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Rapid detection of respiratory picornaviruses in nasopharyngeal aspirates by immunofluorescence assay

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ABSTRACT

Background: Respiratory picornaviruses (enteroviruses and rhinoviruses) are commonly cited as causes of self-limited upper respiratory tract infection. However, it has recently been suggested that they may cause more severe respiratory disease. Immunofluorescence (IF) assays are rapid and inexpensive and are often used for the detection of respiratory viruses.
Objectives: We sought to develop an IF procedure, using commercially available reagents, for the detection of respiratory picornaviruses directly from nasopharyngeal aspirates (NPA).
Study design: From 1st November 2006 until 31st October 2007 all NPA from patients with respiratory infection were stained with the Light Diagnostic Pan-Enterovirus Reagent – "Blend" by IF (IF-ENVPAN). Those specimens which tested positive with this stain were further tested (subject to the availability of frozen specimen) with the xTAG respiratory viral panel, a multiplex PCR directed against respiratory picornaviruses, adenovirus (ADV), respiratory sincytial virus (RSV), influenza viruses A and B (IFA and IFB), parainfluenza virus (PIV) 1–4, human metapneumovirus (HMPV) and coronaviruses.
Results: 241/1122 NPA tested positive by IF-ENVPAN. 143 NPA were available for testing by xTAG respiratory viral panel. The multiplex PCR detected respiratory picornaviruses in 139 NPA, in 126 as the sole viral pathogen.
Conclusions: Our results indicate the potential of IF-ENVPAN for the laboratory detection of respiratory picornaviruses in clinical specimens. As far as we are aware, this is the first publication of such a method.

1. Background

Recent studies using molecular biology approaches suggest a previously unsuspected role for respiratory picornaviruses (enteroviruses and rhinoviruses) in the pathogenesis of severe respiratory tract infections. Acute wheezing (bronchiolitis and acute asthma) and viral pneumonia in children and infants,[1–5] exacerbations of COPD,[2,6–8] asthma,[2,6,9,10] and cystic fibrosis,[2,6] severe and chronic infections in immunologically compromised patients[2,6,11] and severe lower respiratory tract infections in elderly persons[2,12] have been associated with respiratory picornaviruses.

Rapid diagnosis of respiratory viral pathogens is important to help prevent the spread of nosocomial infection, to minimize unnecessary antibiotic usage and to discern the need for antiviral therapy.[13–15] Immunofluorescence (IF) is a widely used method for the detection of many respiratory viruses directly from respiratory samples. IF is a rapid, reliable and cost effective method which provides an estimation of specimen quality and allows screening for several viruses simultaneously.[16] However, IF staining of specimens for detection of respiratory picornaviruses has been considered impossible due to the large number of serotypes involved.[16] Nevertheless, the use of specific monoclonal antibodies against enterovirus has been described for identification purposes in both Shell Vial and conventional culture.[17–19]

We conducted a preliminary investigation of the suitability of Light Diagnostic Pan-Enterovirus Reagent – "Blend" IF (IF-ENVPAN) for IF detection of respiratory picornaviruses directly from nasopharyngeal aspirates (NPA). Although not conceived for this application we frequently observed a specific fluorescent cytoplasmatic granular pattern, which was frequently associated with NPA from children with wheezing. As cross-reactivity with hepatitis A and astrovirus was unlikely in respiratory samples, and adenoviruses could be ruled-out by specific staining, our observations suggested a possible association between this fluorescent pattern and the detection of respiratory picornaviruses.

2. Objectives

In order to asses the suitability of IF-ENVPAN for the detection of respiratory picornaviruses directly from NPA, we prospectively

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tested 1154 NPA specimens from patients with a suspected viral respiratory infection with the IF-ENVPAN stain, further testing available IF-ENVPAN positive specimens with xTAG respiratory viral panel, a commercial multiplex PCR[20,21] directed against many respiratory viruses including respiratory picornaviruses.

3. Study design

3.1. Sampling

All 1154 nasopharyngeal aspirates (NPA) collected over the 1-year period (1 November 2006 to 31 October 2007) from 986 children and 5 adults with suspected respiratory infection were prospectively tested with Light Diagnostic Pan-Enterovirus Reagent – "Blend" IF (IF-ENVPAN) and with our usual IF screening panel for respiratory viruses (ADV, RSV, IFA, IFB, PIV 1–3 and HMPV). When available, excess sample material was frozen at −80 °C.

All IF-ENVPAN positive NPA having sufficient frozen material were further tested with xTAG respiratory viral panel, a multiplex PCR directed against respiratory picornaviruses, ADV, RSV, IFA, IFB, PIV 1–4, HMPV and coronaviruses. The total number of IF-ENVPAN positive NPA tested by multiplex PCR was 143.

Every 3rd IF negative NPA (by both IF screening panel and IF-ENVPAN) collected between 1st November 2006 and 14th September 2007 having sufficient frozen material, was also tested by xTAG multiplex PCR. The total number of IF negative specimens tested by multiplex PCR was 100.

3.2. Patient population

Most of the NPA were collected from children admitted to the emergency room of the Department of Pediatrics of Bern University, Switzerland. Some samples came from children admitted to other hospitals or sent from paediatrician's practices. All 5 adults were admitted to the University Hospital of Bern, Switzerland; 3 of them were immuncompromised.

The patient's ages were as follows: 493 < 1 year, 331: 1–3 years, 115: 4–9 years, 47: 10–18 years, and 5 adults.

3.3. Immunofluorescence assays

The NPA-samples were vortexed and centrifuged at 700 × *g* for 5 min. The cell pellet was re-suspended in PBS and centrifuged again at 700 × *g* for 5 min and was finally re-suspended in PBS to form a slightly cloudy suspension. One drop of this cell suspension was placed on each well of multi-well slides. After drying the slides were fixed in acetone for 10 min. After staining all slides were examined with a fluorescence microscope at 200–400×. Samples containing fewer than 20 ciliated epithelial cells were considered inadequate.

3.3.1. IF screening panel for ADV, RSV, IFA, IFB, PIV 1–3 and HMPV

The wells were stained with Light Diagnostics (Chemicon International, now part of Millipore, Temecula, CA) Respiratory Viral Screen DFA and the single fluorescein-conjugated monoclonal antibody for the detection of ADV, RSV, IFA, IFB and PIV 1–3 and with the Diagnostic Hybrid DFA Metapneumovirus Identification Kit for the detection of HMPV according to the manufacturer's instructions.

3.3.2. IF-ENVPAN

All specimens were also tested with Light Diagnostics Pan-Enterovirus Reagent "Blend" Cat. No. 3360 that contains a blend of two monoclonal antibodies 2E11 and 9D5.[22] Separate slides were used for the IF-ENVPAN as this was the only indirect immunofluorescent antibody assay. One drop of monoclonal antibody was placed on each well. After incubation in a humid chamber at 37° ± 1.5 °C for 30 min slides were washed in PBS-Tween and a drop of Anti-Mouse IgG Conjugate (Light Diagnostics Cat. No. 5008) was added to each well. The slides were then incubated in the same way, washed, dried and examined. A positive result was indicated by the presence of two or more intact cells exhibiting the specific fluorescence pattern: the cytoplasm of positive cells appears enveloped at the surface with a bright apple-green large grained granular fluorescent mantle (Fig. 1). Samples with intra-nuclear or extra-cellular fluorescence were regarded as negative.

3.4. xTAG respiratory viral panel assay

RNA and DNA were extracted with the EasyMAG extractor (bioMérieux) using the generic 1.0.6. protocol. To each 200 μl of NPA, 20 μl of a 10^{-2} dilution of the *Escherichia coli* phage MS2 (ATCC strain 15597-B1) in Universal Transport Medium (Copan) were added as an internal control. The elution volume was set to 110 μl. Extracts were stored at −80 °C.

The xTAG respiratory viral panel assay (Luminex Molecular Diagnostics) comprises the following steps, all of which were carried out according to the manufacturer's instructions: target-specific reverse-transcription PCR, exonuclease/phosphatase treatment, target-specific primer extension, target-specific hybridization of the amplicons to fluorochrome-marked microbeads, detection of the amplicons on the Luminex IS 200 instrument. For a detailed description of the method see Merante et al.[21]

Raw data output files consisting of median fluorescence intensities for all viruses, and subtypes were interpreted using the TDAS RVP-I 1.10 software (Luminex Molecular Diagnostics).

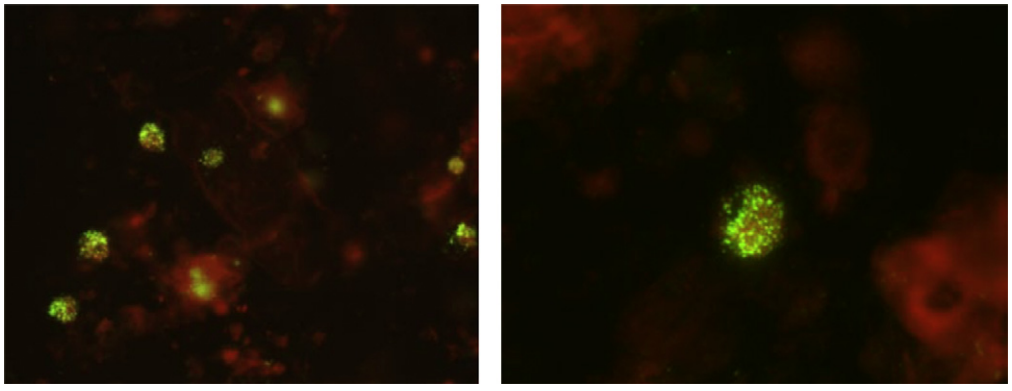

Fig. 1. Examples of respiratory mucosal cells from NPA stained with IF-ENVPAN. The cytoplasm of positive cell appears enveloped at the surface with a bright apple-green large grained granular fluorescent mantle (left 200×, right 630×).

Table 1
Number (%) of IF-ENVPAN and IF screening panel positive NPA according to age.

	<1 years	1–3 years	4–18 years	>18 years	Total
IF-ENVPAN	125 (11.1)	85 (7.5)	30 (2.6)	1	241 (21.4)
RSV	196 (17.4)	64 (5.7)	11 (0.9)	0	271 (24.1)
ADV	8 (0.7)	25 (2.2)	3 (0.2)	0	36 (3.2)
IFA	13 (1.1)	10 (0.8)	9 (0.8)	0	32 (2.8)
PIV 1–3	15 (1.3)	8 (0.7)	5 (0.4)	0	28 (2.5)
HMPV	3 (0.2)	6 (0.5)	1	0	10 (0.9)
Total	360 (32)	198 (17.6)	59 (5.2)	1	618 (55.0)

IF-ENVPAN: Pan-Enterovirus Reagent – "Blend" IF. NPA: nasopharyngeal aspirates.

4. Results

4.1. IF-results

A total of 1122 NPA specimens were available for analysis; 32 samples were rejected because of inadequacy. The IF-results are shown in Table 1.

4.1.1. IF screening panel for ADV, RSV, IFA, IFB, PIV 1–3 and HMPV

271 NPA were positive for RSV (24.1%), 36 for ADV (3.2%), 32 for IFA (2.8%), 28 for PIV 1–3 (2.5%) and 10 for HMPV (0.9%). Co-infection was not observed.

4.1.2. IF-ENVPAN

241 (21.4%) NPA from 220 patients tested positive. A dual infection with another respiratory virus (9 RSV, 1 ADV, 2 PIV 1–3, and 1 HMPV) was found in 13 samples. The result was inconclusive in 12 specimens due to non-specific background staining and a lack of granular clustering.

4.2. xTAG respiratory viral panel—results

A total of 243 NPA (143 IF-ENVPAN positive and 100 negative by both IF-ENVPAN and the IF screening panel) were tested by xTAG multiplex PCR. The comparison IF-ENVPAN/xTAG multiplex PCR results is shown in Table 2.

4.2.1. IF-ENVPAN positive NPA

The multiplex PCR detected respiratory picornaviruses in 139 (in 126 as the sole viral pathogen) of the 143 NPA positive by IF-ENVPAN. Only 4 NPA positive by IF-ENVPAN were negative by multiplex PCR. Due to a lack of frozen material we were unable to clarify these discrepancies.

Six NPA with co-infection (all with RSV) had sufficient frozen material and were tested with xTAG multiplex PCR. The multiplex PCR confirmed the IF-result in all six NPA. xTAG multiplex PCR detected a dual infection in a further 7 specimens (3 RSV, 2 PIV 4 and 2 PIV 3).

4.2.2. IF negative NPA

xTAG multiplex PCR detected 41 respiratory picornaviruses, 5 RSV, 5 IFA, 4 coronaviruses, 2 ADV, 2 PIV 4, 2 PIV 3 and 2 HMPV in 56 out of the 100 IF negative NPA. Multiple viral pathogens were detected in 6 specimens (5 samples with two and one with three respiratory viruses).

Table 2
Comparison of IF-ENVPAN results with xTAG multiplex PCR detection of respiratory picornaviruses.

No. of NPA tested by xTAG multiplex PCR for respiratory picornaviruses	No. of NPA tested by IF-ENVPAN		
	Positive	Negative	Total
Positive	139	41	180
Negative	4	59	63
Total	143	100	243

IF-ENVPAN: Pan-Enterovirus Reagent – "Blend" IF. NPA: nasopharyngeal aspirates.

4.3. Statistical analysis

The calculation of sensitivity and specificity of IF-ENVPAN compared with xTAG multiplex PCR was not possible because of the sampling differences between IF-ENVPAN positive and IF negative NPA as described in the study design. Compared to xTAG multiplex PCR, IF-ENVPAN showed a positive predictive value of 97.2% and a negative predictive value of 59%.

5. Discussion

Our study evaluated an IF procedure for the detection of respiratory picornaviruses from NPA. For this purpose IF-ENVPAN was compared to xTAG multiplex PCR. The multiplex PCR detected respiratory picornaviruses in 139 of the 143 IF-ENVPAN positive NPA, in 126 as the only viral pathogens. The recognition of the positive staining pattern did not present any particular difficulties for experienced laboratory staff. Only 4 IF-ENVPAN positive NPA were negative using xTAG multiplex PCR. Unfortunately, the 4 discrepant specimens could not be retested due to a lack of frozen material. Moreover, xTAG multiplex PCR confirmed the IF-result in all six tested NPA with RSV co-infection. Thus a cross-reaction of IF-ENVPAN with other respiratory viruses appears to be most unlikely. Based on our results, we consider NPA showing the specific cytoplasmatic granular fluorescent pattern following IF-ENVPAN staining to be truly positive for respiratory picornaviruses. The positive predictive value of IF-ENVPAN compared to xTAG multiplex PCR was 97.2%.

Neither IF-ENVPAN nor xTAG PCR can distinguish between enteroviruses and rhinoviruses. Testing with enterovirus and rhinovirus specific PCR or sequencing-studies are necessary to resolve this uncertainty. However, it is probable that both rhinoviruses and enteroviruses are part of a continuous spectrum of related viruses causing related diseases.[23]

Of a total of 1122 tested NPA 241 were positive by IF-ENVPAN for respiratory picornaviruses. When using IF, respiratory picornaviruses were the second most frequently detected viral pathogens in NPA; the first if only subjects older than 1 year are considered. Our IF data supports the suspected high frequency of respiratory picornaviruses as viral pathogens as described by other authors.[3–5,7,8]

xTAG multiplex PCR detected at least one viral pathogen (41 respiratory picornaviruses) in 56 of the 100 IF negative NPA and was thus, when compared to IF, more sensitive for the detection of respiratory viruses, particularly for respiratory picornaviruses. Given the wide genetic variation of respiratory picornaviruses, it is possible that the monoclonal antibody blend does not react with all serotypes present. On the other hand, the enhanced sensitivity of molecular methods compared to IF for the detection of respiratory viruses[20,24,25] should not only be regarded as being advantageous. Respiratory picornaviruses have also been detected by PCR in approximately 20% of subjects lacking respiratory symptoms.[26–29] Moreover, at least two reports have documented the recovery of respiratory picornaviruses by PCR for 2–3 week following acute respiratory illness.[28,29] Does the detection of respiratory picornaviruses nucleic acid represent the presence of virus without correlation with the current clinical symptoms, the persistence of virus genome following previous infection or an active infection?[7,28,30] A major advantage of IF methods is, paradoxically, their relative insensitivity—there has to be a substantial quantity of virus material present to score as positive.[30] An IF positive result correlates well with an active infection.[16] Furthermore, IF is much more flexible and much less expensive than molecular procedures:

the results are available several time a day within 2–4 h and the preparation of new specimens can begin while earlier ones are being completed.[16,31] For all these reasons IF still retains advantages over PCR as a first-line test to guide clinicians.[31]

Summing up, our data support the clinical utility of IF-ENVPAN staining of NPA for the detection of respiratory picornaviruses, particularly in infants and children. The addition of IF-ENVPAN to IF screening, targeting ADV, RSV, PIV, IFA, IFB and HMPV, will provide a more complete diagnosis of respiratory viral infections.

Conflict of interest statement

None.

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