

Viruses and bacteria in sputum samples of children with community-acquired pneumonia

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Abstract

Few comprehensive studies have searched for viruses and bacteria in children with community-acquired pneumonia (CAP). We identified 76 children hospitalized for pneumonia. Induced sputum samples were analysed for 18 viruses by antigen detection and PCR, and for six bacteria by culture and PCR. Viruses were found in 72% of samples, bacteria in 91%, and both in 66%. Rhinovirus (30%), human bocavirus (18%) and human metapneumovirus (14%) were the most commonly detected viruses. Two viruses were found in 22% of samples and three in 8%. The most common bacteria found were *Streptococcus pneumoniae* (50%), *Haemophilus influenzae* (38%), and *Moraxella catarrhalis* (28%). Rhinovirus–*S. pneumoniae* was the most commonly found combination of virus and bacterium (16%). All six children with treatment failure had both viruses and bacteria detected in the sputum. Otherwise, we found no special clinical characteristics in those with mixed viral–bacterial detections. With modern molecular diagnostic techniques, there are high rates of both viral and bacterial identification in childhood CAP. The clinical significance of mixed viral–bacterial infections remains unclear, although we found a potential association between them and treatment failure.

Keywords: Aetiology of pneumonia, children, respiratory viruses, viral-bacterial infection

Original Submission: 31 March 2011; **Revised Submission:** 3 June 2011; **Accepted:** 9 June 2011

Editor: L. Kaiser

Article published online: 14 June 2011

Clin Microbiol Infect 2012; **18**: 300–307

10.1111/j.1469-0691.2011.03603.x

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Introduction

There are approximately 150 million cases of childhood community-acquired pneumonia (CAP) each year [1]. The aetiology of pneumonia has been widely investigated (Table 1), but so far no one has studied all of the most common childhood CAP causative agents. According to previous studies, up to two-thirds of childhood pneumonia cases are associated with viral infection [2–6]. Respiratory syncytial virus (RSV), rhinovirus (RV), human bocavirus (HBoV), human metapneumovirus (HMPV) and parainfluenza viruses (PIVs) are the most common viruses associated with pneumonia [1–10]. With modern molecular diagnostic techniques, an increasing number of microorganisms can be detected, and recent aetiological

studies have reported increasing detection rates for mixed viral–bacterial infections in children with CAP [2,3,5–7].

The aim of this study was to investigate thoroughly the roles of all of the most common respiratory microorganisms in the aetiology of CAP, and to determine the real prevalence of mixed viral–bacterial infections.

Materials and Methods

Sputum specimens were collected from children aged 6 months to 15 years with radiologically verified CAP treated in the Paediatric Infectious Disease Ward of Turku University Hospital (Turku, Finland) from January 2006 to April 2007. Pneumonia was defined as the presence of pneumonic infiltrates (alveolar or interstitial) on the chest radiograph with simultaneous signs and/or symptoms of acute infection. Sputum production was induced by inhalation of 5.0% hypertonic saline solution, and the sputum sample was obtained by aspirating through the nostrils ($N = 69$) or by expectoration

TABLE 1. Viral aetiology of community-acquired pneumonia in children when one or more viruses were searched for by PCR techniques

	Juven et al. [2]	Tsolia et al. [3]	Cilla et al. [4]	Hamano-Hasegawa et al. [7]	Nascimento-Carvalho et al. [5]	Samramsamruajkit et al. [13]	Cevey-Macherel et al. [6]	Wolf et al. [14]	O'Callaghan-Gordo et al. [8]
Country	Finland	Greece	Spain	Japan	Brazil	Thailand	Switzerland	Israel	Mozambique
No. of cases	254	75	338	1700	184	257	99	1296	807
Hospitalized (%)	100	100	53	NG	100	100	100	77	100
Age (years)	Mean 3.8	Median 7.2	Median <3	Range 0–19	Median 1.6	Median 1.3	Mean 2.4	Range 0–4	Range 0–5
Additional methods	Cul, Ag, Serol	None	Cul, Ag	None	Ag, Serol	None	Cul, Ag, Serol	Cul, Ag	None
No. of viruses studied (PCR)	12 (1)	9 (9)	14 (14)	12 (12)	9 (2)	8 (8)	13 (12)	8 (1)	11 (11)
Aetiology detected (%)	85	77	NG	77	78	NS	86	NS	NS
Blood culture-positive (%)	1	3	1	NG	5	NS	1	NS	11
Bacteria (total %)	53	40	NG	50	42	NS	53	NS	NS
Viruses (total %)	62	65	67	43	60	49	67	47	49
≥2 viruses (%) (3 viruses, no.)	14	9 (1)	27 (9)	2	NG	NG	12	5	20 (4)
Viruses and bacteria (%)	30	28	NG	15	23	NS	33	NS	NS
Respiratory syncytial virus (%)	29	3 ^a	20	9	15	16	13	23	11
Rhinovirus (%)	24	45	14	15	21	NS	20	NS	41
Human bocavirus (%)	NS	NS	14	3	NS	NS	NS	NS	NS
Human metapneumovirus (%)	NS	1	12	7	NS	9	13	8	8
Parainfluenza virus types 1 2 and 3 (%)	10	8	11 ^b	7	17	5	13	3	7 ^b
Influenza A or B virus (%)	4	7	7	<2 ^c	9	13	14	3	8
Adenovirus (%)	7	12	3	2	3	7	7	3	21
Coronaviruses (%)	3	NS	7	<2 ^c	NS	NS	7	NS	NS
Enteroviruses (%)	NS	NS	NS	<2 ^c	5	NS	13	NS	4

Ag, antigen detection; Cul, viral culture; NG, not given; NS, not studied; Serol, viral enzyme immunoassay from paired serum samples.

^aStudied children aged 5–14 years.

^bPCR included types 1, 2, 3 and 4.

^cInfluenza A and B viruses, coronavirus OC43, enterovirus.

if the child was old enough to produce an adequate sputum sample ($N = 7$). A good-quality sputum sample with <25 squamous epithelial cells and >25 leukocytes per low-power field was obtained from 76 of 133 children otherwise eligible for the study. The details of the sputum collection method and preliminary microbiological results have been described previously by Lahti et al. [9].

Viral antigen detection (influenza A and B viruses, PIV types 1, 2 and 3, RSV, and adenovirus (AdV)) by time-resolved fluoroimmunoassay, and nucleic acid detection by quantitative RT-PCR for enteroviruses, RV, RSV and HMPV, and quantitative PCR for HBoV, were performed as described previously [9]. We completed these viral studies to maximize the number and variety of viruses recovered from respiratory tract samples. Multiplex RT-PCR (Seeplex RV12 ACE Detection; Seegene, Seoul, Korea) was used for the detection of 12 respiratory pathogens (AdV, influenza A and B viruses, RSV A and B, HMPV, PIV types 1, 2 and 3, RV A and B, and coronaviruses 229E/NL63 and OC43/HKU1) according to the manufacturer's protocol. Sequence analysis was used for RV species determination as described elsewhere [11]. In addition, quantitative RT-PCRs detecting human parechoviruses, PIV4, influenza C virus, and quantitative PCR for AdV, were performed in separate assays. The

assay for human parechovirus has been described in detail elsewhere [12]. The assays for PIV4, influenza C virus, and AdV are described in the Supporting Information.

Gram staining, semiquantitative bacterial culture, quantitative *Streptococcus pneumoniae* PCR on *S. pneumoniae* culture-positive samples, *Mycoplasma pneumoniae* PCR and the IgM immunoassay test for *M. pneumoniae* were previously performed as described by Lahti et al. [9]. We completed these bacterial studies by multiplex PCR (Prove-It Sepsis) searching for respiratory bacteria (*S. pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Streptococcus pyogenes*), performed by the manufacturer at Mobidiag (Helsinki, Finland) for 14 samples with negative results or only normal/mixed flora found in previous bacterial studies. All of the patients were previously immunized against *H. influenzae* type b, and therefore only non-typeable serogroups of *H. influenzae* were studied.

Univariate associations between age and the frequency distribution of viral and bacterial pathogens were analysed with logistic regression. Univariate associations between the distributions of viral, bacterial and mixed viral–bacterial detections and the clinical picture of illness were studied with multinomial logistic regression analysis. The studied predictor variables were the patient's age, a white blood cell count and a serum C-reactive protein level on admission,

and the highest fever during stay at hospital. Thereafter, a multiple multinomial logistic regression analysis was performed with all of the predictor variables simultaneously. Associations between viral, bacterial and mixed viral–bacterial detections and the chest radiograph were studied with Fisher's exact tests. For children with at least one virus, the univariate association between predictor variables and number of viruses was studied with logistic regression analysis. Thereafter, a multiple logistic regression analysis was performed with all of the predictor variables simultaneously. The clinical pictures of the most commonly found combinations of viruses and bacteria (the three most common viruses (RV, HBoV and HMPV) with one or more bacteria, and the three most common bacteria (*S. pneumoniae*, *H. influenzae* and *Moraxella catarrhalis*) with one or more viruses) were compared by using one-way analysis of variance for continuous variables and Fisher's exact tests for chest radiographs. The statistical analyses were performed with SAS (version 9.2; SAS Institute, Cary, NC, USA). p-Values <0.05 were considered to be statistically significant.

The study was approved by the Ethics Committee of the Hospital District of South-West Finland. Signed, informed consent was obtained from parents or guardians before enrolment.

Results

The mean age of the 76 patients was 4.7 years (standard deviation, ± 3.9 years). Respiratory microorganisms were

detected in 74 (97%) of our patients. Viruses were found in 55 (72%) and bacteria in 69 (91%) of the studied children. Proportions of microbiological findings are shown in Fig. 1. One child had six microorganisms (three bacteria and three viruses) simultaneously detected (Fig. 2) and, altogether, seven children had two or three bacteria with two or three viruses co-detected (Table 2).

RV, HBoV, HMPV, AdV, PIV3, coronaviruses and RSV were the most commonly detected viruses (Fig. 1). Two viruses were detected in 17 (22%) of our patients and three viruses in six (8%). HBoV–RV was the most common combination of multiple viruses detected (six cases). Of HBoV-positive samples, 79% were also positive for other viruses. RSV and HMPV were most commonly found as sole viral agents (60% and 64%, respectively), whereas other viruses were found as sole viral agents in $\leq 44\%$ of cases. Sequence analysis of 14 RV-positive samples revealed nine belonging to RV A species and five to RV C species. Of children <5 years of age, 80% had viruses detected. Of HBoV detections, 93% were in children <5 years of age. Eight of 11 HMPV detections were in children aged 2–5 years. The mean age of children with multiple viral findings was 2.6 years (standard deviation, ± 1.6 years).

Evidence of *S. pneumoniae* was found in 50% of our patients. Several cases of *H. influenzae*, *M. catarrhalis* and *S. aureus* infection were also detected (Fig. 1). Thirty-six children were tested for serum IgM antibodies to *M. pneumoniae*, and seven (19%) of them had a positive result (these findings are excluded from our statistics, because this test was performed in only 47% of our study population). By

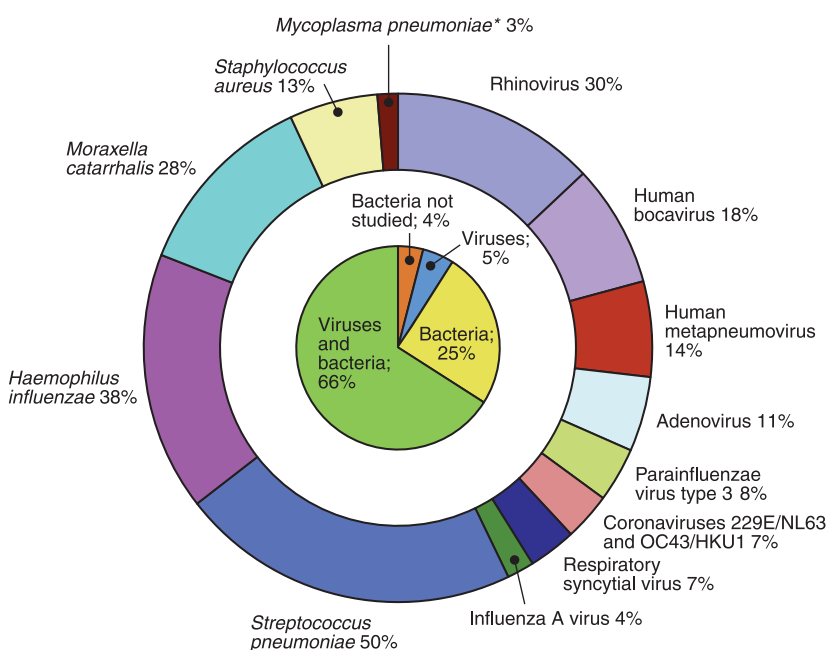


FIG. 1. Proportions of microbiological findings in induced sputum in children with community-acquired pneumonia. Proportions of viral, bacterial and mixed viral–bacterial detections are shown in the inside figure. Proportions of the most commonly found viruses (right side) and bacteria (left side) are shown in the outside figure, and the total percentage is over 100 because of co-detections of multiple microorganisms. Total: 76 patients. **Mycoplasma pneumoniae* was found in 20% of those 36 patients who were studied both serologically and by PCR.



FIG. 2. A chest radiograph of a girl aged 3 years and 9 months with community-acquired pneumonia. Six potential pathogens were detected in the sputum sample: *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, enterovirus, human bocavirus, and rhinovirus. Parenchymal consolidation is seen in the right lung just below the hilus. The clinical picture on admission included fever of 39.8°C, fatigue, poor appetite, rhinitis, cough, right-sided acute otitis media, right-sided fine crackles on auscultation, a C-reactive protein level of 108 mg/L, and a white blood cell count of $35.9 \times 10^9/L$. The patient was treated with intravenous penicillin G. After 1 day of treatment, the girl was afebrile and was discharged from hospital with a course of oral amoxicillin for 7 days.

multiplex PCR, the presence of bacteria was shown in ten (71%) of 14 patients with initially negative culture results. *S. pneumoniae* and *M. catarrhalis* were the most commonly co-detected bacteria (12 cases), and the most commonly found combination of viruses and bacteria was RV–*S. pneumoniae* (12 cases). Of the 38 patients with *S. pneumoniae* in their sputum, 25 (66%) had a concomitant viral detection (Table 2). In 34 (45%) of the studied children, sputum samples were collected after the onset of intravenous antibiotic treatment.

No significant difference in the clinical picture of pneumonia was found, whether it was associated with viruses, bacteria or both (Table 3). Children with interstitial infiltration (14%) on the chest radiograph had evidence of a sole viral or mixed viral–bacterial infection, but not of a sole bacterial infection. Our data did not show any clinical correlation between the clinical picture of pneumonia (the white blood cell count and the serum C-reactive protein level on admission, the highest fever during stay at hospital, and duration of fever $\geq 38^\circ\text{C}$) and whether one or multiple viruses caused it. Mixed viral–bacterial detections including *S. pneumoniae*

with one or more viruses were associated with higher white blood cell counts on admission than *H. influenzae* with one or more viruses ($p 0.001$) (Table 3). Other significant clinical distinctions between different concomitantly detected microorganisms were not found. All of the six children (8%) with treatment failure (fever $\geq 38^\circ\text{C}$ lasting for ≥ 48 h regardless of antibiotic treatment) had evidence of both viruses and bacteria. Of the 19 children with a sole bacterial detection, 100% had alveolar infiltration on the chest radiograph, 89% had a serum C-reactive protein level ≥ 60 mg/L and 68% had a white blood cell count $\geq 15.0 \times 10^9/L$ on admission. Of children with evidence of both viruses and bacteria, only 18% had interstitial infiltration on the chest radiograph, 64% had a serum C-reactive protein level ≥ 60 mg/L and 50% had a white blood cell count $\geq 15.0 \times 10^9/L$ on admission.

Discussion

Our observations suggest that the majority of childhood CAP cases are associated with both viruses and bacteria. Respiratory microorganisms were detected in 97% of the children studied, and 84% of the cases were found to be associated with viral–viral, viral–bacterial or bacterial–bacterial co-detections (Table 2). Rhinovirus–*S. pneumoniae* was the most commonly detected combination of virus and bacterium.

We studied the viral aetiology of childhood CAP by searching for 18 respiratory viruses in sputum specimens, and detected viruses in 72% of the children; this is the highest detection rate of viral aetiology reported [2–6]. Previously, the most comprehensive virological study searched for 14 viruses in nasopharyngeal aspirates from 338 children with pneumonia, and suggested a viral aetiology in 67% [4].

RV, HBoV and HMPV were the most commonly detected viruses in our study. Altogether, these viruses were responsible for up to 57% of all viral detections. RV, either alone or in combination with other viruses or bacteria, was identified in one-third of the studied children. In previous studies, RV has been detected in 14–45% of children with pneumonia, suggesting a significant role for RV in childhood pneumonia [2–8]. Our detection rates for HBoV and HMPV were also in line with earlier studies. Recent studies have identified HMPV in 1–13% of children with pneumonia [3,4,6–8,13,14]. The role of HBoV as a causative agent of pneumonia is more difficult to assess, because, after a primary infection, HBoV may persist for a longer time than other respiratory viruses [15]. In a prospective study by Fry *et al.* [16], HBoV was detected in 12% of hospitalized children <5 years of age who had pneumonia. Moreover, HBoV was recently identified

TABLE 3. Clinical findings of children with community-acquired pneumonia associated with viruses, bacteria, both viruses and bacteria, or the most common combinations of viruses and bacteria

Characteristic	Viruses	Bacteria	Both viruses and bacteria	RV with ≥1 bacterium	HMPV with ≥1 bacterium	<i>Streptococcus pneumoniae</i> with ≥1 virus	<i>Haemophilus influenzae</i> with ≥1 virus
N (%)	4 (5)	19 (25)	50 (66)	10 (13)	7 (9)	14 (18)	14 (18)
Age (years) ^a	2.0 (1.6–2.4)	6.0 (2.9–7.7)	4.4 (1.5–5.8)	5.4 (1.5–5.8)	4.1 (3.0–6.0)	6.4 (2.0–11.4)	3.5 (1.7–4.5)
On admission							
White blood cell count (10 ⁹ /L) ^a	18.6 (14.7–22.6)	20.1 (14.0–26.7)	17.0 (9.3–24.6)	21.0 (11.0–28.9)	11.3 (5.4–11.6)	21.4 ^c (18.4–27.3)	11.3 ^c (7.7–10.3)
Serum C-reactive protein level (mg/L) ^a	82.5 (6.0–159.0)	166.4 (120.0–244.0)	121.8 ^{b1} (32.0–189.0)	129.9 (20.0–212.0)	50.3 (24.0–218.0)	132.6 (58.0–212.0)	99.3 (32.0–136.0)
Fever (°C) ^a	40.1 ^{b2} (39.8–40.4)	39.7 ^{b1} (39.5–40.0)	39.7 ^{b7} (39.6–39.8)	39.6 ^{b1} (39.8–39.8)	39.6 (39.3–39.8)	39.9 ^{b1} (39.8–40.1)	39.7 ^{b2} (39.7–39.8)
Chest radiograph findings							
Alveolar infiltrates, n (%)	3 (75)	19 (100)	43 (86)	9 (90)	7 (100)	12 (86)	12 (86)
Interstitial infiltrates, n (%)	1 (25)	0 (0)	9 (18)	1 (10)	1 (14)	2 (14)	3 (21)

HMPV, human metapneumovirus; RV, rhinovirus.

^aValues are mean (interquartile range).

^bNumber of missing data.

^cSignificant difference ($p < 0.001$) between the groups of *S. pneumoniae* with viruses and *H. influenzae* with viruses. Otherwise, there were no significant differences ($p \leq 0.05$) in the clinical picture of community-acquired pneumonia between different aetiological groups.

Values of C-reactive protein level and fever were not available from all of the children and therefore the number of missing data is shown as superscripts in the table.

serologically in 12% of children with CAP in Italy [17]. Globally, RSV continues to be the major pneumonia causative virus [18]. In our study, RSV was detected at lower rates (7%) than reported by other investigators, (15–29%) [2,4–8,13,14], but this can be explained by our study period being between two RSV epidemics.

With sputum samples as diagnostic specimens, bacteria were demonstrated in 69 of our 76 study children. PCR broadened the detection rate of bacteria substantially. Almost half of our study population had intravenous antibiotic treatment started before the collection of sputum samples, which probably explains why some had negative results in the bacterial culture with concurrent positive results in bacterial PCR. Of bacterial detections, 41% included more than one bacterium and 72% included a concomitant viral detection (Table 2), which complicates investigation of the role of each microorganism in the pathogenesis and clinical features of illness.

Two-thirds of our patients had viral–bacterial co-detections, as compared with rates between 15% and 45% reported in previous aetiological studies of childhood CAP [2,3,5–7,9,10]. A popular view is that primary viral infection is followed by secondary bacterial infection. Consequently, it is difficult to estimate the true incidence of mixed viral–bacterial infections, because the earlier infection may easily be undetected.

One interesting finding in our study was that two viruses were found in 22% of the cases and three viruses in 8%. Multiple viral findings were commonly made among young children, the mean age being 2.6 years. Cilla *et al.* [4] have reported two viruses in 15% of studied children with pneumonia and three viruses in 3%. In our study, HBoV and RV were the most common concomitantly detected viruses. Of

those with HBoV in the sputum, 79% also had other viruses detected. This high rate of HBoV co-infection is in concordance with many previous studies [16,19,20].

The clinical consequences of mixed infections are not fully understood. Some evidence suggests that mixed viral–bacterial infections may potentially induce more severe disease than individual viral or bacterial infections [21–23]. Previously, mixed influenza virus–*S. aureus* infection has been shown to be able to cause severe, fatal pneumonia in children [22–25], and mixed RV–*S. pneumoniae* infection has been shown to be associated with severe pneumonia in adults [26]. In children with invasive pneumococcal disease, viral co-infections are also common and are possibly associated with higher mortality [27]. Limited evidence suggests that viral co-infections may also induce more severe clinical illness than individual viral infections. Cilla *et al.* [4] have reported children with viral co-infections being hospitalized more frequently than those infected with a single virus. In addition, Esposito *et al.* [19] demonstrated that HBoV co-infections with other viruses were associated with greater disease burden (more hospitalizations and loss of school days) in children with respiratory tract infection than in those with HBoV infection alone. In our study, it is of interest that all children (8%) with treatment failure had evidence of mixed viral–bacterial infection.

Our study has many important limitations. The diversity of findings in relation to the size of our study population limits the conclusions that can be drawn. The duration of the study period was short. Only the sputum samples with a high leukocyte count were included in this study. This may have influenced our results, especially in terms of viruses which may not induce leukocytes to the sputum. We did not use

serology, which could have established infections caused by some viruses. It must be stressed that the detection of bacteria or viruses in the sputum sample does not necessarily mean that they are the causative agents of the concomitant lung infection. Bacteria might be contaminants from the nasopharynx, which, in healthy young children, often carries pathogenic bacteria. The detected virus might be found because of the concomitant upper respiratory tract infection. Furthermore, respiratory viruses, especially RV, have been detected in asymptomatic children (up to 60% in children <1 year of age) [28,29], but prolonged shedding of RV is not known. Some respiratory viruses, such as HBoV and enteroviruses, may show prolonged shedding for months after acute infection [16]. Some bacteria, such as *M. catarrhalis*, are known to be less virulent and less likely to cause pneumonia [30]. Some respiratory DNA viruses, such as AdV, may be latent, and may be found by PCR but not be associated with the symptomatic infection. Thus, it is clear that the term 'causative agent' should be used with caution.

In conclusion, our study suggests that viruses and bacteria are commonly detected in children with CAP. Consequently, our findings support the current guidelines that all children with pneumonia should be treated with antibiotics, as the detection of a virus does not allow a concomitant bacterial infection to be ruled out. On the other hand, viral-bacterial co-infection may manifest as an apparent treatment failure. Viral pneumonia should receive greater attention in future treatment and prevention studies.

Acknowledgements

The authors thank T. Ylinen for technical assistance at the Department of Virology. We also thank J. Matomäki for help with data analyses.

Transparency Declaration

This study was financially supported by a special government grant from Turku University Hospital, Finnish Cultural Foundation at Southwestern Finland, and the Paulo Foundation. M. Honkinen, E. Lahti, R. Österback and M. Waris have no conflicts of interest. O. Ruuskanen has been a consultant to Novartis Vaccines and Abbott.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. The assays of RT-qPCR for parainfluenza virus type 4 and influenza C virus, and qPCR for adenovirus.

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