays and standard run times [34]. Nevertheless, the use of hydrolysis probes increases the economic cost of the assay [40–42], so the assay used here could be a cheaper, faster and more effective diagnostic alternative for the diagnosis of CAstV.

The efficiency curve of the assay was 97.3% (Fig. 3A), implying that the results are highly reliable, and that the standard curve can be used for the quantification of CAstV. Moreover, the melting curve obtained showed a single peak at 77.5 °C (Fig. 3C) that could be used to confirm the presence of the virus in the samples. Using this rapid RT-qPCR based on the SYBR® assay we report the detection and quantification of CAstV in the intestinal tract (jejunum) of 7 days old broiler chickens (Figs. 4 and 5), all died presenting enteric problems. Our results

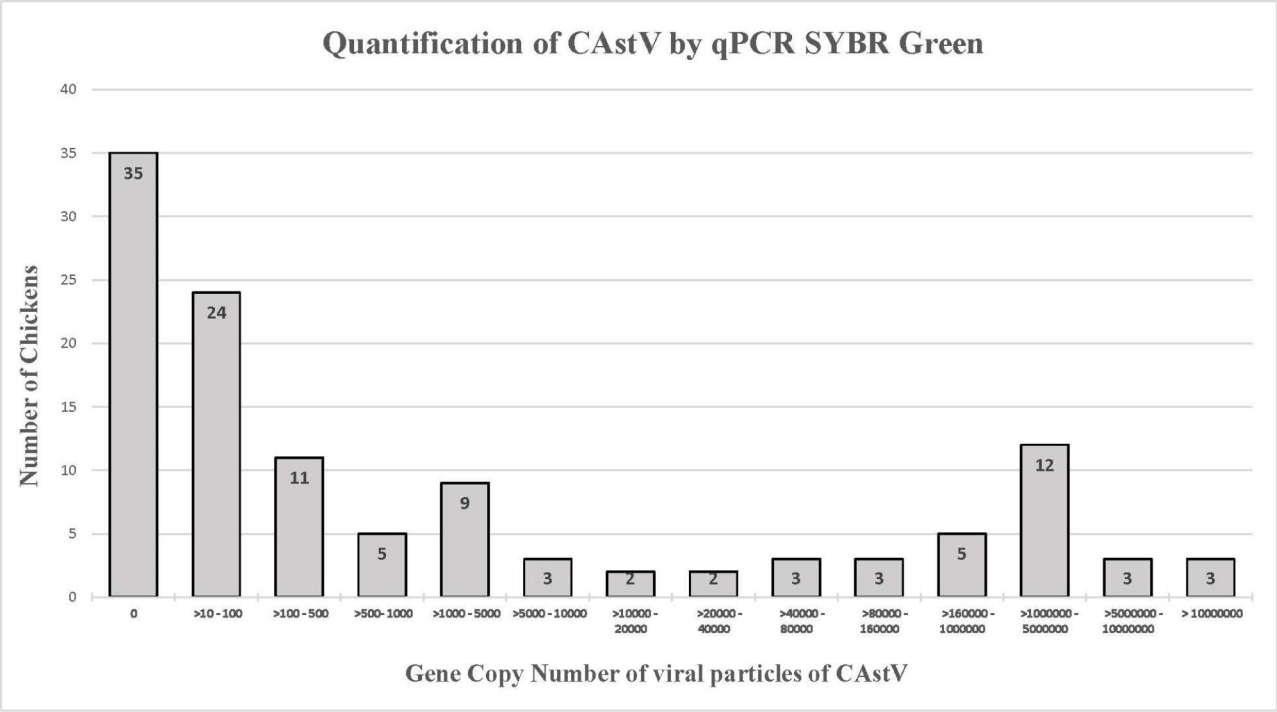

Fig. 5 Quantification of CAstV (GC) and the number of positive birds for CAstV

concur with a previous study carried out by Smyth et al., 2010 [34] that also detected and quantified CAstV in intestinal content samples from broiler flocks with growth problems that could last up to three weeks after the birds are born [36].

Finally, we determined a LoD and LoQ on 10^1 viral particles per µL for our assay showing more sensibility than other assays [43] and were able to detect a concentration as high as 96582242.47 GC in a single sample; thus, indicating that the virus is present in significant concentrations from an early age [8] and also demonstrated limited GC of the virus. In Smyth's study [34], high virus levels (> 105.99 virus copies) were quantified from gut content samples in 67% of broiler flocks that exhibited growth delay, a finding that was similar with to the results of our study where we detected the presence of the virus in 70.83% o the samples in birds that also presented clinical signs of enteric disease. However, a percentage of 29.17% analysed samples tested negative for CAstV detection, showing that other pathogens (ChPV, FAdV, ANV, IBV, AReV or ARoV) beyond or in co-infection with CAstV related to RSS pathogenesis could be responsible of the enteric disease and chicks' mortality [2, 18, 36, 44–49], indicating the significance to continue with future studies aimed at determining the presence or absence of these enteric viruses in birds affected with enteric disease in Ecuador. In the present study some randomly selected samples were sequenced, where RNA of CAstV was identified by proposed RT-qPCR assay. The analysis of the sequences showed high identity with other sequences of the same virus from India, Brazil and Slovakia, depicting that the genetic material identified here belonged to CAstV genome, and indicates a part of the same genome. The phylogenetic analysis showed also that the sequences obtained here are genetically related to viral genetic material from countries from South America, India and Europa, but not to sequences from China.

There is a lack of information regarding the molecular characteristics of CAstV in Ecuador; however, this study reveals that the most closely related viral genetic material originates from India and Brazil, suggesting a potential source of the virus in the country, which requires further investigation. This analysis indicates a diminished genetic relationship between the Chinese and European CAstV sequences. All information is important, nevertheless, more studies need to be carried out in order to determine the genotypes of CAstV that are circulating in the Ecuadorian poultry and which could be related to the genesis of enteric diseases. As previously mentioned, the CAstV based in their capsid diversity could be determined in genotype A and B, related with enteric problems [2, 8, 17, 18, 33, 34, 37], white chicks syndrome [10–12, 28, 29], and visceral gout [20, 38], making the classification of CAstV in Ecuador particularly important.

Consequently, the present study is the first to report, the molecular detection of CAstV in chicks that died from enteric diseases in Ecuador. The proposed assay, performed herein, is economical (not requiring

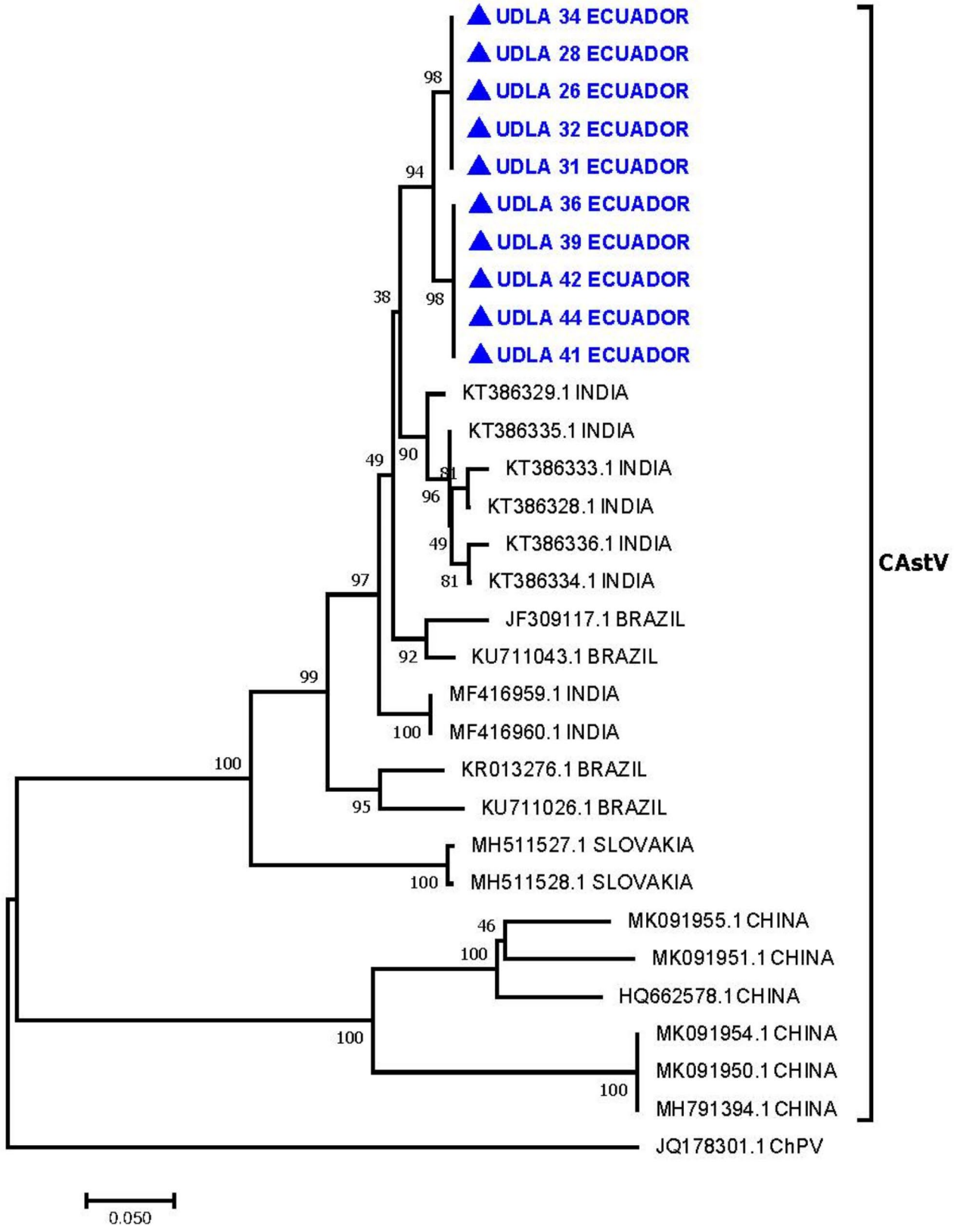

Fig. 6 (See legend on next page.)

(See figure on previous page.)

Fig. 6 Phylogenetic relations between the sequences obtained here of CAstV detected in chickens with enteric diseases and other sequences of CAstV based on a part of ORF 1b gene nucleotide sequences. Sequences were aligned using the CLUSTAL W method in ClustalX2 2.1. The phylogenetic tree was constructed using the MEGA 7 software package. The numbers along the branches refer to the bootstrap values of 1000 replicates. The scale bar represents the number of substitutions per site. Chicken Parvovirus (ChPV) was used as the outgroup. ▲ = Sequences obtained in this study and written in blue

hydrolysis probes), rapid (taking approximately one hour), and highly specific for the molecular diagnosis of CAstV. Notably, none of the other viruses tested using the same protocols were capable of producing gene amplification products.

Conclusion

The present study shows the first report of molecular detection of CAstV in death chicks that have been affected with enteric disease, showing the virus as a potential causal agent of enteric disease circulating in commercial chicken flocks in Ecuador, which may contribute to mortality in the country's chick populations. This factor must be considered when establishing biosecurity measures to mitigate the virus's impact. Furthermore, the developed method for detecting and quantifying CAstV presents a valuable alternative, enabling rapid and effective diagnosis. Given that CAstV is a pathogen responsible for various clinical manifestations in poultry, its swift detection could improve disease prevention and enhance the efficacy of available treatments.

Methods

Samples

The chicks utilized in this investigation were dispatched deceased to the Universidad de Las Americas for the identification of enteric viruses from six farms situated in Pichincha state, where 20 chicks arrive from each farm. The poultry farmers reported that the birds were kept to 4 °C from the time of collection until their arrival at the laboratory. The clinical record indicated that the animals perished due to an intestinal ailment endemic to the farm of their upbringing. This study involved 120 seven-day-old broiler chickens exhibiting clinical signs of enteric disease, including apathy, ruffled feathers, diarrhea, and cloacal pasting, which were sent to the research laboratory of Universidad de Las Americas in Quito, Ecuador, to investigate the presence of CAstV. The deceased animals underwent necropsy, and the jejunum was utilized to ascertain the presence or absence of CAstV, in addition to the standardization and validation of the RT-qPCR assay employing SYBR Green.

RNA extraction

The RNA was extracted from approximately 100 mg of jejunum macerated using stainless steel beads in TissueLyser LT (Qiagen® USA). The macerated samples were then subjected to RNA extraction using the TRIzol reagent (Invitrogen by Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription

The RNA obtained was submitted to a reverse transcription reaction to obtain complementary DNA (cDNA). A volume of 3.5 µL of extracted RNA was incubated at 65 °C for 5 min together with 10 mM of deoxynucleotide triphosphates (dNTPs), 2.5 µM of random and oligo dT primers and 6.5 µL of a mixture containing 2 µL 5X buffer, 1 µL dithiothreitol (DTT), 0.5 µL SuperScript™ III Reverse Transcriptase enzyme (Invitrogen Life Technologies, Carlsbad, CA) and UltraPure™ DNase/RNase-free Distilled dH2O (Invitrogen by Life Technologies, Carlsbad, CA, USA). The reverse transcription reaction was performed under the following conditions: 25 °C for 5 min followed by 50 °C for 60 min and 70 °C for 15 min. The cDNA obtained was submitted to PCR or qPCR.

Molecular detection of CAstV

cDNA cloning and primer design

The obtained cDNA was subjected to an endpoint PCR as described previously by Day et al., 2007 [25]. For the PCR reaction, 23 µL of the reaction mixture was used and complemented with 0.5 µM of the forward and reverse primers (Table 2), 2x Buffer, 5 mM of each dNTP, 37.5 mM of Mg, and 1 U of Platinum Taq DNA polymerase (Thermo Fisher Scientific, Carlsbad, CA, USA), and 2 µL of cDNA. The cDNA of samples UDLA 26 and UDLA 40 were subjected to PCR amplification following the next conditions: one cycle of 95 °C for 5 min to completely denature the DNA followed by 35 cycles of 95 °C for 30 s for template denaturation, 56 °C for 30 s for primer annealing, and 72 °C for 45 s for DNA extension, and lastly, a final extension step at 72 °C for 10 min. The reaction was maintained at 4 °C till stored at – 20 °C. The PCR products were subjected to electrophoresis in a 1.5% agarose gel, and the samples were stained with SYBR® Safe DNA gel stain (Invitrogen by Thermo Fisher Scientific Carlsbad, CA 92008 USA) and compared against a 100-bp molecular ladder (Invitrogen by Thermo Fisher Scientific Carlsbad, CA 92008 USA). The gels were analyzed and photographed using a UV Transilluminator 2000 (BioRad, California 94547, CA, USA). After the aforementioned PCR amplification reaction, the generated product was inserted into a PCR 2.1-TOPO vector (Invitrogen by Thermo Fisher Scientific Carlsbad, CA 92008

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.
1. UDLA 26 ECUADOR		99.45%	99.72%	99.72%	99.45%	97.51%	97.51%	97.24%	97.51%	97.51%	93.83%	92.62%	91.61%	91.93%	91.16%	88.12%	83.99%	90.75%	77.55%	77.55%	37.87%	35.47%	35.47%	35.73%	33.97%
2. UDLA 28 ECUADOR	99.45%		99.72%	99.72%	99.45%	97.51%	97.51%	97.79%	97.51%	97.51%	93.18%	92.00%	91.30%	91.61%	90.61%	87.57%	83.63%	91.10%	77.96%	77.96%	37.87%	35.20%	35.20%	35.47%	34.24%
3. UDLA 31 ECUADOR	99.72%	99.72%		100%	99.72%	97.79%	97.79%	97.51%	97.79%	97.79%	93.51%	92.31%	91.30%	91.61%	90.88%	87.85%	83.63%	91.10%	77.96%	77.96%	37.60%	35.47%	35.47%	35.73%	33.97%
4. UDLA 32 ECUADOR	99.72%	99.72%	100%		99.72%	97.79%	97.79%	97.51%	97.79%	97.79%	93.51%	92.31%	91.30%	91.61%	90.88%	87.85%	83.63%	91.10%	77.96%	77.96%	37.60%	35.47%	35.47%	35.73%	33.97%
5. UDLA 34 ECUADOR	99.45%	99.45%	99.72%	99.72%		97.51%	97.51%	97.24%	97.51%	97.51%	93.51%	92.00%	90.99%	91.30%	90.61%	87.57%	83.27%	90.75%	77.55%	77.55%	37.60%	35.47%	35.47%	35.73%	33.97%
6. UDLA 36 ECUADOR	97.51%	97.51%	97.79%	97.79%	97.51%		100%	99.72%	100%	100%	93.51%	92.00%	90.68%	90.99%	91.99%	87.85%	83.27%	92.88%	77.96%	77.96%	36.80%	34.93%	34.93%	35.20%	34.78%
7. UDLA 39 ECUADOR	97.51%	97.51%	97.79%	97.79%	97.51%	100%		99.72%	100%	100%	93.51%	92.00%	90.68%	90.99%	91.99%	87.85%	83.27%	92.88%	77.96%	77.96%	36.80%	34.93%	34.93%	35.20%	34.78%
8. UDLA 41 ECUADOR	97.24%	97.79%	97.51%	97.51%	97.24%	99.72%	99.72%		99.72%	99.72%	93.18%	91.69%	90.68%	90.99%	91.71%	87.57%	83.27%	92.88%	77.96%	77.96%	37.07%	34.67%	34.67%	34.93%	35.05%
9. UDLA 42 ECUADOR	97.51%	97.51%	97.79%	97.79%	97.51%	100%	100%	99.72%		100%	93.51%	92.00%	90.68%	90.99%	91.99%	87.85%	83.27%	92.88%	77.96%	77.96%	36.80%	34.93%	34.93%	35.20%	34.78%
10. UDLA 44 ECUADOR	97.51%	97.51%	97.79%	97.79%	97.51%	100%	100%	99.72%	100%		93.51%	92.00%	90.68%	90.99%	91.99%	87.85%	83.27%	92.88%	77.96%	77.96%	36.80%	34.93%	34.93%	35.20%	34.78%
11. KT386335.1 INDIA	93.83%	93.18%	93.51%	93.51%	93.51%	93.51%	93.51%	93.18%	93.51%	93.51%		96.75%	92.76%	93.10%	92.53%	89.61%	84.58%	93.39%	76.96%	76.96%	35.83%	34.89%	34.89%	35.20%	34.39%
12. KT386329.1 INDIA	92.62%	92.00%	92.31%	92.31%	92.00%	92.00%	92.00%	91.69%	92.00%	92.00%	96.75%		93.16%	93.49%	90.15%	88.00%	86.07%	93.85%	78.85%	78.85%	37.28%	35.21%	35.21%	35.50%	34.14%
13. MF416959.1 INDIA	91.61%	91.30%	91.30%	91.30%	90.99%	90.68%	90.68%	90.68%	90.68%	90.68%	92.76%	93.16%		99.69%	89.75%	86.34%	84.56%	91.12%	82.51%	82.51%	38.81%	36.12%	36.12%	36.12%	35.37%
14. MF416960.1 INDIA	91.93%	91.61%	91.61%	91.61%	91.30%	90.99%	90.99%	90.99%	90.99%	90.99%	93.10%	93.49%	99.69%		90.06%	86.65%	84.94%	91.51%	82.51%	82.51%	38.81%	36.12%	36.12%	36.12%	35.37%
15. JF309117.1 BRAZIL	91.16%	90.61%	90.88%	90.88%	90.61%	91.99%	91.99%	91.71%	91.99%	91.99%	92.53%	90.15%	89.75%	90.06%		87.02%	82.92%	93.95%	76.33%	76.33%	36.27%	33.33%	33.33%	33.60%	35.33%
16. KR013276.1 BRAZIL	88.12%	87.57%	87.85%	87.85%	87.57%	87.85%	87.85%	87.57%	87.85%	87.85%	89.61%	88.00%	86.34%	86.65%	87.02%		89.68%	85.41%	75.10%	75.51%	36.27%	35.47%	35.47%	35.73%	34.51%
17. KU711026.1 BRAZIL	83.99%	83.63%	83.63%	83.63%	83.27%	83.27%	83.27%	83.27%	83.27%	83.27%	84.58%	86.07%	84.56%	84.94%	82.92%	89.68%		81.49%	74.92%	75.25%	35.06%	35.42%	35.42%	34.77%	36.07%
18. KU711043.1 BRAZIL	90.75%	91.10%	91.10%	91.10%	90.75%	92.88%	92.88%	92.88%	92.88%	92.88%	93.39%	93.85%	91.12%	91.51%	93.95%	85.41%	81.49%		74.35%	74.35%	34.76%	34.23%	34.23%	33.33%	35.59%
19. MH511527.1 SLOV	77.55%	77.96%	77.96%	77.96%	77.55%	77.96%	77.96%	77.96%	77.96%	77.96%	76.96%	78.85%	82.51%	82.51%	76.33%	75.10%	74.92%	74.35%		99.71%	35.87%	36.00%	36.00%	34.60%	34.56%
20. MH511528.1 SLOV	77.55%	77.96%	77.96%	77.96%	77.55%	77.96%	77.96%	77.96%	77.96%	77.96%	76.96%	78.85%	82.51%	82.51%	76.33%	75.51%	75.25%	74.35%	99.71%		35.87%	36.33%	36.33%	34.92%	34.84%
21. HQ662578.1 CHINA	37.87%	37.87%	37.60%	37.60%	37.60%	36.80%	36.80%	37.07%	36.80%	36.80%	35.83%	37.28%	38.81%	38.81%	36.27%	36.27%	35.06%	34.76%	35.87%	35.87%		77.62%	77.86%	77.25%	28.34%
22. MK091954.1 CHINA	35.47%	35.20%	35.47%	35.47%	35.47%	34.93%	34.93%	34.67%	34.93%	34.93%	34.89%	35.21%	36.12%	36.12%	33.33%	35.47%	35.42%	34.23%	36.00%	36.33%	77.62%		99.77%	99.53%	29.59%
23. MK091950.1 CHINA	35.47%	35.20%	35.47%	35.47%	35.47%	34.93%	34.93%	34.67%	34.93%	34.93%	34.89%	35.21%	36.12%	36.12%	33.33%	35.47%	35.42%	34.23%	36.00%	36.33%	77.86%	99.77%		99.30%	29.59%
24. MH791394.1 CHINA	35.73%	35.47%	35.73%	35.73%	35.73%	35.20%	35.20%	34.93%	35.20%	35.20%	35.20%	35.50%	36.12%	36.12%	33.60%	35.73%	34.77%	33.33%	34.60%	34.92%	77.25%	99.53%	99.30%		29.03%
25. JQ178301.1 ChPV	33.97%	34.24%	33.97%	33.97%	33.97%	34.78%	34.78%	35.05%	34.78%	34.78%	34.39%	34.14%	35.37%	35.37%	35.33%	34.51%	36.07%	35.59%	34.56%	34.84%	28.34%	29.59%	29.59%	29.03%	

Fig. 7 Comparison of the nucleotide of Ecuadorian samples of CAstV with other sequences of this virus

Table 2 Primers used in this study

Primer	Gene	Assay	Sequences	Product	Reference
CAS pol 1 F	ORF 1b	RT-PCR	5′-GAYCARCGAATGCGRAGRTTG-3′	362 bp	[25]
CAS pol 1R			5′-TCAGTGGAAGTGGGKARTCTAC-3′		
CASTV AF		RT-qPCR	5′- CGCATTCGRCAGATTAGAT-3′	74 pb	This study
CASTV AR			5′- CAATCAAACAATTCCTTRTTAGCC-3′		

USA) and transformed and cloned into *E. coli* competent cells according to the manufacturer's instructions. Plasmid DNA was extracted from a culture of cloned bacteria using a QIAprep Spin Miniprep Kit (Qiagen, USA). The plasmid DNA was subjected to endpoint PCR as described by Day et al., 2007 [25] to confirm that the PCR fragment was correctly inserted into the vector and sequenced three times in both directions using a Big Dye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems by Thermo Fisher Scientific CA 94404 USA). The sequencing reaction was performed using an ABI 3730 DNA Analyzer (Applied Biosystems by Thermo Fisher Scientific CA 94404 USA). The obtained sequences were edited and aligned using the software package Geneious Prime 2022.1.1 (https://www.geneious.com), and the identity with other CAstV sequences found in GenBank was determined using the BLAST tool. The plasmid DNA where was confirmed that the part of ORF 1b gene was inserted was used as standard DNA. One pair of primers were designed using the software package Geneious Prime 2022.1.1 (https://www.geneious.com) (Table 2) based on the ORF 1b gene alignment built with the sequences KT386330.1, KC633180.1, GU014472.1, OQ685946.1 and the obtained sequences (Fig. 2).

Standard curve construction

To define the sensitivity of the assay, plasmid DNA extracted from the bacterial culture as described above that had the PCR fragment of the ORF 1b gene of CAstV was quantified as described above; and through, the web tool DNA copy number and dilution calculator (Thermo Fisher Scientific CA, USA) a dilution was made with an amount of 10^8 of plasmid DNA and the curve was generated based on nine serial dilutions with a base of 10, resulting in a standard curve with 10^1 to 10^8 plasmid copy numbers. The absolute quantification of CAstV copy numbers was performed based on the standard curve obtained and presented as viral gene copies per mg of tissue. The efficiency of the RT-qPCR reaction was also established using the plasmid serial dilution method.

Limit of detection and quantification

The LoD and LoQ of the method were established using the same standard curve, determining the minimum number of copies of plasmid DNA our assay was able to detect and quantify.

RT-qPCR to detect CAstV

CAstV presence was tested in the intestinal samples of jejunum, where the viral RNA was detected and quantified using the RT-qPCR assay developed to amplify a part of the ORF 1b gene. The reactions were prepared to a final volume of 10 µL using a mixture containing 5 µL of PowerUpTM SYBR® Green Master Mix (2x) (Applied Biosystems by Thermo Fisher Scientific CA 94404 USA), 0.5 µM of each primer (Table 2), 1 µL of cDNA from each sample and UltraPure™ DNase/RNase-free Distilled dH2O to reach the total volume (Invitrogen by Life Technologies, Carlsbad, CA, USA). NTCs (No Template Controls) were prepared by substituting the cDNA with an equal volume of dH2O. The reactions were run using a CFX 96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA) in fast mode with a UDG activation step at 50 °C for 2 min, Dual-Lock DNA polymerase step at 95 °C for 2 min, followed by 40 cycles at 95 °C for 3 s and 60 °C

for 30 s. A dissociation (melting) curve was performed in three steps: 95 °C for 15 s, followed by a decrease in temperature to 60 °C for 1 min, and a gradual increase in temperature (0.3 °C) up to 95 °C. All samples were tested in duplicate, and absolute quantification was performed using the standard curve obtained in each run. A standard plasmid was used as the positive control, ddH2O was used as a negative control and NTCs were run in each assay to ensure functionality.

Repeatability of assay

For assessing the intra-assay and inter-assay repeatability and stability of the RT-qPCR assay, 10-fold serial dilutions of the reference samples of standard curve from 10^8 to 10^4 were prepared for RT-qPCR assay, each of these dilutions was aliquoted separately and stored to -20 C until use. The average value of Ct and the CV were calculated according to the test results, and the stability of the assay was evaluated by CV.

Intra-assay repeatability: five 10-fold serially diluted reference samples of standard curve from 10^8 to 10^4 were prepared and 5 replicates were run for each dilution factor. The RT-qPCR assays were performed simultaneously.

Inter-assay repeatability: five 10-fold serially diluted reference samples of standard curve from10^8 to 10^4 were amplified by RT-qPCR 5 times under the same reaction conditions by two operators on different days. In each amplification a different aliquot of the five serial dilutions was thawed, and checked if there are variations in Cq from 0.5 to 1 at the curving point of each thawed aliquot during each amplification according to the MIQE guidelines [50].

Specificity of RT-qPCR test

In order to determine the specificity of the test, other viruses like Avian pneumovirus (aMPV), AReV, Newcastle diseases virus (NDV), infectious bronchitis virus (IBV), ANV and ARoV were also tested using the assay developed for CAstV detection.

Sanger sequencing and phylogenetic analyses

In order to confirm that the current assay is binding to a part of the Orf 1b gene of CAstV and is capable to diagnose CAstV, eight samples were randomly selected from the positive samples determined in the present study. Therefore, the cDNA obtained previously as described above of these samples were subjected to an endpoint PCR as described by Day et al., 2007 [25], with the conditions described above. The amplicons generated were purified using a ExoSAP-IT™ Express PCR product Cleanup (Applied Biosystems, Santa Clara, CA 95051, USA) as described by the manufacturer. Each purified product was sequenced in the forward and reverse sense using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequencing reaction was carried out using an ABI 3730 DNA Analyzer (Applied Biosystems by Thermo Fisher Scientific CA 94404 USA). The obtained sequences were edited and assembled using the de novo assembly method with Geneious software package 11.0.1 (https://www.geneious.com). The generated sequences were analyzed using the BLAST tool to determine the identity between the sequences and other sequences deposited in the GenBank. The nucleotide sequences obtained here were aligned and compared with other sequences of CAstV from other parts of the world using the CLUSTAL W method available in the ClustalX 2.0.11 software package (European Bioinformatics Institute Saron Walden CB 10 1SD, UK). The phylogenetic analyses were performed in MEGA 7 software [51], then the phylogenetic tree was built with a Neighbor-joining statistical method along with a p-distance substitution model and phylogeny test bootstrap model with 1000 replicates.

Statistical analysis

A descriptive analysis of the data of the analyzed samples was carried out in R software package 4.3.1 version.

GenBank accession numbers

The sequences obtained here from a part of the ORF 1b gene were submitted to the Genbank under the accession number: UDLA 26 (OR545217.1); UDLA 28 (OR545219.1); UDLA 31 (OR545215.1); UDLA 32 (OR545216.1); UDLA 34 (OR545218.1); UDLA 36 (OR545210.1); UDLA 39 (OR545211.1); UDLA 41 (OR545214.1); UDLA 42 (OR545213.1); UDLA 44 (OR545212.1).

Abbreviations

LoD	Limit of detection
LoQ	Limit of quantification
AReV	Avian orthoreovirus
FAdV	Fowl adenovirus
BC	Broiler chicken
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
NTCs	Non template controls
NTC	Non-template control
aMPV	Avian metapneumovirus
NDV	Newcastle disease virus
IBV	Infectious bronchitis virus
ARoV	Avian rotavirus
ANV	Avian nephritis virus
TAstV	Turkey astrovirus
DAstV	Duck astrovirus
CAstV	Chicken astrovirus
ChPV	Chicken parvovirus
NT	Nucleotide
Cq	Cycle quantification
RSS	Runting and stunting syndrome
CV	Coefficient of variation
GC	Gene copies
RdRp	RNA-dependent RNA polymerase
NS	Nonstructural
ORFs	Open reading frames

Author contributions
S.S.P. and L.N. participated in the study design; S.S.P. wrote the manuscript; S.S.P., A.L.G., and L.N. collected the information on study; S.S.P., C.A.F., A.L.G., A.J.P.F. and L.N. carried out the study; S.S.P. statistically analysis, S.S.P., C.A.F., A.L.G., N.C.K., A.J.P.F. and L.N. revised the manuscript.

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Data availability
All data supporting the findings of this study are available within the paper. The sequences obtained here of a part of the ORF 1b gene were submitted to the Genbank under the accession number: UDLA 26 (OR545217.1); UDLA 28 (OR545219.1); UDLA 31 (OR545215.1); UDLA 32 (OR545216.1); UDLA 34 (OR545218.1); UDLA 36 (OR545210.1); UDLA 39 (OR545211.1); UDLA 41 (OR545214.1); UDLA 42 (OR545213.1); UDLA 44 (OR545212.1).

Declarations

Ethics approval and consent to participate
All experimental procedures conducted in the present investigation were in accordance with the guidelines and the approval of the Committee for the Care and Use of Laboratory and Domestic Animal Resources of the Agency of Regulation and Control of Phytosanitary and Animal Health of Ecuador (AGROCALIDAD), under number #INT/DA/019. The study was carried out in compliance with the ARRIVE guidelines. All methods were carried out following relevant guidelines and regulations. Permissions from farm owners for sample collection were obtained verbally or in writing prior to the commencement of the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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