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A novel real-time RT-PCR assay for influenza C tested in Peruvian children

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ABSTRACT

Background: Influenza C virus (ICV) is associated with acute respiratory illness. Yet ICV remains under recognized, with most previous studies using only culture to identify cases.
Objectives: To develop a sensitive and specific real-time RT-PCR assay for ICV that allows for rapid and accurate detection in a clinical or research setting.
Study design: Multiple ICV sequences obtained from GenBank were analyzed, including 141 hemagglutinin-esterase (HE), 106 matrix (M), and 97 nucleoprotein (NP) sequences. Primers and probes were designed based on conserved regions. Multiple primer-probe sets were tested against multiple ICV strains.
Results: The ICV M and NP genes offered the most conserved sequence regions. Primers and probes based on newer sequence data offered enhanced detection of ICV, especially for low titer specimens. An NP-targeted assay yielded the best performance and was capable of detecting 10–100 RNA copies per reaction. The NP assay detected multiple clinical isolates of ICV collected in a field epidemiology study conducted in Peru.
Conclusions: We report a new real-time RT-PCR assay for ICV with high sensitivity and specificity.

1. Background

Influenza C virus (ICV) has been recognized as a cause of acute respiratory illness (ARI) in humans since 1947 [1]. However, since ICV is difficult to culture, the true burden of disease has remained unclear. Seroprevalence of ICV has been reported to be as high as 64% by 5 years of age, suggesting that many infections occur during early childhood [2–5]. Recent RT-PCR studies suggest that ICV may be more commonly associated with ARI than previously thought [6–8]. However, ICV assays are not commercially available or widely tested in children. We sought to develop a new real-time RT-PCR assay for ICV based on all available sequence data. We then used the assay to test specimens collected during a study of Peruvian children with ARI.

2. Study design

2.1. Sequence analysis and primer design

Published HE, M, and NP gene sequences were obtained from GenBank and aligned using MacVector 12 (MacVector). Sequences are listed in Supplemental Tables 1 and 2. Primers and probes were designed using Primer Express 2.0 (Applied Biosystems) targeting conserved regions. We tested a previously published ICV real-time RT-PCR assay targeting the NP gene [7]. Probes were 5′-labeled with 6 ~ FAM, 3′-labeled with Black Hole Quencher (BHQ1a), and purified by HPLC.

2.2. Real-time RT-PCR

Real-time RT-PCR was performed in 25-μL reactions containing 0.8 μM forward and reverse primers, 0.2 μM probe, and 5 μL of extracted RNA on an ABI StepOnePlus (Thermo Fisher) using the AgPath-ID One-Step RT-PCR kit (Thermo Fisher). Cycling conditions were 50 °C

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Table 1
Primer and probe sequences tested.

Assay	Gene	Forward primer (5′–3′)	Reverse Primer (5′–3′)	Probe (5′–3′)
M2	M	ACAGCTTGGTTGAGATGCAA	CAAGGCCAGTAATACCAGCA	AGCCAGCACAGCAATGAAYGAAA
M3	M	AATGAGGGCCTTYAGAGATG	TTGCATCTCAACCAAGCTGT	TGATCAYCCAGACGAYTACACACCA
NP1 [7]	NP	GCRTGCTTTGGRCTTGCTTATG	ARTTTCCTATTTTCATTCTGTTTCTCAAC	TTTGGTYTCTGCYATGGTYAGCCAYCCTCT
NP2	NP	TTTGTAAGGAAGTGGGCCTT	TGGGCKTCYCTTGCTTTCTTT	CCAGKATCAACATTTCATCGCCA
NP4	NP	CCGYTCAAGAATTGTTCAAA	CTTGCTGCRTTTCTTCCTCT	TCGGCTTCTCWGCACTCTTYGCTTC

for 30′, 95 °C for 10′minutes, and 45 cycles of 15″ at 95 °C and 30″ at 55 °C. Fluorescence was measured during the 55 °C annealing/extension step. Nuclease-free water served as negative control in all experiments.

2.3. Assay optimization, sensitivity, and specificity

Primers and probes targeting M gene sequences were tested against plasmids containing the segment 6 coding region of C/Ann Arbor/1/50 (pCM2AA) and C/Taylor/1233/47 (pCM2Tay) [9]. Assays were then tested against RNA extracted from cultures of C/Ann Arbor/1/50 (C/Ann Arbor) and C/Taylor/1233/47 (C/Tay), prototype ICV strains [1,10]. Sensitivity and linearity of assays were determined using RNA runoff transcripts. To generate RNA runoff transcripts, the assay target gene segment was cloned into pGEM (Promega), transcribed *in vitro* from *Hinc*II-digested plasmids using T7 RNApol (NEB), purified, and quantified by spectrophotometry. The assay was tested in multiple replicates against serial 10-fold dilutions of RNA transcripts. Specificity of the assay was determined by testing against RNA from influenza viruses A H3N2, B, and 2009 H1N1, human metapneumovirus (MPV), respiratory syncytial virus (RSV), human rhinovirus (HRV), parainfluenza virus (PIV) types 1, 2, and 3, adenovirus (AdV), and coronaviruses (NL63, OC43, 229E, and MERS).

2.4. Clinical specimens

The NP4 assay was tested on nasal swab (NS) specimens from the study of Respiratory Infections in Andean Peruvian children (RESPIRA-PERU), a prospective cohort study designed to evaluate the epidemiology of respiratory viruses in ARI among young Andean children fully described elsewhere [11,12]. Briefly, the study was conducted in the rural province of San Marcos in the Peruvian northern highlands. A dynamic cohort of ~500 children < 3 years was followed from May 1, 2009 through September 30, 2011 through weekly active household-based ARI surveillance. ARI was defined as the presence of cough or fever, and an ARI was considered severe ARI/pneumonia if any sign or symptom of lower respiratory infection was present, including tachypnea, wheezing, nasal flaring, retractions, grunting, stridor, or cyanosis, or any general World Health Organization danger sign (inability to drink, lethargy, *etc.*) [13]. Newborns in study households were enrolled and children followed until their third birthday, loss to follow-up, withdrawal of consent, death, or end of study. Trained field workers visited the home of each enrolled child weekly to collect information on respiratory signs and symptoms over the preceding week with a standardized questionnaire and obtained a NS from any child with ARI. NS were also obtained from study children during asymptomatic periods. NS were placed in Remel M4RT® transport medium, transported on ice to the local research laboratory within 8 h, and frozen at −70 °C. RNA was extracted from thawed specimens with the MagMAX-96 Viral RNA Kit (Applied Biosystems). Specimens had been tested previously for influenza A/B, RSV, MPV, and PIV [14–18] while the ICV assay was under development. After the influenza C PCR assay was optimized and validated, the assay was performed on RNA extracts from specimens previously used to test for the other specified respiratory viruses. All specimens were tested by real-time RT-PCR for RNAse P to ensure RNA integrity and exclude PCR inhibition [19]. The study was approved by the IRBs at Vanderbilt University and the Instituto de Investigacion Nutricional.

The clinical features and prevalence of co-detection with other respiratory viruses of ICV were compared with influenza A (IAV), the most frequently detected influenza type in the parent study using Chi-squared/Fisher exact tests for categorical variables and *t*-tests for continuous variables.

3. Results

3.1. Sequence analysis

We analyzed 139 hemagglutinin-esterase (HE) genes from GenBank. There was substantial diversity among the HE genes, and highly conserved primers and probes could not be identified (not shown). In contrast, ICV nucleoprotein (NP) and matrix (M) genes are highly transcribed and well conserved among different lineages of the virus [20]. Published full-length NP (n = 97) and M (n = 105) gene sequences obtained from GenBank were aligned with MacVector 12 to design multiple primer and probe combinations.

3.2. Assay testing and selection

Two new assays targeting M (M2 and M3), 2 new assays directed against NP (NP2 and NP4), and 1 published NP assay (NP1) [7] were tested (primer and probe sequences listed in Table 1). Each of the primer and probe sequences had minimal mismatches with the aligned viral gene sequences (not shown). The 2 assays targeting M, M2 and M3, were initially tested using serial dilutions of pCM2AA and pCM2Tay DNA plasmids as template. Both M2 and M3 assays detected dilutions of both plasmid DNA with comparable threshold cycle (Ct) values (Fig. 1**A**). However, the intensity of fluorescence (ΔRn) in the M3 assay was much lower than that detected for M2 and highly variable between the 2 plasmids, while the fluorescence intensity for M2 was identical for both plasmids (Fig. 1**B**).

The M2 and M3 assays targeting M and the NP1, NP2, and NP4 assays targeting NP were then tested against 5 μL of RNA extracted from 1:100 dilutions of the C/Ann Arbor and C/Taylor stock viral cultures to evaluate Ct and ΔRn. M3 had the lowest and most variable fluorescence emission intensity. Compared with M2, the M3 assay also had less sensitivity in detecting C/Taylor, although it had a comparable Ct for C/Ann Arbor. Assay NP2 showed sensitivity similar to M2, NP1, and NP4; however, the magnitude of the fluorescence signal in the NP2 assay was much lower than that of the remaining assays (not shown). M3 and NP2 were therefore not tested further.

RNA from 9 ICV-positive specimens previously collected in Nashville was extracted and tested with assays M2, NP1, and NP4. All specimens were detected by all 3 assays with similar Ct values between assays and thus comparable sensitivity (not shown); therefore, we anticipated that the M2, NP1, and NP4 assays would successfully detect diverse strains of ICV.

To determine whether the assays could successfully detect lower concentrations of RNA, assays M2, NP1, and NP4 were tested against serial dilutions of RNA extracted from undiluted C/Taylor stock culture.

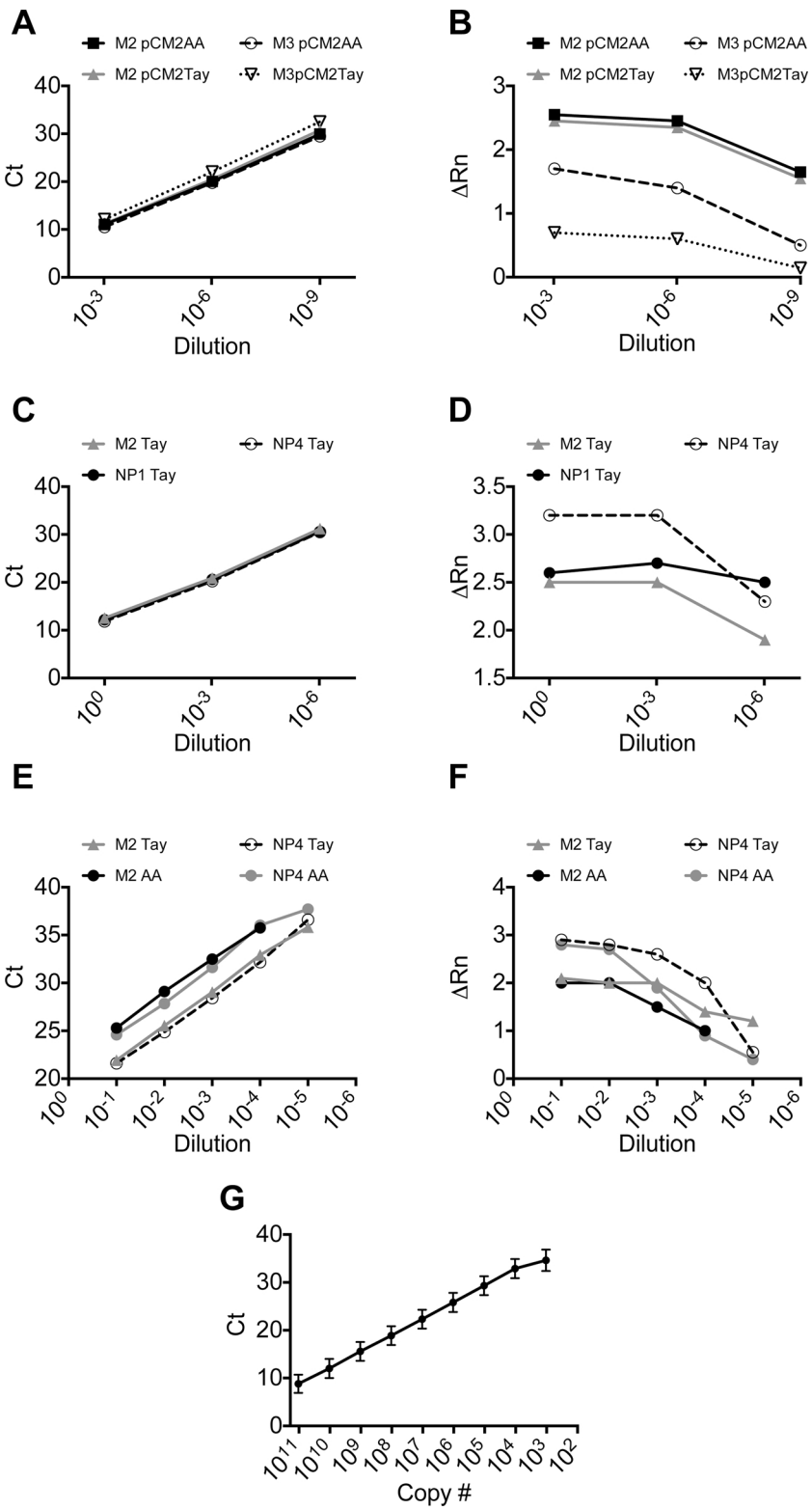

Fig. 1. Performance of assays tested against prototype ICV strains. Plots of cycle threshold (Ct) versus RNA 10-fold dilutions. **A**) Ct vs. RNA dilution and **B**) ΔRn vs. RNA dilution of M2 and M3 assays tested against DNA plasmids encoding the M gene of C/Ann Arbor (pCM2AA) or C/Taylor (pCM2Tay). **C**) Ct vs. RNA dilution and **D**) ΔRn vs. RNA dilution of M2, NP1, and NP4 assays tested against RNA from C/Taylor (Tay). **E**) Ct vs. RNA dilution and **F**) ΔRn vs. RNA dilution of M2 and NP4 assays tested against RNA from C/Ann Arbor (AA) and C/Taylor (Tay). G) Ct vs. RNA copy number of NP4 assays tested against serial 10-fold dilutions of RNA runoff transcript. Error bars = SEM of three separate runs.

While the Ct for all three assays were similar (Fig. 1**C**), the ΔRn for the NP4 assay was superior to the NP1 assay and thus NP1 was not tested further (Fig. 1**D**). Assays NP4 and M2 were subsequently tested against serial dilutions of RNA extracted from C/Ann Arbor and C/Taylor. Both assays exhibited slightly lower Ct against C/Taylor (Fig. 1**E**). However, with the exception of the 10^{-5} dilution of C/Taylor, NP4 demonstrated increased ΔRn and was consistently more sensitive in the detection of both strains than M2 (Fig. 1**F**). Due to the reliability and the sensitivity of the assays using NP4, we decided to further characterize this assay.

To determine the linear performance and limit of detection of the NP4 assay, we generated RNA runoff transcripts of the target gene segment. The assay demonstrated linear performance over eight 10-fold serial dilutions (Fig. 1**G**). The limit of detection of the assay was between 10 and 100 RNA copies per reaction.

To ensure specificity, we analyzed the NP4 primers and probe sequences through NCBI BLAST to detect genetic sequence similarities to

Table 2
Clinical features of Influenza A and C ARI.

	Influenza A (n = 153)	Influenza C (n = 39)	p*
Age in months at ARI onset, mean (SD)	15.9 (9.7)	16.5 (10.5)	0.725
Fever, No. (%)	**139 (91.0)**	**24 (61.5)**	**< 0.001**
Cough, No. (%)	136 (88.9)	34 (87.1)	0.765
Rhinorrhea, No. (%)	138 (90.2)	35 (89.7)	0.933
Decreased appetite, No. (%)	**81 (52.9)**	**9 (23.1)**	**0.001**
Malaise, No. (%)	**96 (62.7)**	**16 (11.5)**	**0.014**
Co-detection with ≥1 other virus, No. (%)	**31 (20.3)**	**17 (43.6)**	**0.003**
Pneumonia, No. (%)	7 (4.6)	1 (2.6)	1.000
Tachypnea, No. (%)	3 (1.9)	1 (2.6)	0.641
Nasal flaring, No. (%)	2 (1.3)	0 (0.0)	0.605
Stridor, No. (%)	1 (0.6)	0 (0.0)	0.779
Wheezing, No. (%)	2 (1.3)	0 (0.0)	0.605

* Chi-squared/Fisher exact tests were used for categorical variables as appropriate, and *t*-tests were used for continuous variables. *p*-values < 0.05 were considered statistically significant.

other respiratory viruses; none were seen. Then, the NP4 assay was tested in triplicate against known influenza viruses A H3N2, B, and 2009 H1N1, MPV, RSV, HRV, PIV 1, 2, and 3, AdV, coronaviruses (NL63, OC43, 229E, and MERS), ICV, and negative controls. All samples were negative except for the ICV samples (not shown).

3.3. Clinical features of ICV infections

In total, 892 children were enrolled into the RESPIRA-Peru study. There were 4475 ARI episodes during 755 observed child-years at risk [21]. Of these, 3957 nasal swabs were collected from children with ARI and tested for viruses by RT-PCR. Thirty-nine/3957 (1.0%) of these tested positive for ICV (Table 2). Median ARI duration was 6 days (IQR 4–10) for ICV infections. Fever, cough, and rhinorrhea occurred commonly, in 24/39 (61.5%), 34/39 (87.1%), and 35/39 (89.7%) of ICV infections, respectively. Only one ICV infection was classified as pneumonia, based on the presence of age-specific tachypnea in a 22 month-old child. None of the children with ICV infection exhibited nasal flaring, stridor, or wheezing.

3.4. Comparison of clinical features of ICV with influenza A

Of ARI with NS tested for viruses, 153/3957 (3.9%) tested positive for IAV [21]. Fever was significantly more common with IAV vs. ICV infection (91% vs. 61.5%, p < 0.001), as were decreased appetite and malaise (Table 2).

3.5. Co-detection of influenza C virus with other viruses

Co-detection with at least one other respiratory virus occurred significantly more frequently with ICV (17/39, 43.6%) than with IAV (31/153, 20.3%; p = 0.003). ICV was co-detected with one other virus in 10/39 (25.6%) ARI episodes and with 2 other viruses in 7/39 (18.0%) ARI episodes. HRV or PIV3 were the viruses most frequently co-detected with ICV, in 13/39 (33.3%) and 5/39 (12.8%) ARI episodes, respectively. The ICV infection classified as pneumonia was associated with co-detection of PIV3 and AdV.

3.6. Asymptomatic influenza C detections

We previously reported that detection of influenza viruses was rare during asymptomatic periods in our cohort, with only 6/859 (0.7%) asymptomatic respiratory samples positive for influenza virus [16]. Three of these were positive for IAV, and 3 were positive for ICV.

4. Discussion

There have been relatively few reports on the burden and the clinical features of ICV infections, particularly among young children, in whom symptomatic ICV infection is thought to be most frequent [8,22–24]. Lack of recognition of ICV infection seems to be attributable not only to difficulties in isolating the pathogen, but also to the mild symptoms associated with ICV compared to other influenza types. We report a new real-time RT-PCR assay for accurate detection of ICV. Although other RT-PCR assays for ICV have been previously described, those were usually developed based on a few sequences [6–8]. In contrast, we designed a novel NP4 ICV assay based on all available sequences in GenBank, including sequences from the original C/Taylor strain in 1947. Thus, the NP4 assay is likely to remain capable of detecting future circulating strains. We found that the NP gene is the most highly conserved ICV gene, and the new NP4 assay was able to detect diverse strains of ICV with high sensitivity and specificity.

We used our NP4 assay to characterize ICV infections among young children from the Peruvian Andes. The 1% detection of ICV among children with ARI in our study is comparable to previous studies, outside of outbreak settings, where molecular diagnostic methods were used. In these settings ICV was typically detected in < 1% of subjects with ARI, although those studies were not all restricted to pediatric populations, and some included assessments of hospitalized as well as outpatient subjects [23–25]. Most previous studies have reported that symptoms of ICV infection are mild with fever, cough, and rhinorrhea occurring most frequently [6,23–26]. Those results are similar to our findings from children followed through household surveillance. However, in another study from Japan, where respiratory tract specimens were collected from symptomatic children who visited one of four clinics or three hospitals from 1990 to 2004 (84,946 specimens), overlapping with a nationwide ICV epidemic in 2004 [20–27], 21/170 (12.3%) ICV detections were associated with pneumonia or bronchiolitis [24]. Another study of hospitalized children in Japan reported that ICV was detected in 10% of children hospitalized with lower respiratory tract illness, and these infections were associated with a similar proportion of pneumonia when compared to human metapneumovirus (MPV) infection [28]. These studies and others of hospitalized children [29] and children with pneumonia [30] suggest that while the typical clinical symptoms of ICV infection could be characterized as a common cold-like illness, complicated, lower respiratory tract infection requiring hospitalization may also occur [22,23,31].

Other studies have compared clinical manifestations among infections with different influenza types. Some studies reported similar clinical features and severity associated with ICV and IAV [30]. However, similar to our findings, an earlier study found that fever was more common and the duration of fever was longer among children with IAV compared to ICV [24]. Another study of ICV among military recruits in Finland demonstrated a substantial difference in the proportion of fever with IAV (41%) compared to ICV (11%) [6]. Other groups have also found that co-detection with other viruses occurs more frequently with symptomatic ICV infection than with other influenza types, although co-detections were not associated with more severe symptoms [29,31]. Additionally, we have demonstrated that ICV detection is rare among asymptomatic children, indicating that, when present, the virus likely contributes to the pathogenesis of respiratory illness [16].

Our study has several important strengths. Ours is one of few reports of children with ARI followed via longitudinal, community, household-based surveillance, capturing a relatively milder spectrum of disease than other reports of pediatric ICV infections that study features of illness from among children that present or are admitted to health care facilities. Therefore, these findings expand our understanding of the spectrum of ICV disease. Our study is also subject to a few limitations. We did not test all published primer/probe sets, and some might have performed well despite nucleotide mismatches; furthermore, *in silico* analysis does not always predict *in vitro* performance. However, we

included one assay that has been used in studies of ICV epidemiology as a comparator. All assays performed well, though with sequence-based modifications the performance on the NP4 assay was enhanced. Validation with specific instruments and kits is necessary for all molecular assays, and additional assessments of the assays in other settings would be helpful. Finally, ICV was frequently associated with detection of other respiratory viruses during ARI episodes. Clarifying the role of ICV as a causative agent of those illnesses is difficult and additional research is needed on this topic.

In summary, we developed a new and accurate ICV real-time RT-PCR assay based on comparison of multiple ICV gene sequences. The assay exhibited linear performance, detected viruses from diverse lineages over time, and enhanced detection of ICV in clinical specimens. We demonstrated ICV infections among young Peruvian children with ARI. These ICV infections were infrequently associated with lower respiratory tract illness in this household-based surveillance study. This new assay should be useful for future epidemiologic studies in diverse populations.

Conflict of interest information

JVW serves on the Scientific Advisory Board of Quidel and an Independent Data Monitoring Committee for GlaxoSmithKline. MRG has received grant funding from MedImmune. KME has received research funding and serves on a Data Safety and Monitoring Board for Novartis. CGG has served as consultant for Pfizer and Merck. CFL is an advisor to Takeda Vaccines Division. All other authors have no potential competing interests to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2017.08.014.

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