

# Detection of Rhinovirus RNA in Middle Turbinate of Patients With Common Colds by In Situ Hybridization

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Human rhinovirus 14 RNA was determined by in situ hybridization from middle turbinate biopsies in 32 patients with diagnosed common colds and in five control individuals. Twenty-two (69%) biopsies from common colds patients but none of the five control biopsies showed reactivity for human rhinovirus 14 antisense probe. The signal was detected both in the respiratory epithelium and in mucosal inflammatory cells. In situ hybridization of the middle turbinate tissue yielded more positive results than RT-PCR (47%) or virus culture (34%) assayed from nasopharyngeal aspirates, but no statistical significant differences were observed ( $P=0.265$ ,  $P=0.425$ , respectively). The results indicated that in situ hybridization procedure was slightly more sensitive than PCR assays and classical culture for the detection of human rhinovirus infection of upper respiratory tract. However, in situ hybridization procedure appeared to be an interesting methodology to investigate the physiopathology of respiratory tract infection by rhinoviruses. **J. Med. Virol.** 70:319–323, 2003. © 2003 Wiley-Liss, Inc.

**KEY WORDS:** rhinovirus; respiratory infection; in situ hybridization

## INTRODUCTION

Human rhinoviruses are the most common cause of acute respiratory illnesses in humans and have a global distribution. Recent studies using sensitive PCR protocols to detect rhinovirus RNA have confirmed further the importance of these viruses in the etiology of common colds [Arruda et al., 1997; Mäkelä et al., 1998; Steininger et al., 2001], but the pathogenesis of rhinovirus infections and the factors controlling sites of virus replication remain incompletely defined.

At the beginning of a rhinovirus infection, viruses invade the nasopharynx and interact with epithelial cells. Rhinoviruses are recovered from the nasopharynx earlier, more frequently, and longer than from more anterior sites in nasal cavities [Winther et al., 1986; Arruda et al., 1995]. Although the initial event in cold production is viral infection of the nasal epithelia, morphologic studies have not detected significant changes in the lymphocyte content of the nasal mucosa during rhinovirus colds and nasal biopsy studies have shown little, if any, cell damage [Winther, 1994]. Rhinovirus replication cycle time in humans is similar to that in cell culture and some symptoms begin within 12 hr of exposure [Harris and Gwaltney, 1996]. Virus titres peak 2–3 days after infection and may persist at low levels for up to 3 weeks [Winther et al., 1986]. Because rhinovirus does not cause a marked destruction of the epithelial lining, infection has considered to be limited only certain areas in the nasal epithelium. This has been confirmed in the few earlier experimental studies using in situ hybridization, where rhinoviruses were shown to cause only focal infection of the nasal epithelium [Bruce et al., 1990; Arruda et al., 1991, 1995; Bardin et al., 1994]. In contrast to the above studies, we found in our earlier study on patients with acute maxillary sinusitis a large number of sinus epithelial cells positive for rhinovirus by in situ hybridization [Pitkäranta et al., 2001]. These findings led us to study middle turbinate biopsies from patients with naturally occurring common colds by in situ hybridization to further evaluate the course of

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natural rhinovirus infection, its pathogenesis, and spread of rhinoviruses through the respiratory tract.

## PATIENTS AND METHODS

### Patients and Clinical Samples

Two hundred young adults were enrolled in a common cold study in Turku University Hospital as described previously [Mäkelä et al., 1998]. From those 200 patients, middle turbinate biopsies were obtained from 32 patients (21 female and 11 male, mean age 24 years) on day 7. From nine of these patients, middle turbinate biopsies were also obtained on day 1. The samples were collected during a 10-month period. The study protocol was accepted by the ethical committee of the Turku University Hospital. A signed informed consent was obtained from each patient. The patients contacted the study physician within 24 to 48 hr after the onset of symptoms of the common cold. For inclusion in the study, patients had to self-diagnose the common cold based on their earlier experience of symptoms. In addition, the study physician confirmed clinical evidence of acute rhinorrhea, nasal congestion, and/or sore throat. Patients with tonsillitis and with previous histories of allergic rhinitis, any chronic illness, or use of regular medication were excluded. Middle turbinate biopsy was undertaken with atraumatic Gerritsma biopsy forceps from the mucosa of anterior part of middle turbinate. The specimens were immediately fixed in formalin and stored for later process at room temperature [Fokkens et al., 1988]. Control middle turbinate biopsies were obtained from five adult patients who were admitted to the hospital because of nasal fracture. None of the patients had common cold symptoms. The Otorhinolaryngological Ethical Committee of the Helsinki University Central Hospital accepted the study plan and a signed informed consent was obtained from each of these patients.

### In Situ Hybridization

For preparation of the rhinovirus 14 probe a region representing part (nucleotides 332–572) of human rhinovirus 14 5'-untranslated region (5'-UTR) was amplified, by PCR, from a cDNA template [Stanway et al., 1984] and cloned into pGEM-4Z plasmid (Promega, Madison, WI) between HindIII and XbaI sites. Human rhinovirus 14 represent genetically the human rhinovirus B-species [Savolainen et al., 2002]. The 240 nt fragment was cloned in both orientations under the control of T7 promoter. Digoxigenin-labelled RNA probes were synthesised using T7 RNA polymerase and the DIG RNA labelling kit (Roche Molecular Biochemicals, Espoo, Finland). Two sections (sense and antisense) from each patients were available. The sections for in situ hybridization were 5 µm thick and were mounted in ribonuclease-free conditions on heat-treated Super frost (+) slides (Menzel-Gläser<sup>®</sup>, Braunschweig, Germany). Two sections (sense and antisense) from each patient were examined.

In situ hybridization was carried out using an automated Ventana Gen II in situ hybridization/immunohistochemistry Slide Stainer (Ventana Medical Systems, Inc., Tucson, AZ). The program used was "Ventana Regular" protocol. The sections were first deparaffinised in three changes of xylene for 15 min and then hydrated in two changes of absolute, 96% and 70% ethanol, 3 min each. After rinsing in distilled water containing with 0.1% diethylpyrocarbonate, the slides were kept in "Ventana alkaline phosphatase solution." The sections were treated for 8 min with "Ventana Protease 3 Reagent" before hybridization at 45°C for 14–15 hr. The hybridization mixture contained 50% formamide and 5× standard saline citrate (SSC). Three washes after hybridization were performed at 65°C for 8 min in 1× SSC, 0.5× SSC, and 0.1× SSC, respectively. Monoclonal anti-digoxigenin antibody (Clone DI-22; Sigma Chemical Co., St. Louis, MO) was incubated with the sections for 28 min. The probe was detected with the Ventana Basic DAB (3,3'-diaminobenzidine tetrahydrochloride) biotin avidin detection kit. The sections were dehydrated and mounted with coverslips with Eukitt (O. Kindler, GmbH, Freiburg, Germany) and analysed with an Olympus light microscope. The in situ hybridization results were evaluated in a blinded manner from coded slides and the evaluators did not have any information about the PCR, or the culture or the clinical group. Positive hybridization signal was defined visible cytoplasmic reactivity in more than 10% of the respiratory epithelial cells.

Rhinovirus RT-PCR analysis and virus culture from nasopharyngeal aspirates (NPA) at days 1 and 7 were also carried out as described earlier [Mäkelä et al., 1998].

## RESULTS

The specificity of the in situ hybridization signal was verified by in vitro infection of HeLa cells with rhinovirus 14. Analysis of the infected cells with the protocol used for clinical samples demonstrated reactivity of the infected cells with antisense but not with the sense probe (Fig. 1A,B). Uninfected HeLa cells were negative both by the antisense and by the sense probe (data not shown).

Out of 32 middle turbinate biopsies 22 (69%) were positively in situ hybridization with the antisense probe (Table I). Rhinovirus RNA was mainly localized in the cytoplasm of the respiratory epithelial cells and in the mucosal inflammatory cells. Instead, the mucosal glands were always negative (Fig. 1C,D). A relative large number of in situ hybridization-positive cells were present in some of the middle turbinate biopsies (Fig. 1C). All five biopsies from control patients were negative for rhinovirus by in situ hybridization (Fig. 2).

In situ hybridization yielded more positive results than the PCR (15/32, 47%) or virus culture (11/34, 34%) determined from NPAs (Table I) but statistical significant differences were not observed ( $P = 0.265$ ,  $P = 0.425$ , respectively). In situ hybridization results on days 1 and

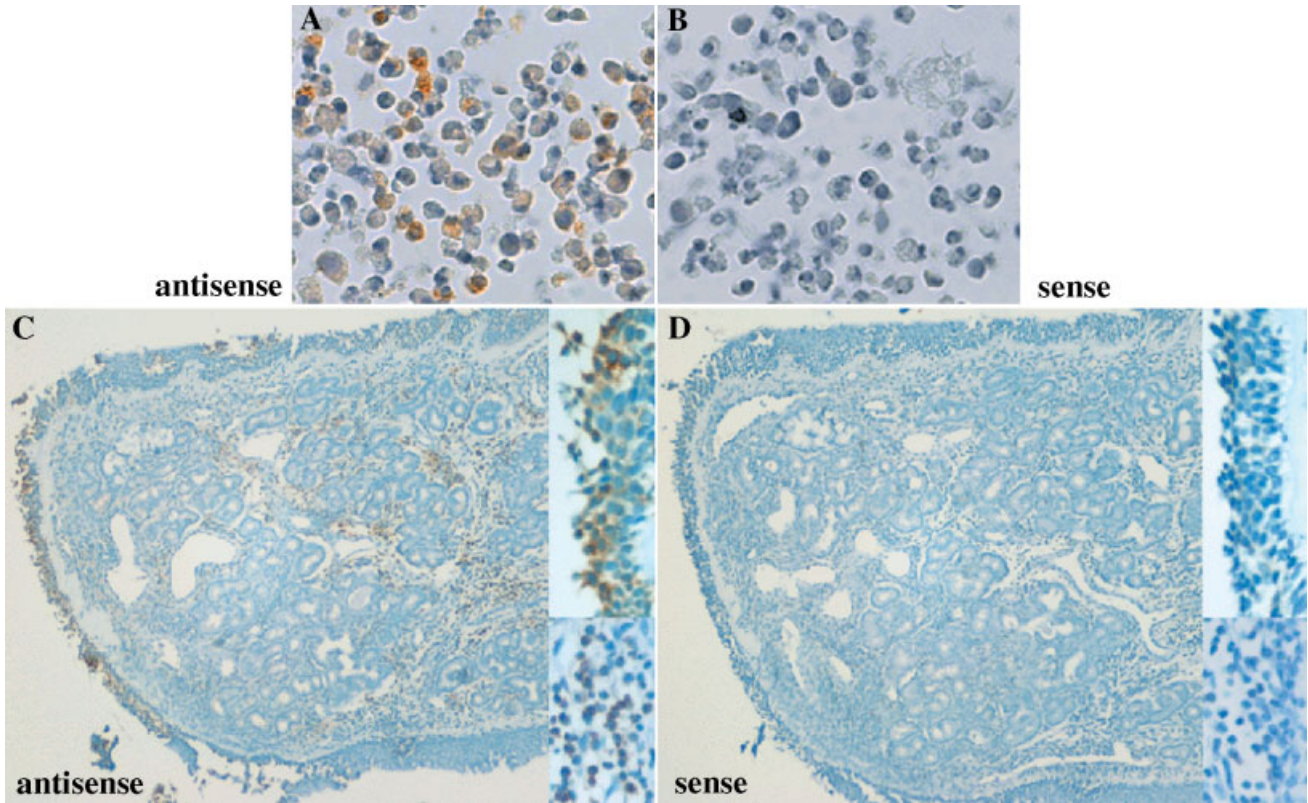


Fig. 1. In situ hybridization of rhinovirus-14 infected HeLa cells shows positive reactivity with the antisense probe (A) but not with the sense probe (B). Detection of rhinovirus RNA from a middle turbinate biopsy by in situ hybridization. (C) Antisense probe demonstrates

positive reactivity in the respiratory epithelium and scattered mucosal mononuclear inflammatory cells. (D) Control sense probe shows no reactivity. The top insets show epithelial cells and the bottom insets show inflammatory cells at a higher magnification.

7 showed that out of eight rhinovirus-positive patients, viral RNA was detected in five for up to 7 days after onset (Table II).

**DISCUSSION**

In the present study, it was shown that rhinovirus RNA can be detected commonly in the nasal epithelium of patients with common colds by in situ hybridization.

The large number of positive nasal epithelial cells in our study is in contrast to earlier studies [Bruce et al., 1990; Arruda et al., 1991, 1995; Bardin et al., 1994], where only a small fraction of nasal epithelial cells were rhinovirus positive by in situ hybridization. One reason for this may be methodological. Radioactively labelled probes used in earlier studies [Bruce et al., 1990; Arruda et al., 1991, 1995] may not be as sensitive as digoxigenin-labelled probes that we used, as has been shown in earlier studies with human papillomavirus [Morris et al., 1990]. The other reason for the different spread of rhinovirus infection may be the nature of the infec-

TABLE I. Detection of Rhinovirus in 32 Common Cold Patients by In Situ Hybridization in Middle Turbinate Biopsies and RT-PCR and Culture in the Nasopharyngeal Aspirates on day 7\*

	In situ hybridization		
	+	-	
Total in situ hybridization	22 (69)	10 (31)	
PCR			Total
+	12 (38)	3 (9)	15 (47)
-	10 (31)	7 (22)	17 (53)
Culture			Total
+	9 (28)	2 (6)	11 (34)
-	13 (41)	8 (25)	21 (66)

\*In ten rhinovirus negative subjects, influenza C virus was found in two, parainfluenza type 2 virus in two, coronavirus in one, respiratory syncytial virus in one, and influenza A virus in one common-cold subject. One rhinovirus positive (by PCT and in situ) patient was also positive for coronavirus by PCR.

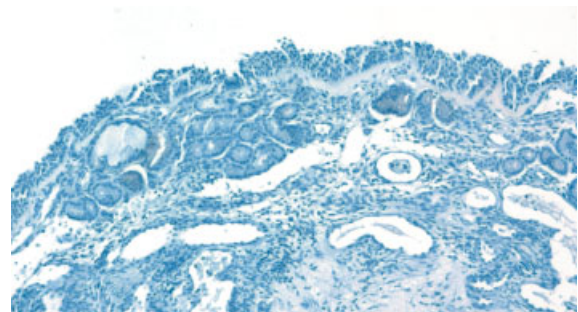


Fig. 2. Lack of rhinovirus reactivity in a middle turbinate biopsy specimen obtained from the middle turbinate from the uninfected patient during nasal surgery. Reactivity with the antisense probe is shown.

TABLE II. Detection of Rhinovirus by In Situ Hybridization on Days 1 and 7 From the Middle Turbinate Biopsies and by Rhinovirus Culture on Day 1 From the Nasopharyngeal Aspirates in Patients With Common Colds

Number of patients	In situ hybridization		Virus culture
	Day 1	Day 7	Day 1
4	+	+	+
3	+	-	+
1	+	+	-
1	-	-	-

tion. Earlier studies have either been carried out in vitro [Arruda et al., 1991; Bates et al., 1997] or in vivo with volunteers inoculated with rhinovirus [Bruce et al., 1990; Arruda et al., 1995]. Artificially induced rhinovirus infection may not infect as high number of positive nasal epithelial cells as natural infection. This has been shown in a study [Bardin et al., 1994], where nasal biopsies obtained from four subjects with inoculated rhinovirus infection and from two subjects with natural rhinovirus colds were tested. Both biopsies from patients with natural colds yielded positive results, while only one rhinovirus-inoculated subject was positive for rhinovirus by in situ hybridization [Bardin et al., 1994]. Of note, also in the present study the number of positive epithelial cells varied.

In situ hybridization of the middle turbinate tissue detected the rhinovirus infection more frequently than PCR. However, these results cannot be compared directly since the tests measure different parameters and middle turbinate biopsies and nasopharyngeal aspirates were taken from different anatomical loci. However, PCR is still today the practical method of choice for diagnosis of rhinovirus infection, while in situ hybridization, as an invasive and slow method, is more useful for studies on pathogenesis and spread of rhinovirus infections in the respiratory area.

In the present study, it was not tested how broadly rhinovirus 14 probes react with other rhinoviruses. We used human rhinovirus 14 probe, which consist 249 nucleotides from the 5'-untranslated region. These nucleotide sequences are very similar in all human rhinoviruses [Kallajoki et al., 1990]. Human rhinovirus-14 represents genetically the human rhinovirus-B species (25 serotypes) [Savolainen et al., 2002] and it is probable that the probe we used recognises at least the members of this cluster, if not all rhinovirus serotypes.

It is possible that by using in situ hybridization, we detect previous rather than acute ongoing infection. According to the epidemiological studies, rhinovirus shedding in nasal fluid can persist for up to 3 weeks in patients with rhinovirus colds and subclinical infections may occur. Since we did not have a baseline (and post-infection) samples from the patients, we cannot be sure if the higher in situ hybridization detection rates than PCR and culture could be due to non-replicative, perhaps even persistent, rhinovirus infection. Although rhinovirus persistence in upper respiratory track has

been described earlier in children [Marin et al., 2000], the situation in adults may differ from that. In addition, our adult controls were all rhinovirus negative by in situ hybridization. However, the potential persistence of rhinoviruses in upper respiratory track may be important because it could be linked either to the development of bacterial superinfection [Waner, 1994] or to the development of chronic asthma by chronic inflammatory mechanisms as has been suggested [Freytmuth et al., 1999; Marin et al., 2000].

In conclusion, our study demonstrates that rhinovirus can frequently be detected in middle turbinate epithelial cells in natural common colds. Based on the results, in situ hybridization is a useful tool for studies on the pathogenesis and spread of rhinovirus infections through the respiratory tract. Our data imply that during naturally acquired infection, the spread of rhinovirus on the respiratory epithelium of the upper airways may be wider than assumed earlier.

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