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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: 241/1 122 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.

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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,6,7,8 asthma,2,9,10 and cystic fibrosis,2,6,11 severe and chronic infections in immunologically compromised patients2,6,12 and severe lower respiratory tract infections in elderly persons2,12 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 assess the suitability of IF-ENVPAN for the detection of respiratory picornaviruses directly from NPA, we prospectively
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 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 immunocompromised.

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 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×).
56 out of the 100 IF negative NPA. Multiple viral pathogens were RSV, IFA, coronaviruses, ADV, PIV 4, PIV 3 and HMPV in 4.2.2. IF negative NPA detected a dual infection in a further 7 specimens (3 RSV, 2 PIV 3).

PCR confirmed the IF-result in all six NPA. xTAG multiplex PCR clarified these discrepancies.

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

### Table 1

| Age Group | IF-ENVPAN | IFA | PIV 1–3 | ADV | RSV | Total |
|-----------|-----------|-----|---------|-----|-----|-------|
| <1 years  | 125 (11.1)| 8   | 30      | 11  | 196 | 362   |
| 1–3 years | 85 (7.5)  | 25  | 3       | 11  | 64  | 187   |
| 4–18 years| 80 (6.2)  | 10  | 5       | 11  | 64  | 187   |
| >18 years | 30 (2.6)  | 2   | 4       | 11  | 1   | 57    |
| Total     | 360 (32)  | 198 | 59      | 30  | 271 | 618   |

4. Results

#### 4.1. IF-results

A total of 1122 NPA specimens were available for analysis; 32 specimens 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 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.

#### 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).

#### 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%.

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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.

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 should not only be regarded as being advantageous. Respiratory picornaviruses have also been detected by PCR in approximately 20% of subjects lacking respiratory symptoms.

Moreover, at least two reports have documented the recovery of respiratory picornaviruses by PCR for 2–3 week following acute respiratory illness. 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? 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. An IF positive result correlates well with an active infection. Furthermore, IF is much more flexible and much less expensive than molecular procedures.

#### 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** | 139 | 41 | 180 |
| **No. of NPA tested by IF-ENVPAN** | 143 | 100 | 243 |

IF-ENVPAN: Pan-Enterovirus Reagent – “Blend” IF. NPA: nasopharyngeal aspirates.
the results are available several a day within 2–4 h and the preparation of new specimens can begin while earlier ones are being completed.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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References

1. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. BMJ 1995; 310:1225–9.
2. Hayden FG. Rhinovirus and the lower respiratory tract. Rev Med Virol 2004; 14:17–31.
3. Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den Hoogen B, Osterhaus AD, et al. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute respiratory wheezing in children. Emerg Infect Dis 2004; 10:1095–101.
4. Sanderson G, Lampe F, Smith S, Johnston SL, Bednarski WP, Pattemore PK, et al. Association of respiratory picornaviruses with acute bronchiolitis in French infants. J Clin Virol 2006; 35:463–6.
5. Chung JY, Han TH, Kim SW, Hwang ES. Respiratory picornavirus infections in Korean children with lower respiratory tract infections. Scand J Infect Dis 2007; 39:250–4.
6. Greenberg SB. Respiratory consequences of rhinovirus infection. Arch Intern Med 2003; 163:278–84.
7. Roehde C, Wiethege A, Borg I, Kauth M, Bauer TT, Gillissen A, et al. Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case–control study. Thorax 2003; 58:37–42.
8. Beckham JD, Cadena A, Lin J, Piedra PA, Glezen WP, Greenberg SB, et al. Respiratory viral infections in patients with chronic, obstructive pulmonary disease. J Infect 2005; 50:322–30.
9. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. BMJ 1993; 307:982–6.
10. Mos M, Sanañ M, Soja J, Olechnowicz H, Busse WW, Szczeklik A. The presence of rhinovirus in lower airways of patients with bronchial asthma. Am J Respir Crit Care Med 2008; 177:1082–9.
11. Kaiser L, Aubert JD, Pache JC, Deffner C, Rochat T, Garbino J, et al. Chronic rhinoviral infection in lung transplant recipients. Am J Respir Crit Care Med 2006; 174:1392–9.
12. Louie JK, Yagi S, Nelson FA, Kiang D, Glaser CA, Rosenberg J, et al. Rhinovirus outbreak in a long term care facility for elderly persons associated with unusually high mortality. CID 2005; 41:262–5.
13. Hayden FG, Osterhaus AD, Treanor JJ, Fleming DM, Aoki FY, Nicholson KG, et al. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza virus infections. N Engl J Med 1997; 337:874–80.
14. Wos M, Sanak M, Soja J, Olechnowicz H, Busse WW, Szczeklik A. The presence of rhinovirus in lower airways of patients with bronchial asthma. Am J Respir Crit Care Med 2005; 171:1218–20.
15. Madeley CR, Peiris JS. Methods in virus diagnosis: immunofluorescence revisited. J Clin Virol 2002; 25:121–34.
16. Madeley CR, Peiris JS. Methods in virus diagnosis: immunofluorescence revisited. J Clin Virol 2002; 25:121–34.
17. Klespies SL, Cebula DE, Kelley CD, Gablehouse D, Maurer CC. Detection of enteroviruses from clinical specimens by spin amplification shell vial culture and monoclonal antibody assay. J Clin Microbiol 1996; 34:1465–7.
18. Buck GE, Wiesemann M, Stewart T. Comparison of mixed cell culture containing genetically engineered BGMK and CaCo-2 cells (Super E-Mix) with RT-PCR and conventional cell culture fort he diagnosis of enterovirus meningitis. J Clin Virol 2002; 25(Suppl. 1):S13–8.
19. She RC, Crist G, Billedeaux E, Langer J, Petti CA. Comparison of multiple shell vial cell lines for isolation of enteroviruses: a national perspective. J Clin Virol 2006; 37:151–5.
20. Mahony J, Chong S, Merante F, Yaghoubian S, Sinha T, Lisle C, et al. Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. J Clin Microbiol 2007; 45:2965–70.
21. Merante F, Yaghoubian S, Janezczko R. Principles of the xTAG™ respiratory viral panel assay (RVP assay). J Clin Virol 2007; 40(Suppl. 1):S31–5.
22. Yagi S, Schunn D, Lin J. Spectrum of monoclonal antibodies to coxsackievirus B-3 includes type- and group-specific antibodies. J Clin Microbiol 1992; 30:2498–501.
23. Carman WF, Mahony JRB. The pathogens. J Clin Virol 2007; 40(Suppl. 1):S55–10.
24. Rovida E, Percivalle E, Zavattoni M, Torsellini M, Sarasin A, Campanini G, et al. Monoclonal antibodies versus reverse transcription-PCR for detection of respiratory viruses in a patient population with respiratory tract infections admitted to hospital. J Med Virol 2005; 79:336–47.
25. Fox JD. Nucleic acid amplification tests for detection of respiratory viruses. J Clin Virol 2007; 40(Suppl. 1):S153–21.
26. Nakos-Koivisto J, Kilpinen T, Hovi T, Vitikainen O. Human picornavirus and coronavirus RNA in nasopharynx of children without concurrent respiratory symptoms. J Clin Virol 2007; 40(Suppl. 1):S153–21.
27. Wright PF, Deatly AM, Karron RA, Belshe RB, Shi JR, Gruber WC, et al. Comparison of results of detection of rhinovirus by PCR and viral culture in human nasal wash specimens from subjects with and without clinical symptoms of respiratory infection. J Clin Virol 2007; 38:644–50.
28. Jartti T, Lehtinen P, Vuorinen T, Koskenvuo M, Ruuskanen O. Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children. J Med Virol 2004; 78:605–9.
29. Winther B, Hayden FG, Hendley JO. Picornavirus infections in children diagnosed by RT-PCR during longitudinal surveillance with weekly sampling; association with symptomatic illness and effect of season. J Med Virol 2008; 80:644–50.
30. Madeley CR. “Is it the cause?”—Robert Koch and viruses in the 21st century. J Clin Virol 2008; 43:9–12.
31. Landry ML, Cohen S, Ferguson D. Prospective study of human metapneumovirus detection in clinical samples by use of light diagnostics direct immunofluorescence reagent and real-time PCR. J Clin Microbiol 2008; 46:1098–100.